COFFEE BOOK
on
STEM CELL STUDIES

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Foreword

Dear Readers,

With immense pleasure, I present you this Coffee Book, which is a compilation of articles on stem cell research executed at Neuroscience Research Lab (NRL), a GLP compliant laboratory. The lab has investigated differentiation, regeneration, and renewal of stem cells in degenerative disorders such as Alzheimer's disease, retinal degeneration, and retinal ischemia. Various studies have demonstrated that embryonic and adult stem cells can produce progenitor cells for tissue renewal and regeneration of damaged tissues. The NRL deals with patient samples and animal models to study the pathophysiology of a neurodegenerative disease characterized by amyloid beta deposition and memory loss. Researchers at NRL have reported significant contribution and expertise of stem cells in their potency to rescue\reverse disease progression. The authors published not only the role of stem cells in the rescue of memory loss in amyloid beta injured mice but also included isolation and characterization of various stem cell types, the potential for their manipulation, and the possibilities for future therapeutic uses in experimental models and human diseases such as neurodegenerative disorders.

Most importantly, the researchers of this lab explored the stem cells isolated from various sources such as bone marrow, human umbilical cord blood, and tail vein from mice and have shown that stem cells from different sources have different outcomes in the rescue of injury. These achievements have been published in PubMed indexed and peer reviewed journals.

This Coffee Book is dedicated to the visionary and devoted Principal Investigator, Professor Akshay Anand, and all the authors for their hard work. The published work was part of exciting yet challenging projects that were taken up by research scholars, and helped them to obtain a PhD degree.

I extend my thanks to Dr. Akshay Anand for his knowledge, encouragement, support, enthusiasm, and patience in providing the vast expertise and knowledge under one roof. Our lab has significantly contributed to enhancing knowledge and generating evidence-based data that has recognized us at the National and International levels. I am sure that NRL will continue its growth and accomplishments by encouraging young scientists and researchers for benefit of the society and the scientific community.

I hope that the Stem Cells Coffee Book will provide valuable and authoritative information to aid those seeking answers to the unanswered questions.

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CLINICAL TRIAL REPORT

Common Yoga Protocol Increases Peripheral Blood CD34+ Cells: An Open-Label Single-Arm Exploratory Trial

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Purpose: Physical inactivity can be a cause of various lifestyle disorders including atherosclerosis, diabetes, hypertension, and cardiovascular diseases (CVDs). Lifestyle modification by the inclusion of Yoga and similar activities has shown beneficial effects on disease prevention and psychological management. However, the molecular mechanism at the cellular level is unknown. This study aims to identify the molecular response at systemic level generated after three months of Common Yoga Protocol (CYP) practice.

Methods: A total of 25 healthy adult females were recruited for this study (25 to 55 years). After the drop out of 6 participants at baseline and 2 participants after 1 month; blood samples of 17 participants were assessed. Blood samples were assessed for lipid profile, CD34+ cell enumeration and angiogenesis markers (ie, VEGF, Angiogenin and BDNF) at baseline (before intervention), after one month and after three months of Common Yoga Protocol (CYP) practice. The psychological health of the participants was assessed at baseline and after three months of CYP practice. The psychological tests used were General Health Questionnaire (GHQ), State-Trait Anxiety Inventory (STAI), Trail Making Test A & B, Digit symbol test, Digit symbol substitution test.

Results: After 3 months of intervention, blood samples of 17 participants were collected and following results were reported (1) percentage of CD34+ cells increased significantly after 3 months of CYP practice (from 18.18±7.32 cells/μL to 42.48±18.83 cells/μL) (effect size: W, 0.40; 95% CI, $p = 0.001$) (2) neurogenesis marker, ie, BDNF showed a significant change with time after 3 months of CYP intervention (effect size: W, 0.431, 95% CI; $p = 0.002$), (3) HDL showed an increasing trend (non-significant) after three months of CYP practice (53.017±1.28 mg/dl to 63.94±5.66 mg/dl) (effect size: W, 0.122; 95% CI; $p = 0.126$) (4) General Health score (10.64 ± 3.53 to 6.52 ± 3.12) (effect size: d, 0.98; 95% CI; $p = 0.001$) along with visual and executive function improved (69.94±26.21 to 61.88±28.55 (time taken in seconds)) (effect size: d, 0.582; 95% CI; $p = 0.036$), also stress and anxiety showed reduction (effect size: d, 0.91; 95% CI; $p = 0.002$) (5) a significant positive correlation was found between: HDL with VEGF ($r = 0.547$, $p = 0.023$) and BDNF ($r = 0.538$, $p = 0.039$) after 3 months of intervention; also, a significant positive correlation was found between VEGF with BDNF ($r = 0.818$, $p \leq 0.001$) and Angiogenin ($r = 0.946$, $p \leq 0.001$), also, BDNF was also positively correlated with Angiogenin ($r = 0.725$, $p = 0.002$) at both 1 month and 3 months after intervention. Also, VEGF and BDNF showed a significantly negative correlation with stress and anxiety questionnaire after the intervention.

Conclusion: The current study provides insights into the molecular response to CYP practice at systemic level. The results suggest that CYP practice indeed increased CD34+ cells in peripheral blood and BDNF also showed a significant change after the intervention. An overall improvement in general health and psychology of the participants was also observed.

Keywords: common yoga protocol, stem cells, angiogenesis, psychology, lipid profile

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Introduction

Physical inactivity can have serious implications leading to various lifestyle-related disorders and the incidence of these disorders increases with age. This may occur due to the dysregulation of the metabolic and molecular pathways, which may cause Non-Communicable Diseases (NCDs) either via overactivation or via inhibition of the molecules involved. To cite an example, in cancer overactivation of angiogenic mechanism occurs while via inhibition there is a reduced blood vessel development, halted stem cell proliferation and cardiac complications. The reason for these conditions can be physical inactivity along with other contributing factors. It has been shown that one-third of the adult population in the world is leading a sedentary life with insufficient physical activity.[1–3] In India, this population is also high, and women are more prone to physical inactivity.[4] As per a report published in 2019, on a global average, women are more physically inactive in comparison to men (31·7% for inactive women vs 23·4% for inactive men),[5] which makes women more prone to developing NCDs, with an impact on their quality of life[6] and cognition,[7,8] these studies underscore the importance of awareness and practice of physical activity among women globally.

Angiogenesis is described as the sprouting of new blood vessels from preexisting ones, and this process is mediated through molecular signals like VEGF and Angiogenin.[9] Angiogenesis is known to influence neurogenesis mechanism mediated via cross-talk between VEGF and BDNF.[10,11] Alteration in angiogenesis-related pathways is pivotal to the development of lifestyle disorders like cancer, diabetes, hypertension, atherosclerosis, stress and depression which is cross linked to the dysregulated angiogenesis.[12–17] Physical activity is known to primarily regulate angiogenesis molecular mechanism and therefore may help in prevention of NCDs.[18,19] Increased vascular density after exercise enhances cognition and quality of life.[19] The major molecular players involved in this mechanism are markers of angiogenesis (VEGF and Angiogenin),[20–22] Neurogenesis (BDNF),[23] Lipid profile,[24] stem cell mechanisms (CD34+ Hematopoietic stem cells (HPCs))[25] which lead to the development of new cells in the system and these altogether lead to an overall development of better health.

CD34+ cells are a type of hematopoietic stem cell (HPCs) with the potential of developing into endothelial cells.[26,27] These bone marrow-derived cells have been found in circulating peripheral blood, and their role in pro-angiogenic therapies has been studied extensively.[25] Circulating HPCs enhance the regenerative potential of blood and tissue cells, more specifically in circulation, which shows that these correlate with vascular endothelial function.[28] Studies have also shown that a deteriorating number of CD34+ along with angiogenic markers increases the risk of cardiovascular diseases (CVDs) reflecting reduced vascular capacity.[25] Also, physical exercise has been known to enhance the mobilization of CD34+ cells into circulation.[29]

Lipid metabolism is associated with CVDs like atherosclerosis, coronary heart disease, etc.[30–32] An increase in lipid metabolites above the normal range can inhibit the process of angiogenesis and further lead to blockage in arteries which is a significant cause of CVDs.[33] Together, these molecular responses to physical activity are known to influence and improve general health and cognition.[34,35]

Yoga is a branch of physical activity that focuses on the mind and body, and evokes relaxation through stress and anxiety resistance techniques. It leads to an overall enhancement in physiological, psychological, and physical health. Studies have shown that the practice of Yoga reduces inflammatory markers, improves immune responses and T effector cell function, and improves the overall quality of life and psychosocial health.[35] Wu et al in 2020 reported an increased proportion of CD34+ cells after Innovative Mind-Body easy exercise.[36] Another similar study reported that sustained one-year Tai chi practice showed significant elevation in peripheral CD34+ cell number in young adults.[37] In our previous study, we have reported an increase in angiogenesis markers, ie, Angiogenin and VEGF after 1 month of Common Yoga Protocol (CYP) intervention along with and elevation in HDL, as an extension of previous study we wanted to explore the effects of CYP practice for a longer time duration, ie, 3 months and also wanted to explore and correlate the effects of CYP through angiogenesis and stem cell mechanisms.[38]

The risk factor for high disease frequency in females affecting their quality of life is the prevalence of sedentary lifestyle which is high worldwide and also in India. Therefore, we primarily aimed to identify the angiogenic response of Common Yoga Protocol, CYP[39] (a generalized yoga protocol introduced by Govt. of India on International Yoga Day for the general population) in sedentary adult women by evaluation of CD34+ cells, angiogenesis markers and lipid profile in

peripheral blood as angiogenic mechanism is the preliminary response of cell proliferation and growth. We also aimed to identify the psychological response to the involvement of CYP in daily lifestyle amongst these sedentary adult females. The study's hypothesis was to identify the response of inclusion of CYP in daily routine to improve and manage overall health and to identify the psychological aspects in response to CYP practice in sedentary adult women. This study was planned to identify the potential of CYP as an adjunct therapy in daily lifestyle for overall health management and decipher the molecular response associated with the practice of CYP in sedentary adult females.

Methodology

Study Design

This study is an open-label single-arm exploratory trial to investigate the effects of CYP practice for three months.

Subject's Characteristics

A total of 25 healthy female subjects (without any co-morbidity) between the age group of 25–55 years were recruited for this 3-month yoga intervention study based on their willingness to participate (Figure 1), after 3 months eight participants dropped out and 17 participants gave their follow-up samples. Recruitment of participants was based on self-reported sedentary lifestyle of the participants since last 1 year, those who were not performing any vigorous/moderate physical activity were recruited for the study (these details were acquired at the time of recruitment). The participants were residents of urban areas of Chandigarh city, India. The participant's primary language was Hindi, with an understanding of the basic English language. The education of all the participants was above secondary school.

Recruitment of participants was done between January 2021 and June 2021 (all the participants were recruited at different time points). All the recruited participants were informed about the purpose of the study and informed consent was obtained from all individual participants.

The participant's blood samples were taken at three time points: Baseline (before the intervention), after 1 month, and after 3 months. Blood obtained was used for lipid profile assessment, assessment of angiogenesis markers, and also for CD34+ cell enumeration. Ethical approval was obtained from the PGIMER, Chandigarh Ethical Committee (IEC No. IEC-03/2020-1541). The study was registered in CTRI (CTRI No. CTRI/2020/09/027747). The study complies with principles of Declaration of Helsinki.

Intervention

45–50 minutes of Yoga intervention (Common Yoga Protocol) (Table 1) was given to the participants 5 days/week for 3 months through online interface (Google meet). Common Yoga Protocol (CYP), which includes Loosening practices, Asanas (standing, sitting, prone and supine), Pranayama, and Meditation practice, was used[39] as an intervention. The intervention was given in the morning (6–6:45 am) and evening (5:30–6:15 pm) timings depending upon the suitability of the participant. Daily attendance of the participants was recorded.

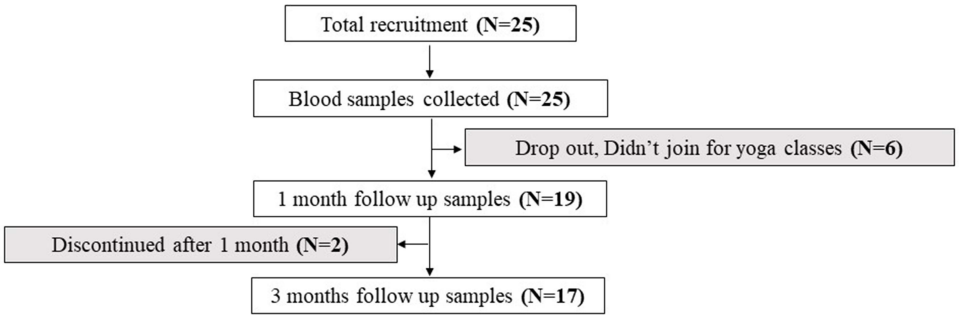

Figure 1 Participant information.

Table 1 Shows the Details of Common Yoga Protocol (CYP) Intervention

S. No.	*ASANAS*	Protocol
1.	Invocation/ Prayer (1 minute)	To enhance the benefits of practice.
2.	Loosening practices (5 minutes)	• Neck bending • Shoulder's movement • Trunk movement (*Katishaktivikasak*) • Knee movement
3.	*Yogasanas* (Yoga postures) (1 minute per asana)	**Standing postures** • *Tadasana* (Palm tree pose) • *Vrksasana* (Tree posture) • *Padahastasana* (The hands and feet posture) • *Ardhacakrasana* (The half wheel posture) • *Trikonasana* (The Triangle Posture) **Sitting postures** • *Bhadrasana* (The firm auspicious posture) • *Vajrasana* (Thunderbolt posture) • *Ardhaustrasana* (The half camel posture) • *Ustrasana* (Camel Posture) • *Sasakasana* (The Hare posture) • *Uttana mandukasana* (Stretched up frog posture) • *Vakrasana* (The spinal twist posture) **Prone postures** • *Makarasana* (The Crocodile posture) • *Bhujangasana* (The Cobra posture) • *Salabhasana* (The Locust Posture) **Supine postures** • *Setubandhasana* (The Bridge Posture) • *Uttanapada Asana* (Raised feet posture) • *Ardha Halasana* (Half plough posture) • *Pavanamuktasana* (The Wind releasing Posture) • *Savasana* (The Dead body Posture)
4.	Kapalbhati (3–4 minutes)	Forceful exhalation by contracting the abdominal muscles (30 strokes/round)
5.	*Pranayama* (2 minutes each)	• *Nadishodhana* or *Anulom Viloma Pranayama* (Alternate nostril breathing) • *Sitali pranayama* • *Bhramari pranayama*
6.	*Dhyana* (Meditation) (5–10 minutes)	For stress free deep relaxation and silencing of mind.
7.	*Sankalpa* (1 minute)	Commitment to be healthy, happy, peaceful and joyful human being.
8.	*Shanti path* (1 minute)	Prayer for happiness, health and peace for all.

Outcome Measures

Attendance Rate

Daily attendance of the participants was recorded.

Anthropometric Assessment

Age, weight, height, and BMI of the participants were recorded before and after the intervention.

Biochemical Assessment

One mL blood sample of the participants was assessed for lipid profile at baseline, after 1 month, and after 3 months of intervention.

Blood Serum Isolation

A fasting blood sample (approx. 3mL) was collected in a clot activator SST tube and was kept at room temperature for 30 minutes to allow clotting of the sample. The sample was processed at 2500 rpm for 30 minutes in a density gradient centrifuge. The upper yellowish layer was separated, aliquoted, and stored at −80°C.

CD34+ Cells Enumeration by Flow Cytometry

The flow cytometry sample preparation and gating strategy was based on published protocols (ISHAGE).

Briefly, 4mL of blood from the participants was collected in EDTA-coated vials. These vials were kept at room temperature for 45–60 minutes. The upper layer containing plasma was then layered on Hisep 1077 (Hi-Media) and centrifuged in a density gradient centrifuge at 1500 rpm for 30 minutes. The middle buffy layer containing PBMCs was then separated. From the separated PBMCs, approximately 1 million cells were suspended in 20 µL of Fc Blocker and kept at 4°C for 30 minutes. CD45 FITC and CD34 PE Fluorochrome labeled antibodies were added per requirement and incubated at 4°C for 1 hour. Washing of the cells with 1X PBS was then done, and finally, the pellet was resuspended in 200 µL of PBS and analyzed on FACS Calibur (BD Bioscience, USA) for 4 hours of processing.[40]

The cells were enumerated using ISHAGE guidelines. The acquisition was mainly based on forward, and side scatters analysis, including lymphocytes excluding debris. Manual gating procedure was followed keeping a negative (cells alone) control for each sample to avoid autofluorescence and false detection. After gating, CD34/CD45 dim cells were analyzed. ISHAGE guidelines were used to calculate estimates of CD34 concentration.

Elisa

Assessment of angiogenesis markers, ie, VEGF (Vascular Endothelial Growth Factor), Angiogenin, and BDNF (Brain-Derived Neurotrophic Factor) in blood serum of the participants was done by sandwich enzyme-linked immunoassay technique ELISA (Kinesis Dx). The procedure was followed as per the manufacturer's instructions. Briefly, after adding the samples, a biotinylated antibody was added, and the plate was incubated for 1 hour. The plate was then washed, the substrate was added, and the plate was again incubated for 10 minutes, after which stop solution was added. Reading was taken at 450 nm with an ELISA reader (Bio-Rad Laboratories). A standard curve was plotted for each experiment, and the respective protein concentration was calculated. $R^2 \geq 0.98$ was considered for the analysis.

Total protein assessment was done to normalize the concentration of the target protein. The Bradford method was used for total protein, and BSA was considered standard. Serum samples were diluted at a concentration of 400X. Coefficient of Variation (CV%) for total protein intra assay assessment was measured using formula $(\sigma/\mu)*100$. For ELISA, samples were assayed in singlets hence no CV% is reported for that.

Neuropsychological Assessment

Participants were assessed with neuropsychological tests before and after three months of intervention. The tests used were as follows: DST (Digit Substitution Test), which measures attention and verbal memory,[41] DSST (Digit Symbol Substitution Test), which measures the information processing capacity of the participant,[42] TMT A & B (Trail Making Test A & B) which measures the visual attention and task switching,[43] SLCT (Six Letter Cancellation Test).[42]

State-Trait Anxiety Inventory, which assesses the anxiety of the participant,[44] and the Short General Health Questionnaire (GHQ-12) to assess the participant for mental health and overall general health of the participant[45] were also administered to the participants. These tests were selected as these tests are quick, short, and reliable for research studies based on the general population.[46,47]

Statistics

Statistical analysis was performed by using SPSS 21 (IBM corp.). Shapiro Wilk test was used to test the normality of the data. Friedman test was used to analyze the non-parametric repeated measures data (lipid profile, CD34+ cell enumeration, and ELISA data). For the parametric pre-post data (psychological assessment, weight, and BMI) paired *t*-test was used. Correlation assessment was done using spearman's rho test to correlate the change between CD34+ cells, lipid

parameters, and angiogenesis markers after 1 month and 3 months of CYP practice. The effect size was reported using Kendall's W concordance coefficient for repeated measures of non-parametric data.

Using formula $W = X2/N(K-1)$, where W is Kendall's W value; X2 is the Friedman test statistic value; N is the sample size. k is the number of measurements per subject.

For the parametric pre-post data of psychological assessment along with weight and BMI assessment, Cohen's d effect size calculation was done using the formula

$$t/\sqrt{N}$$

Where t represents the t value, and N represents the number of participants.[48]

Results

Demographic and Anthropometric Characteristics

A total of 25 healthy females who met the inclusion criteria were recruited for the study. Six participants did not join the yoga classes and were thus excluded, and two discontinued after one month; therefore, 17 were assessed for the final analysis. The mean age of the participants was 40.82 ± 10.11 years.

All the participants were of Indian nationality recruited from Chandigarh city of India with education status of Higher Secondary School or above and an understanding of basic Hindi and English. The socio-economic status of all the study participants was above middle income.

The weight and BMI of the participants were assessed at baseline and after 3 months of CYP intervention, and it was observed that both weight (p = 0.019) and BMI (p = 0.017) reduced significantly after 3 months of intervention (Table 2).

Attendance Rate of the Participants for Intervention

For the present study, a total of 25 participants were recruited, out of which six did not join the yoga class after showing their willingness at the time of recruitment, and two others dropped out after 1 month of intervention. Two dropout participants attended classes even after 1 month for the next 15–20 days, after which they discontinued without giving any apparent reason. Participants with attendance ≥30 (43%) out of 70 classes were excluded from the study.

Table 2 Demographics and Anthropometric Details. Data Was Analyzed Using Paired *t*-Test. N = 17, **p <0.01

Demographics of the Participants					
Average age (in years)	40.82 ± 10.11				
Occupation	11 working professionals, 6- non professional				
Ethnicity	Asian Indian				
Nationality	Indian				
Education	Higher secondary school or above				
Primary Language	Primary Language: Hindi Understandable Language: English				
Socio economic status	All the participants belonged to middle income salary status (Monthly income ≥Rs 45,000/-)				
Co morbidity	2 had migraine				
Diet (self- declared)	4 non-vegetarians, 13 vegetarians				
	Pre (Mean ± SD)	Post (Mean ± SD)	*p* value	t value	Effect size (95% CI)
Weight (kg)	66.48 ± 7.82	65.32 ± 6.92	0.019**	2.607	0.16 (2.16 to 2.10)
BMI (kg/m^2)	26.36 ± 3.05	25.92 ± 2.82	0.017**	2.659	0.16 (0.090 to 0.80)

Daily attendance was recorded; 13 out of 17 participants attended more than 60% of the classes, whereas the other 4 participants joined 60% and 50% of classes (Figure 2).

Quantification of Absolute CD34+ Cell Count

After CYP practice, the CD34+ cell number increased over time. CD34+ cell number increased significantly after 3 months of CYP intervention (42.48±18.83 cells/μL) as compared to baseline (18.18±7.32 cells/μL) ($p = 0.001$), which is more than two times increase. However, this change was not observed at 1 month follow-up time point (Figure 3).

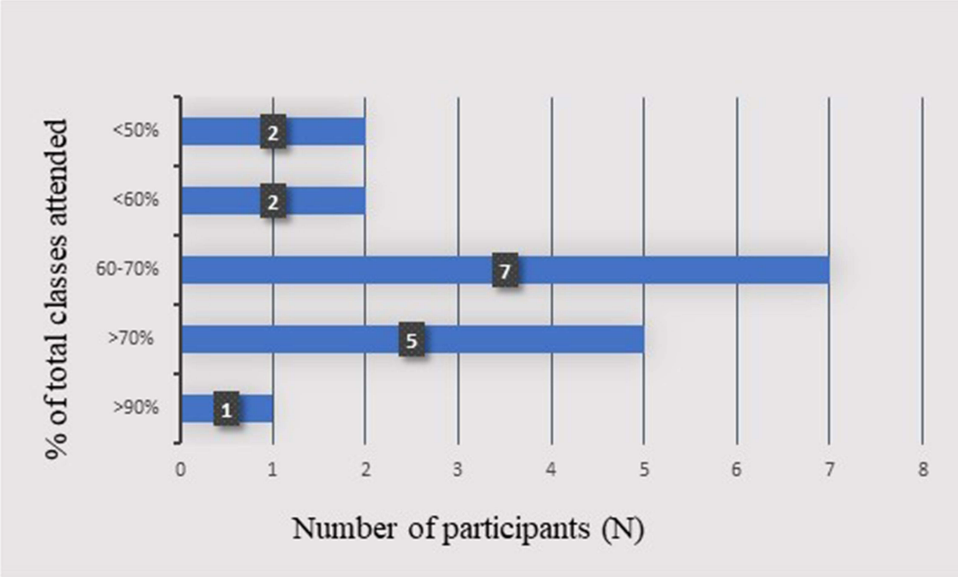

Figure 2 Figure showing attendance record of the participants for 3 months of intervention.

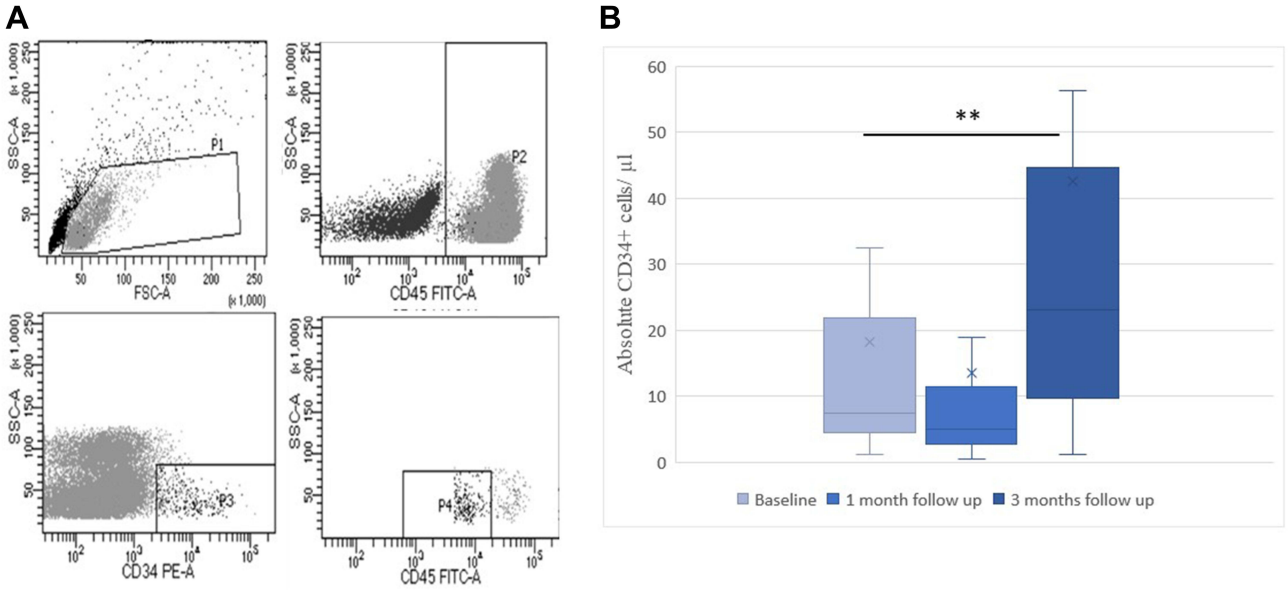

Figure 3 Enumeration of CD34+ cells before and after 1 month and after 3 months of CYP practice. (**A**) Scatter plot of enumeration CD34+ cells total blood cells, P1 shows total leukocyte population, P2 depicts the total CD45+ population, P3 is the CD34 population from the total CD45 population and P4 is the dim CD45+ (**B**) Box plot depicting quantified CD34+ cell population. **$p \leq 0.01$, Degrees of Freedom=2, Effect Size, W= 0.405.
Abbreviations: FSC, Forward Scatter; SSC, Side Scatter; PE, Phycoerythrin; FITC Fluorescein isothiocyanate.

Quantitative Biochemical Measurement

The lipid profile of the participants revealed no significant difference before and after the intervention. However, mean HDL (Baseline 53.01±5.28, 1-month follow-up 54.48±4.37, 3 months follow-up 63.93±23.33) has shown an increasing trend after 3 months of CYP practice (Effect Size, W = 0.122 (p = 0.126)) (Figure 4).

Protein Expression Quantification

ELISA assessment for angiogenesis markers was done from blood serum samples obtained from the participants. VEGF and Angiogenin showed a non-significant trend of increment after 1 month and 3 months of CYP practice. BDNF, which is a marker of neurogenesis, showed a significant decreasing trend following the intervention (Effect size = 0.431, p = 0.002) (Table 3). CV% for intra assay total protein assessment was 8.96%.

Psychological Assessment

Psychological assessment of participants was done at baseline and after 3 months of intervention. Participants showed a significant improvement in general health score (Effect size, d = 0.98; p = 0.001) and a reduced anxiety score post-intervention (Effect size, d = 0.91; p = 0.002). Also, participants showed a significant improvement in Trail making test B (Effect size, d = 0.582; p = 0.036), a visual and executive function parameter. Participants also showed an increasing trend in SLCT, DST, TMT A (parameters of attention and information processing) tests, though not significant, which are parameters of attention and information processing (Table 4).

Correlation Analysis

To determine the effects of change in CD34+ cell count on Angiogenesis markers and lipid profile of the participants, we correlated the values obtained after 1 month and 3 months of CYP practice for all the parameters mentioned above. We found a positive correlation of VEGF with BDNF and Angiogenin after both 1 month (Table 5) and after 3 months

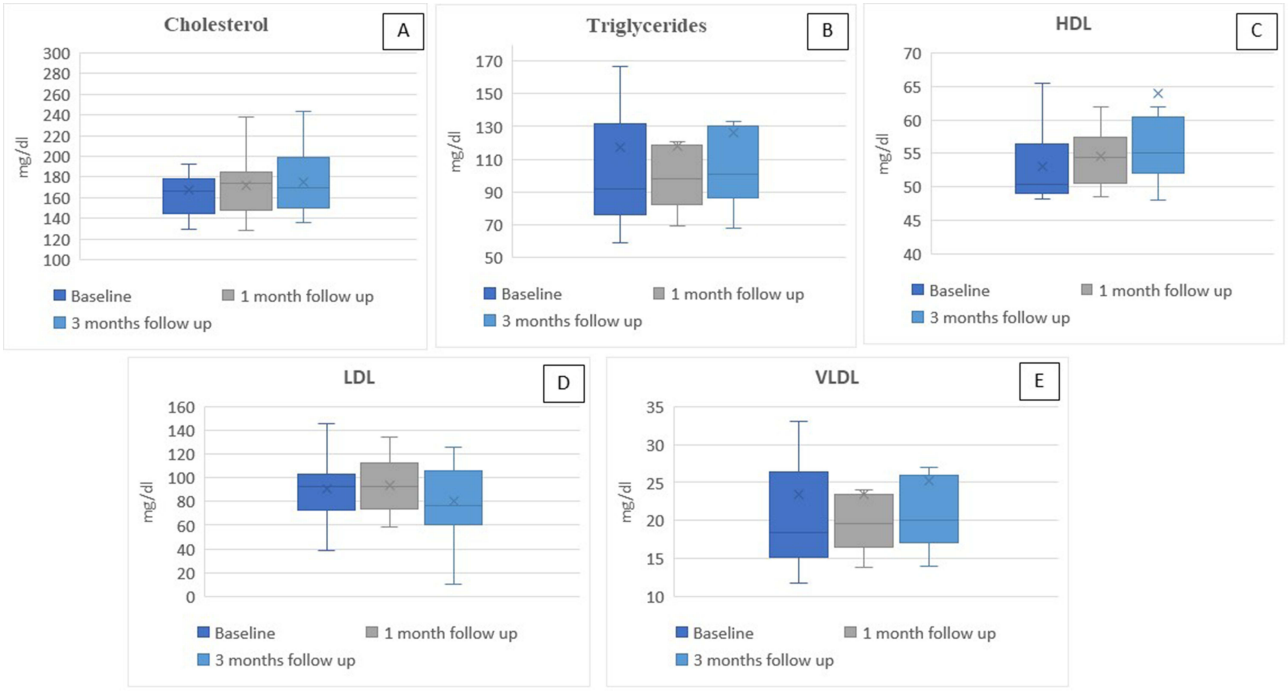

Figure 4 Box plot of Lipid profile at baseline, after 1 month and after 3 months of CYP practice (**A**) Cholesterol, Degree of Freedom = 2; Effect size, W = 0.027 (p = 0.630). (**B**) Triglycerides, Degree of Freedom = 2, Effect size, W = 0.064, p = 0.336. (**C**) HDL, Degree of Freedom = 2; Effect size, W = 0.122 (p = 0.126). (**D**) LDL Degree of Freedom = 2; Effect size, W = 0.010 (p = 0.838). (**E**) VLDL Degree of Freedom = 2, Effect size, W = 0.064, p = 0.336. Data was analysed by using SPSS Friedman K related samples test, N = 17.

Abbreviations: HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; VLDL, Very Low-Density Lipoprotein; CYP, Common Yoga Protocol.

Table 3 Table Showing Levels of Angiogenesis Markers, ie, VEGF, Angiogenin and BDNF at Baseline, After 1 Month and After 3 Months of CYP Practice. Data Was Analyzed Using SPSS Friedman Related Sample KS Test. N = 17, **p = 0.002

	VEGF (pg/µg)			Angiogenin (pg/µg)			BDNF (ng/µg)		
	Baseline	After 1 Month of CYP Practice	After 3 Months of CYP Practice	Baseline	After 1 Month of CYP Practice	After 3 Months of CYP Practice	Baseline	After 1 Month of CYP Practice	After 3 Months of CYP Practice
Median	30×10^{-4}	45×10^{-4}	39×10^{-4}	71×10^{-5}	69×10^{-5}	70×10^{-5}	14×10^{-6}	13×10^{-6}	7×10^{-6}
Minimum	3×10^{-4}	18×10^{-4}	16×10^{-4}	15×10^{-5}	30×10^{-5}	20×10^{-5}	8×10^{-6}	6×10^{-6}	4×10^{-6}
Maximum	7×10^{-2}	7×10^{-2}	6×10^{-2}	14×10^{-3}	23×10^{-3}	19×10^{-3}	12×10^{-5}	15×10^{-5}	12×10^{-5}
25th percentile	2×10^{-3}	3×10^{-3}	2×10^{-3}	39×10^{-5}	49×10^{-5}	36×10^{-5}	93×10^{-7}	80×10^{-7}	55×10^{-7}
75th percentile	20×10^{-3}	12×10^{-3}	14×10^{-3}	33×10^{-4}	32×10^{-4}	54×10^{-4}	58×10^{-6}	56×10^{-6}	40×10^{-6}
Chi square	1.412			1.529			12.933		
***p* value**	0.494			0.465			0.002**		
Degree of freedom	2			2			2		
Effect size (Kendall's W)	0.042			0.045			0.431		

Table 4 Table Showing Pre and Post Effects of Psychological Assessment. Data Was Analyzed Using SPSS Paired Sample *t*-Test. N = 17, *p < 0.05, **p <0.01

Psychological Assessment	Before Intervention (Mean ± SD)	After 3 Months of CYP Practice (Mean ± SD)	t value	Effect Size (Cohen's d)	95% CI		Degree of Freedom	*p* value
					Lower	Upper		
General Health Questionnaire score (GHQ-12)	10.64 ± 3.53	6.52 ± 3.12	4.07	0.98	1.97	6.259	16	0.001**
State Trait Anxiety Inventory (STAI)	29.23 ± 8.62	23.11± 3.40	3.78	0.91	2.69	9.542	16	0.002**
DST (Digit Span Test) Score (forward and backward)	16.88±5.06	16.70±4.52	0.65	0.15	−17.36	32.778	16	0.524
SLCT (Six Letter Cancellation Test)	30.82±12.26	32±13.23	0.23	0.05	−1.42	1.774	16	0.818
DSST (Digit Symbol Substitution Test) Time in seconds	241.17±87.5	233.47± 103.1	−0.49	−0.11	−6.27	3.917	16	0.631
TMT A (Trail Making Test A)	33.41±13.53	31.41±11.16	0.71	0.17	−3.93	7.932	16	0.485
TMT B (Trail Making Test B)	69.94±26.21	61.88±28.55	2.28	0.55	0.582	15.534	16	0.036*

(Table 6). Also, BDNF and Angiogenin were found to be significantly correlated. After 1-month, lipid parameters were found to be correlated with each other (Table 5). A positive correlation of HDL with VEGF and BDNF was also observed after 3 months (Table 6). Also, neuropsychological assessment parameter STAI showed a significant negative correlation with BDNF and VEGF (Table 6). Parameters of neuropsychological assessment were found to be inter correlated (Table 6).

Table 5 Table Showing Correlation Between the Change in CD34+ Cells, Lipid Parameters and Biochemical Parameters After 1 Month of CYP Practice. Data Was Analyzed Using SPSS Spearman Correlation Analysis. N = 17, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. the table alignment should be proper, the empty boxes can be deleted.

CD34 F1	ρ=1.000								
Cholesterol F1	ρ=−0.087 P=0.740	ρ=1.000							
Triglycerides F1	ρ=0.218 p=0.400	ρ=0.524* p=0.031	ρ=1.000						
HDL F1	ρ=0.342 p=0.179	**ρ=0.549** **p=0.022***	ρ=0.447 p=0.072	ρ=1.000					
LDL F1	ρ=−0.368 p=0.146	**ρ=0.888** **p≤0.001****	ρ=0.191 p=0.462	ρ=0.291 p=0.257	ρ=1.000				
VLDL F1	ρ=0.200 p=0.442	**ρ=0.533** **p=0.028***	**ρ=0.999** **p≤0.001****	ρ=0.438 p=0.079	ρ=0.201 p=0.439	ρ=1.000			
VEGF F1	ρ=0.262 p=0.309	ρ=0.120 p=0.646	ρ=0.196 P=0.451	ρ=0.053 p=0.841	ρ=0.050 p=0.848	ρ=0.197 p=0.448	ρ=1.000		
BDNF F1	ρ=0.189 P=0.499	ρ=0.173 p=0.537	ρ=0.300 p=0.277	ρ=0.048 p=0.864	ρ=0.154 p=0.585	ρ=0.302 p=0.274	**ρ=0.929** **p≤0.001****	ρ=1.000	
Angiogenin F1	ρ=0.257 P=0.319	ρ=0.155 p=0.554	ρ=0.324 p=0.205	ρ=0.082 p=0.754	ρ=0.070 p=0.790	ρ=0.326 p=0.201	**ρ=0.917** **p≤0.001****	**ρ=0.896** **p≤0.001****	ρ=1.000
	CD34 F1	**Cholesterol F1**	**Triglycerides F1**	**HDL F1**	**LDL F1**	**VLDL F1**	**VEGF F1**	**BDNF F1**	**Angiogenin F1**

Note: Parameters showing significant correlation is represented in bold.

Discussion

The present study demonstrates that the inclusion of CYP practice in the daily lifestyle routine provides physiological health benefits by enhancing the level of hematopoietic and endothelial progenitor cells, CD34+; and influences angiogenesis markers (VEGF, Angiogenin and BDNF). We have previously also reported a similar trend with respect to CYP practice in sedentary adults after 1 month.[38] We also report an improvement in general health, reduced anxiety and improved visual and executive function after 3 months, which could be attributed to the change in BDNF after 3 months of CYP practice and a negative correlation of STAI with BDNF and VEGF, other cognitive parameters did not show any significant improvement after CYP practice, which implies a longer duration of CYP practice could be assessed for cognitive parameters. Overall, these results depict an improvement in the physical, physiological, and general well-being of the participants with the inclusion of a standardized yoga protocol, ie, CYP, in the participants daily routine.

The average age of the participants was 40.82±10.11 years. In the Indian context, this age group of females has high prospects of high BMI and abnormal lipid profile,[49] indicating the need for lifestyle moderation. Studies have shown that women in their middle age are prone to more sedentary and leisure activities make them susceptible to various lifestyle-related disorders.[50,51] Furthermore, it has been seen that women in their middle age have deteriorated quality of life compared to men of the same age, which accentuates the need for lifestyle modification in women's lifestyles.[52,53]

In the present study, we have found a significant reduction in body weight (66.48 ± 7.82 to 65.32 ± 6.92) (p = 0.019) and BMI (26.36 ± 3.05 to 25.92 ± 2.82) (p = 0.017) after 3 months of CYP practice, the BMI of the participants has moved towards overweight from the obese category.[54] Since the recruited participants did not have any co morbid condition, their lipid profile did not show any significant change, except for HDL, which has shown an increasing trend, though not significant, it has a role in cardiovascular repair mechanism and is also associated with an increase in CD34+.[55,56]

CD34+ cell population was consistent with previous studies, which shows that the detection technique and enumeration method followed were similar to other studies.[57] A significant increase in the total CD34+ population in peripheral blood after 3 months of CYP practice shows an enhancement in the cells regenerative potential and angiogenesis.[36,58] Also, a decrease in CD34+ cells represent a marker of aging; we found the enhancement in CD34+ cells, which depicts a curtailed cellular aging.[56,59]

Table 6 Table Showing Correlation Between the Change in CD34+ Cells, Lipid Parameters and Biochemical Parameters After 3 Months of CYP Practice. Data Was Analyzed Using SPSS Spearman Correlation Analysis. N = 17, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001the table alignment is not proper.

CD34 F2	ρ=1.000										
Cholesterol F2	ρ =- 0.083 P=0.750	ρ=1.000									
Triglycerides F2	ρ= 0.267 P= 0.300	ρ=0.254 P=0.326	ρ= 1.000								
HDL F2	ρ= −0.145 P=0.578	**ρ= 0.661 P=0.004****	ρ= 0.044 P= 0.866	ρ=1.000							
LDL F2	ρ= 0.065 P=0.804	ρ=0.381 P=0.132	ρ = −0.082 P= 0.754	ρ= −0.033 P= 0.899	ρ=1.000						
VLDL F2	ρ= 0.245 *P*=0.344	ρ=0.285 P=0.267	**ρ= 0.997 P≤.001*****	ρ= 0.074 P= 0.778	ρ= −0.065 P=0.804	ρ=1.000					
VEGF F2	ρ= −0.020 P=0.940	ρ=0.150 P=0.567	ρ=0.000 P=1.000	**ρ=0.547 P=0.023***	ρ= −0.163 P= 0.531	ρ= 0.005 P= 0.985	ρ=1.000				
BDNF F2	ρ= −0.275 P=0.321	ρ=0.146 P=0.603	ρ = −0.089 P= 0.752	**ρ=0.538 P=0.039***	ρ= −0.342 P=0.213	ρ= −0.086 P=0.761	**ρ= 0.818 P≤0.001*****	ρ=1.000			
ANGIOGENIN F2	ρ= 0.125 P=0.633	ρ=0.197 P=0.448	ρ= 0.088 P= 0.736	ρ=0.407 P=0.105	ρ= −0.061 P= 0.815	ρ= 0.086 P= 0.743	**ρ= 0.946 P≤ 0.001*****	**ρ= 0.725 P= 0.002****	ρ=1.000		
GHQ POST	ρ= −0.359 P=0.158	ρ= −0.257 P=0.319	ρ= −0.022 P= 0.932	ρ= −0.116 P=0.656	ρ= 0.082 P= 0.754	ρ= 0.002 P= 0.992	ρ= −0.305 P= 0.234	ρ= −0.480 P= 0.070	ρ= −0.449 P= 0.070	ρ= 1.000	
STAI POST	ρ= −0.118 P=0.651	ρ= −0.180 P= 0.489	ρ= −0.024 P= 0.928	ρ= −0.305 P= 0.234	ρ= 0.184 P= 0.480	ρ= −0.006 P= 0.981	**ρ= −0.483 P= 0.049***	**ρ= −0.585 P= 0.022***	ρ= −0.458 P= 0.064	ρ= 0.481 P= 0.051	ρ= 1.000

(*Continued*)

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Table 6 (Continued).

DSST POST	ρ= 0.104 P=0.690	ρ= 0.117 P =0.654	ρ=0.299 P=0.243	ρ= 0.260 P= 0.313	ρ= −0.038 P=0.885	ρ= 0.286 P= 0.267	ρ= 0.211 P= 0.416	ρ= 0.071 P= 0.800	ρ= 0.180 P= 0.488	ρ= 0.061 P= 0.816	ρ= −0.509* P=0.037	ρ= 1.000				
DST POST	ρ= −0.290 P=0.260	ρ=0.271 P=0.292	ρ= −0.152 P= 0.561	ρ= 0.197 P= 0.449	ρ= −0.018 P=0.946	ρ= −0.123 P= 0.638	ρ= 0.144 P= 0.581	ρ= 0.382 P= 0.160	ρ= 0.100 P= 0.703	ρ= −0.258 P= 0.317	ρ= 0.089 P= 0.734	**ρ= −0.711 P=0.001*****| ρ= 1.000			
SLCT POST	ρ= −0.243 P= 0.348	ρ= −0.172 P= 0.510	ρ= −0.278 P= 0.279	ρ= −0.478 P=0.052	ρ= 0.157 P= 0.549	ρ= −0.278 P= 0.280	ρ= −0.240 P= 0.353	ρ= 0.038 P= 0.894	ρ= −0.210 P= 0.419	ρ= −0.148 P= 0.570	ρ= 0.219 P= 0.399	**ρ= −0.786 P≤0.001*****| **ρ= 0.678 P=0.003****	ρ= 1.000		
TMT A POST	ρ= 0.244 P=0.346	ρ= 0.220 P= 0.397	ρ= 0.154 P= 0.555	ρ=0.324 P=0.205	ρ= 0.070 P= 0.791	ρ= 0.169 P= 0.516	ρ= 0.142 P= 0.588	ρ= −0.160 P= 0.569	ρ= 0.167 P= 0.521	ρ= 0.062 P= 0.814	ρ= −0.218 P= 0.401	**ρ= 0.742 P= 0.001*****	**ρ= −0.593 P=0.012***	**ρ= −0.861 P≤0.001*****	ρ= 1.000	
TMT B POST	ρ=0.075 P=0.775	ρ= −0.057 P= 0.827	ρ= 0.246 P= 0.341	ρ=0.255 P=0.323	ρ= −0.339 P= 0.183	ρ= 0.247 P= 0.338	ρ= 0.176 P= 0.499	ρ= 0.079 P= 0.780	ρ= 0.080 P= 0.760	ρ= 0.152 P= 0.560	ρ= −0.264 P=0.306	**ρ= 0.760 P≤0.001*****	**ρ= −0.662 P=0.004****	**ρ= −0.788 P≤0.001*****	**ρ= 0.735 P=0.001*****	ρ=1.000
	CD34 F2	**Cholesterol F2**	**Trigly cerides F2**	**HDL F2**	**LDL F2**	**VLDL F2**	**VEGF F2**	**BDNF F2**	**ANGIO GENIN F2**	**GHQ POST**	**STAI POST**	**DSST POST**	**DST POST**	**SLCT POST**	**TMT A POST**	**TMT B POST**

Note: Parameters showing significant correlation is represented in bold.

Our study has found a significant positive correlation of HDL with VEGF and Angiogenin after 3 months of CYP practice, which signifies that CYP induces a mechanism of elevation in angiogenesis and cardiovascular repair. Furthermore, a positive correlation was also found between VEGF, BDNF, and Angiogenin after both 1 month and 3 months of CYP practice (Table 5 and Table 6) which signifies that the response is interrelated between these angiogenesis and neurogenesis molecules. No positive correlation was detected for CD34+ cells with any other markers analyzed. Furthermore, a negative correlation of STAI assessment with BDNF and VEGF was reported (Table 6) which signifies that with decrease in stress and anxiety after the CYP practice, an increment in VEGF and BDNF could be anticipated. However, studying these responses with a larger sample size and longer duration would depict more precise information.

Yoga may be beneficial with aging by increasing the CD34+ cells and angiogenesis, thereby reducing the risk of CVDs. Yoga may influence this response by immediate induction of intermittent hypoxia through breathing techniques and thereby sympathetic response and increasing blood flow at the time of practice.[37,60] Consequently, in the present study, we found a significant increase in CD34+ cells, and VEGF and Angiogenin followed an increasing trend after CYP for 3 months which is consistent with our previous study. BDNF showed a significant decreasing trend which may be due to the inverse response of resting BDNF levels to the long term of practice.[61]

The present study employs CYP as the standardized Yoga intervention (recommended by Govt. of India for International Yoga Day) as an adjunct inclusion to the daily routine of the recruited adult sedentary females who did not participate in any physical activity in their daily routine. Through this study we confer that CYP protocol mediates its health benefits through angiogenic mechanism via activating the endothelial stem cell niche and further activating the angiogenic molecular response to the practice of CYP. An enhancing level of HDL further enhances the angiogenic activation response via its function in cardiovascular repair. We also found an improvement in general health, reduced stress and anxiety score, and increment in information and visual processing cognitive function, however overall neuro psychology did not show any significant improvement after the intervention.

Overall, the current study shows that the practice of a validated and standardized 45–50 minutes of Yoga protocol, ie, CYP (which is freely available on AYUSH Ministry website) which includes the practices, can be performed by individuals of any age

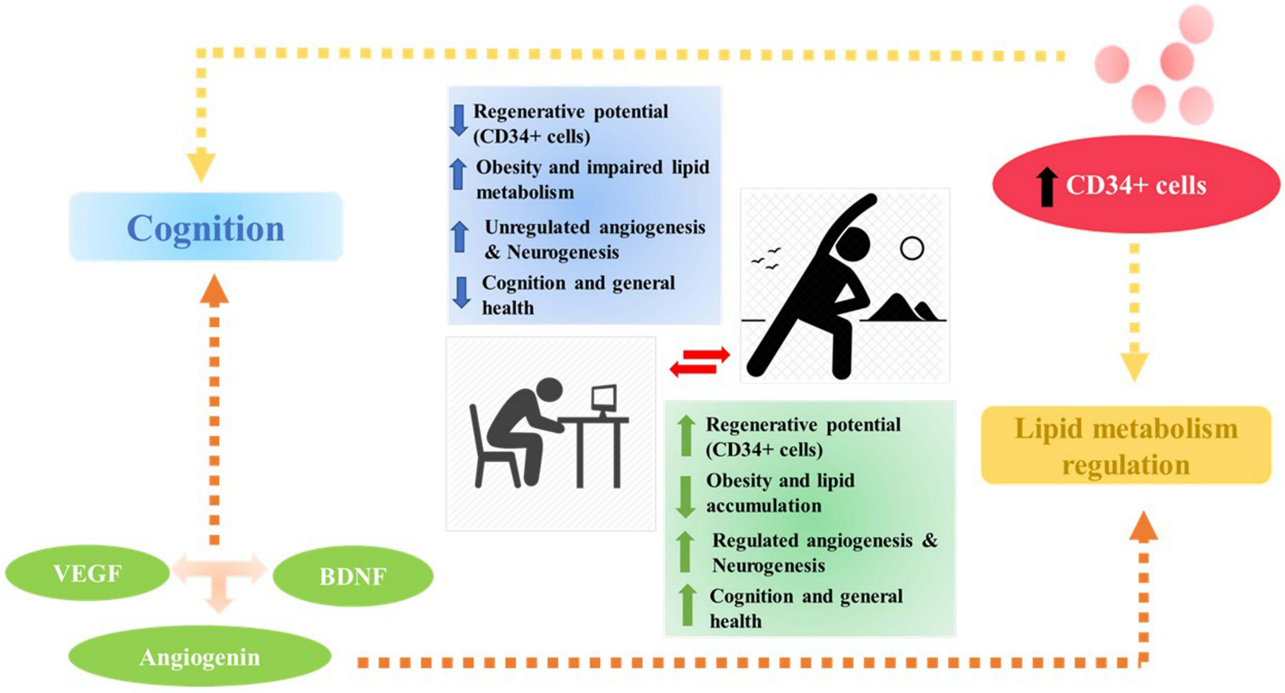

Figure 5 Schematic showing effects of Yoga induced through the regulation of neurogenesis and angiogenesis pathways via CD34+ cells number and Lipid metabolism. ↑ depicts increase, ↓ depicts decrease,----► depicts the pathway.
Note: the arrows in the figure are distorted and not properly alinged

(with no comorbid condition) as an adjunct in daily lifestyle. This can enhance the overall quality of life by boosting general health and improving cognition and also may be beneficial in prevention of NCDs (Figure 5). The possible mechanism of these benefits could be the intermittent hypoxic mechanism activated with the practice of Yoga which activates the stem cell niche from the bone marrow into the peripheral blood. This would also enhance the growth of blood vessels through angiogenesis activation.

Limitations

Small sample size and lack of a control group were the significant limitations of the present study.

Conclusion

We demonstrated that when sedentary adults included 45–50 minutes of Yoga practice in their daily lifestyle, it led to an overall physical, physiological, and psychological health benefits. These health benefits could be escalated through stem cell proliferation prompted by the intermittent hypoxia induced by the Yoga practice. Hence, including CYP as a daily lifestyle habit may provide health benefits and may prevent NCDs.

Data Sharing Statement

The authors confirm that the data confirming the findings of the study are available within the article. The raw data that supports the finding of the study can be made available from corresponding author (AA) on reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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Brief Article

Randomized Controlled Trial of Isha Kriya versus Observation to Improve Quality of Life in Hematopoietic Cell Transplantation Recipients

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A B S T R A C T

Hematopoietic cell transplantation (HCT) impacts recipients' quality of life (QoL). Few mindfulness-based interventions (MBI) in HCT recipients have shown feasibility, but heterogeneous practices and outcome measures have called into question the actual benefit. We hypothesized that self-guided isha kriya, a 12-minute guided meditation based on the principles of yoga focusing on breathing, awareness, and thought, as a mobile app would improve QoL in the acute HCT setting. This single-center, open-label, randomized controlled trial was conducted in 2021 to 2022. Autologous and allogeneic HCT recipients age ≥18 years were included. The study was approved by our Institutional Ethics Committee and registered at the Clinical Trial Registry of India, and all participants provided written informed consent. HCT recipients without access to smartphones or regular practitioners of yoga, meditation, or other mind-body practices were excluded. Participants were randomized to the control arm or the isha kriya arm at a 1:1 ratio stratified by type of transplantation. Patients in the isha kriya arm were instructed to perform the kriya twice daily from pre-HCT to day +30 post-HCT. The primary endpoint was QoL summary scores as assessed by the Functional Assessment of Cancer Therapy-Bone Marrow Transplantation (FACT-BMT) and the Patient-Reported Outcomes Measurement Information System Global Health (PROMIS-GH) questionnaires. The secondary endpoints were the differences in QoL domain scores. The validated questionnaires were self-administered before the intervention and at days +30 and +100 post-HCT. The analysis of endpoints was done on an intention-to-treat basis. Domain and summary scores were calculated for each instrument as recommended by the developers. A P value $< .05$ was considered to indicate statistical significance, and Cohen's d effect size was used to determine clinical significance. A total of 72 HCT recipients were randomized to the isha kriya and control arms. Patients in the 2 arms were matched for age, sex, diagnosis, and type of HCT. The 2 arms showed no differences in pre-HCT QoL domain, summary, and global scores. At day +30 post-HCT, there was no difference between the arms in the mean FACT-BMT total score (112.9 ± 16.8 for the isha kriya arm versus 101.2 ± 13.9 for the control arm; $P = .2$) or the mean global health score (global mental health, 45.1 ± 8.6 versus 42.5 ± 7.2 [$P = .5$]; global physical health, 44.1 ± 6.3 versus 44.1 ± 8.3 [$P = .4$]) in the 2 groups. Similarly, there were no differences in physical, social, emotional, and functional domain scores. However, the mean bone marrow transplantation (BMT) subscale scores, which addresses BMT-specific QoL concerns, were statistically and clinically significantly higher in the isha kriya arm (27.9 ± 5.1 versus 24.4 ± 9.2; $P = .03$; Cohen's d = .5; medium effect size). This effect was transient; mean day +100 scores showed no difference (28.3 ± 5.9 versus 26.2 ± 9.4; $P = .3$). Our data indicate that the isha kriya intervention did not improve the FACT-BMT total and global health scores in the acute HCT setting. However, practicing isha kriya for 1 month was associated with transient improvement in the FACT-BMT subscale scores on day +30 but not on day +100 post-HCT.

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INTRODUCTION

The hematopoietic cell transplantation (HCT) procedure is associated with significant morbidity and mortality risks from regimen-related toxicity, infections, and graft-versus-host disease. These risks cause significant distress and impact the health-related quality of life (HR-QoL) of HCT recipients [1]. Up to 22% to 43% of patients report difficulty living with uncertainty, fear of recurrence, loneliness, memory concerns, and somatic preoccupation [2]. Several studies of mindfulness-based interventions (MBIs) in HCT recipients have shown the feasibility and usefulness of such interventions [3,4]; however, heterogeneous intervention practices with low uptake and tools to measure outcomes have called into question the tangible benefits of MBIs [5,6]. We hypothesized that a culturally acceptable self-guided meditation method as a mobile app will have greater acceptance and adherence and a clinically significant impact on HCT recipients' QoL in short-term follow-up post-transplantation. Isha kriya is based on the science of yoga and incorporates actions aimed at promoting a feeling of connection with universal existence. The practice essentially focuses on breath, awareness, and thought. It reminds practitioners to not be identified by their body and mind and to be a watcher to cease bodily suffering. Isha kriya has been shown to reduce stress levels and improve immunity in healthy volunteers and other disorders by modifying the immune system [7]. The present study aimed to evaluate the impact of an MBI with isha kriya on HCT recipients' HR-QoL in the acute post-HCT setting.

METHODS

This single-center, open-label, randomized controlled trial (RCT) was conducted at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, from January 2021 to December 2022. PGIMER is a large-volume HCT center and an academic institution of national importance under the Indian Ministry of Health. The PGIMER Institutional Ethics Committee approved the study, which was registered at the Clinical Trial Registry of India (CTRI/2021/09/036667). All included patients provided written informed consent.

Autologous and allogeneic HCT recipients age $\geq$18 years were included in the study. HCT recipients without access to smartphones or regular practitioners of yoga, meditation, or other MBI practices were excluded. One study author assessed the patients for study eligibility, and another author was involved in patient randomization and intervention administration. Recipients were randomized to the isha kriya arm or control arm at a 1:1 ratio using a table of random numbers. Randomization was done using a fixed block size of 4 and stratified by type of transplantation. In this open-label study, both the patients and the investigators were aware of the allocation.

Isha kriya is 12-minute guided meditation available as a free app in several regional languages in the Apple and Android stores [8]. Patients in the isha kriya arm were instructed to perform the kriya twice daily (at least once daily) from pre-HCT to day +30 post-HCT. The nursing team ensured adherence to the kriya at least once daily while the patients were admitted and during once-weekly outpatient visits or phone calls after discharge from the transplantation ward.

The study's primary endpoint was the QoL summary score at day +30 post-HCT as assessed by the Functional Assessment of Cancer Therapy-Bone Marrow Transplantation (FACT-BMT) and the Patient-Reported Outcomes Measurement Information System Global Health (PROMIS-GH) questionnaires. The secondary endpoints were the QoL domain scores at days +30 and +100 post-HCT and summary score at day +100 post-HCT. The QoL questionnaires FACT-BMT version 4 [9] and PROMIS-GH Scale version 1.2 (mental and physical) [10] are validated in Indian languages and were self-administered on paper at baseline before the intervention (pre-HCT) and at days +30 and +100 post-HCT.

The sample size of 36 patients per arm was determined based on an assumption of an actual difference in the experimental and control means of 10 with a standard deviation of 15, a type I error probability of .05, power of 80% [5]. The analysis of endpoints was done on an intention-to-treat basis. Domain and summary scores were calculated for each instrument as recommended by the developers. The QoL scores were compared between the 2 arms using the unpaired *t* test. A P value $< .05$ was considered to indicate statistical significance, and Cohen's d effect size was used to determine clinical significance.

RESULTS

A total of 90 HCT recipients were screened for eligibility over the study period, of whom 72 were randomized 1:1 to the isha kriya arm or control arm (Figure 1). There were 4 deaths in the isha kriya arm and 2 deaths in the control arm before day +100; these patients were included in the analysis, which was done on an intention-to-treat basis.

The median patient age was 32.5 years (interquartile range [IQR], 27 to 49 years) in the isha kriya arm and 31 years (IQR, 22 to 49 years) in the control arm (P = .6) (Table 1). There was a male predominance in both arms (61% versus 64%; P = 1.0). The proportions of patients who underwent HCT for myeloma (28% versus 31%), lymphoma (28% versus 31%), and leukemia (36% versus 30%) were similar in the 2 arms (P = 1.0), and were the proportions of patients undergoing autologous HCT (53% versus 56%) and allogeneic HCT (47% versus 44%; P = 1.0). There were no differences between the 2 arms in the pre-HCT QoL domain (physical, social, emotional, functional well-being, and bone marrow transplantation [BMT] subscales), summary (FACT trial outcome index [TOI], General [G] total, and BMT total) scores, and global (mental and physical) scores (Table 2). At post-HCT day +30, there also were no differences between the arms in the mean FACT-BMT total score (112.9 $\pm$ 16.8 versus 101.2 $\pm$ 13.9; P = .2) or mean global health scores (global mental health, 45.1 $\pm$ 8.6 versus 42.5 $\pm$ 7.2 [P = .5], global physical health, 44.1 $\pm$ 6.3 versus 44.1 $\pm$ 8.3 [P = .4]) and no differences in the physical, social, emotional, and functional domain scores. However, the mean BMT subscale score was statistically significantly higher in the isha kriya arm compared with the control arm (27.9 $\pm$ 5.1 versus 24.4 $\pm$ 9.2; P = .03). The difference was clinically significant, with a Cohen's d of .5 implying a medium effect size. The mean difference of 3.5 points also was clinically meaningful within the 10-item BMT subscale [9]. The FACT-TOI, which is a sum of physical, functional and BMT subscale scores, was nonsignificantly higher in the isha-kriya arm at day +30 (66.2 $\pm$ 16.3 versus 61.5 $\pm$ 18.6; P = .3). The FACT-G total score, which is a sum of the physical, social, emotional, and functional scores, also was nonsignificantly higher in the Isha-kriya arm at day +30 (84.9 $\pm$ 13.4 versus 76.8 $\pm$ 23.1; P = .09). This effect of isha-kriya on the BMT subscale was transient, as the day +100 scores showed no difference (28.3 $\pm$ 5.9 versus 26.2 $\pm$ 9.4; P = .3). There were no differences between the arms in the FACT-BMT total/domain scores or PROMIS global scores at day +100 post-HCT (Table 2).

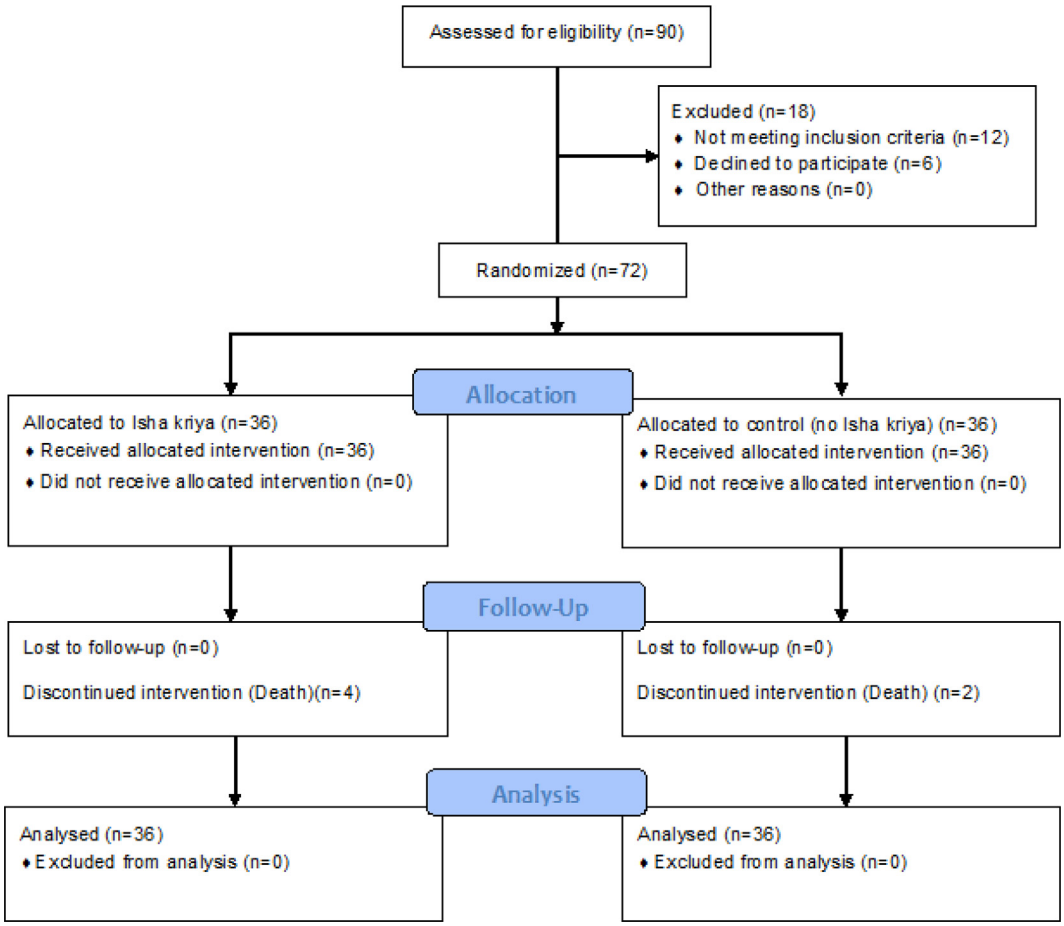

Figure 1. Enrollment, allocation, follow-up, and analysis of study participants.

DISCUSSION

Conducting RCTs of MBI in the HCT setting has several inherent challenges. Foremost are the choice of intervention and acceptance of and adherence to the prescribed intervention. The heterogeneity of tools for assessing QoL, distress, anxiety, and depression makes it difficult to compare studies and draw meaningful conclusions. In one of the largest RCTs conducted by the Blood & Marrow Transplant Clinical Trials Network, a mix of exercise and stress management training programs was found to be ineffective [5]. However, smaller RCTs either have been plagued by low intervention uptake [6] or have shown some benefit in QoL [4].

For this small RCT, we consciously chose a culturally acceptable meditation, isha kriya, which is based on the science of yoga and is available as a mobile app for self-guided practice. We also chose the Indian language validated FACT-BMT and PROMIS-GH tools to assess the domain and total scores for a comprehensive assessment of the impact of isha kriya. Although the practice of isha kriya for a short (1-month) period did not lead to differences between the study arms in

Table 1
Patient Characteristics

Characteristic	Isha Kriya Arm (N = 36)	Control Arm (N = 36)	*P* Value
Age, yr, median (IQR)	32.5 (27-49)	31 (22-49)	.6
Sex, n (%)			
Male	22 (61)	23 (64%)	1.0
Female	14 (39)	13 (36%)	
Disease, n (%)			
Myeloma	10 (28)	11 (31%)	1.0
Lymphoma	10 (28)	11 (31%)	
Leukemia	13 (36)	11 (30%)	
Aplastic anemia	3 (8)	3 (8%)	
Type of HCT, n (%)			
Autologous	19 (53)	20 (56%)	1.0
Allogeneic	17 (47)	16 (44%)	

Table 2
QoL Scores in the Isha Kriya and Control Arms

QoL Domain	Pre-HCT	Day +30 Post-HCT	Day +100 Post-HCT	Pre-HCT	Day +30 Post-HCT	Day +100 Post-HCT	*P* Value, Isha Kriya vs Control, Pre-HCT	*P* Value, Isha Kriya vs Control, Day +30	*P* Value, Isha Kriya vs Control, Day +100
Physical well-being	19 ± 6.4	19.7 ± 6.3	20.6 ± 5.7	19.6 ± 6.2	19.3 ± 7.8	20.7 ± 8.0	.7	.8	.3
Social well-being	24.1 ± 2.6	24.2 ± 3.5	22.9 ± 6.0	23.3 ± 4.2	21.5 ± 7.2	22.5 ± 8.1	.9	.1	.9
Emotional well-being	19.5 ± 3.8	21.1 ± 2.7	19.7 ± 6.2	18.3 ± 5.7	18.6 ± 6.5	18.1 ± 7.3	.7	.2	.3
Functional well-being	19.3 ± 6.2	19.9 ± 5.5	20.7 ± 6.6	19.3 ± 5.1	17.5 ± 7.1	18.7 ± 7.5	.9	.1	.3
BMT subscale	26.9 ± 6.9	27.9 ± 5.1	28.3 ± 5.9	26.7 ± 5.4	24.4 ± 9.2	26.2 ± 9.4	.8	.03	.3
FACT-BMT TOI	41.3 ± 13.0	66.2 ± 16.3	68.0 ± 18.0	41.1 ± 10.5	61.5 ± 18.6	64.2 ± 23.7	.9	.3	.7
FACT-G total score	81.9 ± 13.9	84.9 ± 13.4	82.7 ± 20.6	80.6 ± 16.3	76.8 ± 23.1	78.5 ± 27.9	.8	.09	.8
FACT-BMT total score	108.9 ± 19.5	112.9 ± 16.8	109.0 ± 29.3	107.3 ± 20.1	101.2 ± 13.9	103.9 ± 37.2	.7	.2	.8
PROMIS-global mental health	45.7 ± 7.3	45.1 ± 8.6	45.9 ± 7.3	44.6 ± 6.6	42.5 ± 7.2	43.8 ± 7.2	.7	.5	.4
PROMIS-global physical health	45.5 ± 6.4	44.1 ± 6.3	44.6 ± 7.8	46.8 ± 7.5	44.1 ± 8.3	46.1 ± 8.9	.6	.4	.3

Data are mean ± SD. Bold type indicates statistical significance.

the primary endpoint of FACT-BMT total and PROMIS-GH scores, there was a clinically meaningful difference in the FACT-BMT domain, which addresses BMT-specific QoL concerns. The FACT-BMT scale is sensitive to psychosocial interventions and allows the evaluation of treatment effects in a clinical trial [11]; however, in our cohort, the impact of the kriya was transient during the intervention period, with no difference seen at day +100 post-HCT. The PROMIS global health questionnaire contains questions overlapping with global assessment of the physical, social, emotional, and functional health subscales of the FACT-BMT questionnaire, possibly explaining the lack of differences in global scores.

A limitation of this study was the difficulty of ensuring adherence to the isha kriya practice twice daily. Another limitation was the lack of measurements of symptom burden and psychological outcomes, which are more likely to improve using MBI in cancer survivors [12,13]. The intervention period needs to be longer and could be continued at home after discharge to observe an effect in other QoL domains and global health [14]. Mobile health technology can be used to administer QoL interventions in cancer survivors [15]. It is also important to consider patient preference in the design of clinical trials [16] and suitable endpoints in subsequent RCTs to confirm the effect of MBI on QoL in HCT recipients.

In conclusion, this study demonstrates that the app-guided practice of isha kriya is feasible and acceptable for HCT recipients and shows promise in managing BMT-related stress and improving some aspects of the QoL of HCT recipients.

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Data availability statement**:** Data will be made available on reasonable request by contacting the corresponding author.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jtct.2023.05.010.

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Original Article

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Transplantation Efficacy of Human Ciliary Epithelium Cells from Fetal Eye and Lin-ve Stem Cells from Umbilical Cord Blood in the Murine Retinal Degeneration Model of Laser Injury

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Abstract

A number of degenerative conditions affecting the neural retina including age-related macular degeneration have no successful treatment, resulting in partial or complete vision loss. There are a number of stem cell replacement strategies for recovery of retinal damage using cells from variable sources. However, literature is still deficit in the comparison of efficacy of types of stem cells. The purpose of the study was to compare the therapeutic efficacy of undifferentiated cells, i.e., lineage negative stem cells (Lin-ve SC) with differentiated neurosphere derived from ciliary epithelium (CE) cells on retinal markers associated with laser-induced retinal injury. Laser-induced photocoagulation was carried out to disrupt Bruch's membrane and retinal pigmented epithelium in C57BL/6 mouse model. Lineage negative cells were isolated from human umbilical cord blood, whereas neurospheres were derived from CE of post-aborted human eyeballs. The cells were then transplanted into subretinal space to study their effect on injury. Markers of neurotropic factors, retina, apoptosis, and proliferation were analyzed after injury and transplantation. mRNA expression was also analyzed by real-time polymerase chain reaction at 1 week, and 3-month immunohistochemistry was evaluated at 1-week time point. CE cell transplantation showed enhanced differentiation of rods and retinal glial cells. However, Lin-ve cells exerted paracrine-dependent modulation of neurotrophic factors, which is possibly mediated by antiapoptotic and proliferative effects. In conclusion, CE transplantation showed superior regenerative outcome in comparison to Lin-ve SC for rescue of artificially injured rodent retinal cells. It is imperative that this source for transplantation may be extensively studied in various doses and additional retinal degeneration models for prospective clinical applications.

Keywords

subretinal, laser injury, lineage negative stem cells, ciliary epithelium cells, umbilical cord blood, retinal degeneration

Introduction

Degenerative conditions of the retina, viz. retinitis pigmentosa, age-related macular degeneration, diabetic retinopathy, glaucoma, etc., do not have any successful treatment to reverse the vision loss. Their widespread use in industrial, medical, and military fields has caused severe vision loss in a number of individuals[1]. Laser injuries are also a part of severe retinal damage, as a result of occupational eye injuries[2].

Cell-based therapies may provide treatment avenues for injuries as well as degenerative disorders by using

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reproducible laser injury models. Current ongoing stem cell work findings have also raised hopes of individuals suffering from such untreatable disorders[3–5]. Stem cells from different sources such as neural stem cells, retinal progenitors, hematopoietic, mesenchymal, embryonic, and induced pluripotent stem cells (iPSCs) have been studied for their differentiation potential into neuronal, retinal as well as glial cell lineages[3,6,7]. Stem cell therapies for targeting such chronic diseases may provide effective solutions. However, the field suffers from lack of comparative analysis of the various types of stem cells even though there is overemphasis on iPSCs[8] and embryonic stem cells[9] without any comparative studies between the two and/or other types of stem cells.

Recent studies show that stem cell transplantation at the site of neural injury or neuronal niche may facilitate differentiation into neurons, resulting in functional improvements in animal models[10,11]. Although the use of stem cells derived from umbilical cord blood (UCB) has been employed with several injury/disease models involving the central nervous system, it is limited as compared to the burgeoning number of cord banks being set up in the Asian countries[12]. The study tested the effect of UCB-derived stem cells in laser-induced injury in rabbit trabecular meshwork[13]. Similarly, the efficacy of transplantation of ciliary epithelium (CE) cells has not been adequately investigated let alone compared with other cell types despite repeated failure to regenerate the damaged or diseased retina. However, several groups have identified and continued with studies of transplantation of CE stem cells in retinal injury models[14,15].

In our previous study, we characterized human umbilical cord blood (hUCB)-derived lineage negative stem cells (Lin-ve SC) on the basis of morphology and cell surface marker expression[16]. We identified these cells under scanning electron microscopy and reported homogenous morphology, i.e., size, shape, and structure as compared to lineage positive and mononucleated cells. Lin-ve SC showed significantly increased expression of hematopoietic stem cells expression, i.e., CD117 and CD34, which ameliorated amyloid-induced memory loss upon transplantation in mouse model. The presence of CD34+ and CD117+ in Lin-ve SC showed 99% positive expression of CD45 marker[17]. These cells exerted neurotrophic factors mediated paracrine effects causing anti-apoptotic activity and amyloid clearance by the activation of astrocytes[18,19].

As lasers have been used for creating the models of retinal degeneration in fish, rodents, and primates[20,21], we established a modified animal model, injuring the Bruch's membrane and the surrounding retinal pigment epithelium (without causing choroidal neovascularization). CE cell–derived neurospheres were cultured under in vitro conditions using recombinant fibroblast growth factor (rhFGF) and recombinant epidermal growth factor (rhEGF), which showed increased size and number on sixth day of culture[22]. These neurospheres were dissociated and transplanted in the subretinal space of mice after laser injury. This resulted in marked increase in neurotrophic marker expression.

This study describes the comparative efficacy of differentiated human fetal CE-derived neurospheres and undifferentiated Lin-ve hUCB-derived stem cells, in the subretinal space of laser-induced retinal injury mouse model. We wanted to understand which source provides effective outcomes. Our results showed that the transplantation of CE cells presumably differentiated it into rods and retinal ganglion cells (RGCs) after migration from subretinal space. However, Lin-ve SC rescued the retinal damage by inducing neurotrophic factors, which may exert antiapoptotic and proliferation activity. Hence, we conclude that differentiated (CE cells) cells can be a superior source for cell transplantation therapy in comparison to undifferentiated (Lin-ve SC) cells after laser injury.

Materials and Methods

Animals

C57BL/6 J syngeneic mice ($N = 3$ per group) of 6- to 8-week-old male were used in the study after the approval from Institutional Animal Ethics Committee (IAEC), Post Graduate Institute of Medical Education and Research, Chandigarh, India [71(69)/IAEC/423]. Animals were kept in animal house facility in a 12-h light/dark cycle (LD 12:12). Mice were fed with standard chow diet and access them freely to clean drinking water.

Laser-Induced Retinal Injury in Mouse Model

The laser-induced retinal injury was established using Argon green laser (532 nm, Iris Medical, USA). The C57 mouse was anesthetized with xylazine hydrochloride (10 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) and ketamine hydrochloride (100 mg/kg) (Nirlife, Sachana, Gujarat, India) in 1:10 ratio. Mice were kept over heating pad to prevent cold cataract. The local anesthesia, i.e., lignocaine solution, was given to the cornea and 1% tropicamide solution (Akums Drugs and Pharmaceuticals Ltd, Haridwar, Uttarakhand, India) applied for pupil dilation. The fundus shots were obtained through slit lamp by placing the anesthetized mice in front of laser photo-coagulator (IRIDEX, Mountain View, CA, USA). Eight even laser spots in circular fashion were subjected around optic disk in both the eyes. The standardized parameters used in laser photocoagulation were spot size of 100 μm, power of 200 mW, and duration of pulse of 100 ms. Sham control group was operated for subretinal injection without injecting any liquid/vehicle.

Isolation of Lin-ve Stem Cells from hUCB for Subretinal Transplantation

Human UCB samples were obtained after the approval from Institutional Committee for Stem Cell Research and Therapy (IC-SCRT: Approval No. PGI-ICSCRT-53-2014/1469), Post Graduate Institute of Medical Education and Research, Chandigarh-India. Pregnant women of age 20–35 years and

≥28 weeks of gestation period were included in the study. UCB was taken from the umbilical cords of newborns after filling proper informed consent, in the presence of an independent witness. The collected UCB was layered over histopaque solution (Sigma-Aldrich) for enrichment of MNCs using density gradient centrifugation. These MNCs were then subjected to magnetic associated cell sorter (MACS) (Miltenyi Biotech, Bergisch Gladbach, Germany) for the isolation of Lin-ve SC using human Lin-ve isolation kit (Miltenyi Biotech). The kit works on the basis of biological affinity of biotin and streptavidin. The primary antibody cocktail consisted of biotinylated monoclonal antibodies, which include CD123, CD235a (Glycophorin A), CD56, CD19, CD16, CD15, CD14, CD11b, CD3, and CD2.

MNCs were suspended in MACS-BSA buffer and 10 µl of biotin antibody cocktail was added per 107 cells. These were incubated for 15–20 min at 4–8°C and tapped/gently agitated regularly. The cells were then incubated with the second solution, i.e., streptavidin coated magnetic microbeads. Twenty microliters of these microbeads per 107 cells was added and incubated again with gentle tapping for 20–30 min at 4–8°C. The cells were then diluted with 1–2 ml of the buffer, mixed well, and washed twice at 1,500 rpm for 5 min at 4°C. Supernatant was completely pipetted out. Up to 10^8 cells were suspended in 500 µl of freshly made PBS/MACS-BSA buffer. The cells were then subjected to magnetic separation. The magnetic separation unit along with magnetic separation column was kept in a laminar hood and the column was washed with appropriate amount of buffer solution twice. This was shifted to a fresh collection tube, and the cell suspension was passed through the column at a very slow and steady rate, taking care that there are no air spaces/bubbles inside the column. One milliliter of the buffer was then passed through the column twice to completely elute the cells of interest. Turbid white suspension was obtained as flow through.

Ciliary Epithelial Isolation and Culture

Human fetal eye globes were obtained from abortus after legal termination of pregnancy up to 20 weeks of gestation in accordance to ethical guidelines approved by IC-SCRT (Approval no. PGI-ICSCRT-53-2014/1469). The samples were collected from the terminations at the MTP-OT, SLR, and the Emergency-OT, PGIMER, Chandigarh.

Appropriate informed consents were obtained for every donor, on a prescribed consent form, and all the detailed procedures and objectives related to sample collection were explained to them. All the donors were screened and the following conditions were noted before collection of eye samples:

Inclusion Criteria. The samples were obtained of mid-trimester and up to 20 weeks of fetal abortions. This was on suggestion from the experts at both Department of Obstetrics and Gynecology and the Institutional Committee on Stem Cell Research (IC-SCR), PGIMER, Chandigarh.

Exclusion Criteria.

- Hepatitis virus B and C
- Human immunodeficiency virus
- Samples bearing congenital abnormalities
- Any malformation of fetus effecting head
- Evidence of chorioamnionitis (fever, foul smell liquor)
- Intrauterine fetal death

The fetal eyes were carefully enucleated immediately after abortion and transported in an ice cold sterile Hanks' Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY, USA). The surgical procedure was performed under stereozoom microscope (Leica EZ4, Leica Microsystems, Wetzlar, Germany) in order to isolate CE. Eye was held with forceps and a cut at the anterior edge of pars plana was made in order to carefully get the strip of ocular tissue containing CE. Ciliary rings were isolated and washed with sterile HBSS without taking nonpigmented epithelium, retinal pigmented epithelium (RPE), iris as well as retina. CE was dissociated first mechanically using blade and trypsinized using 4–5 ml of 0.25% trypsin (Gibco, Life Technologies, Burlington, ONT, Canada) with ethylenediaminetetraacetic acid at 37°C for 20–30 min. The action of trypsin was further neutralized with advanced Dulbecco's modified Eagle's medium/F12 (Gibco). The unwanted debris was removed by 0.70 µm cell strainer (BD Biosciences, Discovery Labware, Durham, NC, USA) and then centrifuged at 800G for 10 min. The number of pigmented cells was estimated with hemocytometer (Hausser Scientific, Horsham, PA, USA) and also with automatic cell counter (Millipore, Darmstadt, Germany). Finally, 3,000 cells/well were cultured in 96-well plate using retinal culture medium containing advanced DMEM/F12 (Gibco), 2 mM L-glutamine (Gibco, Paisley, Scotland, UK), N2 supplement (Gibco, Life Technologies), 100 U penicillin–streptomycin (Gibco), and fungizone (Gibco, Paisley, Scotland, UK). The cells were provided with proliferative growth factors, i.e., rhEGF (20 ng/ml; R&D Systems, Minneapolis, MN, USA) and rhFGF basic (20 ng/ml; R&D Systems). The size, shape, and structure of culture neurospheres were estimated using Image J software. The sixth day CE cultured neurospheres were harvested for subretinal transplantation in laser injured retina of mouse.

Transplantation of hCE-Derived Neurospheres and hUCB Lin-ve Stem Cells

We labeled our transplanted cells with fluorescent dye–carboxyfluorescein succinimidyl ester (CFDA-SE) (Sigma-Aldrich) in order to track the Lin-ve SC population and the CE-derived neurospheres after transplantation. For CFDA

Table 1. List of Antibodies Used in Immunohistochemical Experiments Real-Time PCR.

Antibody		Make	Dilution
Primary antibodies			
1.	Rhodopsin	Santa Cruz Biotechnology, USA	1:100
2.	Thy1	Santa Cruz Biotechnology, USA	1:100
3.	BDNF	Santa Cruz Biotechnology, USA	1:100
4.	CNTF	Santa Cruz Biotechnology, USA	1:100
Secondary antibodies			
1.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200
2.	Cy3 conjugated Donkey α Mouse	Jackson Immunoresearch, USA	1:200
3.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200
4.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200

BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; PCR: polymerase chain reaction.

staining, cells were counted and 50,000 were suspended in 1 µl of PBS, then transplanted after 24 h of injury through transcorneal subretinal route. The endpoint analysis was carried out at 1 week and 3 months after transplantation. The subretinal injection in mice was performed under surgical microscope after giving anesthesia. The prick was made at the cornea–scleral junction with beveled 31G insulin needle and pressure was released without injuring the cornea. A microsyringe (EXMIRE, Fuji, Shizouka, Japan) of 33G was introduced through the pricked area and moved behind the lens and moved till it touched the retina without exerting pressure on it. One milliliter suspension of 50,000 cells was injected carefully and bleb formation was observed. This served as a marker for successful subretinal delivery.

Immunohistochemistry

The eye balls were enucleated after sacrifice and frozen at −80°C and cryosectioned (Leica Cryostat, Buffalo Grove, IL, USA). Immunohistochemistry was performed to analyze the protein expression changes in the retinal layers viz. rhodopsin (Santa Cruz Biotechnology, Dallas, TX, USA), Thy1 (Santa Cruz Biotechnology), brain-derived neurotrophic factor (BDNF; Santa Cruz Biotechnology), and ciliary neurotrophic factor (CNTF; Santa Cruz Biotechnology). The sections were fixed using HistoChoice (Sigma-Aldrich) and then incubated with primary antibody (1:100 dilution) at 4°C overnight. Next day, the sections were kept in Cy3 labeled secondary antibody solution (1:200 dilution) (Jackson Immunoresearch, West Grove, PA, USA) for half an hour and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) (1:1,000 dilution) (Table 1). The sections were subsequently imaged under a confocal microscope to analyze the protein expression.

Real-Time Polymerase Chain Reaction

Relative fold change in the expression of the mRNA levels was detected using real-time polymerase chain reaction (PCR). The RNA was isolated from the dissected retinae of the enucleated eye balls, and converted to cDNA libraries, using standard kit protocols (Qiagen, Velno, The Netherlands and Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed using primers for rhodopsin (Eurofins, Bangalore, India), Thy1 (Eurofins), BDNF (Sigma-Aldrich, New Delhi, India), CNTF (Sigma-Aldrich, New Delhi, India), BCl2 (Eurofins), and Ki67 (Eurofins). β-Actin (Sigma-Aldrich, New Delhi, India) was used for an endogenous housekeeping control (Table 2). Expression levels were quantified and analyzed using StepOne, Applied Biosystems real-time PCR software (Thermo Fisher Scientific).

Statistical Analysis

Data were analyzed by calculating mean ± standard error of the mean, and normality of data was analyzed using 1-KS sampling test. The data were analyzed by one-way analysis of variance (ANOVA) followed by LSD and Scheffe and Dunnett's test for post hoc analysis. Statistical analysis of results was analyzed using 16.0 version of SPSS. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ were considered as statistically significant.

Results

CE Cell Transplantation Enhances the Formation of Rod Cells

We used laser-induced retinal injury mouse model without causing CNV. For this, we targeted eight shots in a circular pattern around the optic disc. These shots were focused at the Bruch's membrane, which resulted in the damage to RPE and caused the breach of blood retinal barrier. We validated this model using fundus fluorescein angiography showing leakage in the retinal blood vessels as well as electroretinogram that showed changes in the wave pattern[22]. RPE plays crucial role in providing nutrients to the retina and phagocytosis of photoreceptor's outer segment for its renewal[23]. Therefore, we wanted to analyze the rhodopsin expression, a pigment that makes up rod photoreceptors. The immunohistochemistry for its expression showed significant reduction

Table 2. List of Genes Analyzed and Their Primer Sequences Used for Real-Time PCR.

Sl no.	Gene	Primer sequence	
1.	Rho (mouse)	Forward	5′-CAGTACTCGGAATGCAGCAA-3′
		Reverse	5′-CAGTCTTCAGGGGCTCTGTC-3′
2.	Thy1 (mouse)	Forward	5′-ATTCAGGCCTGCCGGGGTAC-3′
		Reverse	5′-AGTTCTTTCGTGAGCATGGA-3′
3.	BDNF (mouse)	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
4.	CNTF (mouse)	Forward	5′-GCGAGCGAGTCGAGTGGTTGTCTG-3′
		Reverse	5′-TTAGCTTTCGGCCACCAGAGTGGAGAATTC-3′
5.	Ki67 (mouse)	Forward	5′-CAGTACTCGGAATGCAGCAA-3′
		Reverse	5′-CAGTCTTCAGGGGCTCTGTC-3′
6.	Bcl-2 (mouse)	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′

BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; PCR: polymerase chain reaction.

in rhodopsin levels in mice injured with laser, in comparison to the control mice. We then transplanted two types of cells, i.e., sixth day dissociated neurospheres differentiated from CE cells or undifferentiated Lin-ve SC derived from human UCB. The protein expression of rhodopsin at 1 week was found to be significantly higher in the retina transplanted with CE differentiated cells in comparison to the group transplanted with Lin-ve SC (Fig. 1A, B). The mRNA expression of rhodopsin was also estimated at 1 week and 3 months after transplantation. We found increased expression of rhodopsin in CE cells transplanted group as compared to Lin-ve SC transplanted group (Fig. 1C).

Differentiated CE Neurospheres Increase the Retinal Ganglion Cells (RGCs) of Retina

RGCs are a type of neurons present on the inner layer of retina. They transmit the visual stimulus at photoreceptor layer by engaging two types of neurons, i.e., bipolar and amacrine cells[24]. As rhodopsin was found to be significantly altered by laser-induced injury therefore, we wanted to analyze the effect of injury as well as transplantation of Lin-ve SC on RGCs. We found reduction of Thy1 protein expression after laser-induced retinal injury. However, transplantation of CE cells induced the upregulation of Thy1 expression (in comparison to control and laser injured mice) evident from quantitative analysis of immunohistochemical staining carried out at 1 week after transplantation (Fig. 2A, B). The mRNA expression of Thy1 showed significant increase after Lin-ve SC transplantation at 1 week, but this expression was abolished significantly when analyzed at 3 months after transplantation (Fig. 2C).

Neurotrophic Modulation by Paracrine Effects Exerted by Undifferentiated hUCB Lin-ve SC Transplantation

The existing literature of stem cell transplantation suggests that these cells either differentiate or aid integration in the host tissues, while others argue that stem cells act by providing neuroprotection mediated by the release of paracrine factors[25]. In the light of paracrine effects, we analyzed the expression levels of BDNF as well as CNTF in all the groups. We report that BDNF levels were upregulated more by transplantation of undifferentiated Lin-ve SC as compared to differentiated CE cells, when analyzed at 1 week (Fig. 3A, B). The mRNA expression of BDNF was concomitantly found increased after Lin-ve SC transplantation (in comparison to laser injured mice at 1 week). However, this effect was again diminished when analyzed at a longer time duration, i.e., 3 months (Fig. 3C).

CNTF is a ciliary neurotrophic factor and regarded as a survival factor for various neuronal types[26]. We estimated CNTF by immunohistochemistry, using confocal microscopy (Fig. 4A). By quantitative analysis (by Image J software), we found significant CNTF expression in laser injured mice. CNTF protein expression was also found upregulated in mice transplanted with CE cells in comparison to Lin-ve SC transplanted mice (Fig. 4B). The mRNA expression of CNTF, analyzed by real-time PCR, showed significant increase in Lin-ve SC transplanted mice in comparison to all other groups at 1-week after transplantation. However, this expression was diminished after 3 months of transplantation (Fig. 4C).

hUCB Lin-ve SC Transplantation Enhances Antiapoptotic and Proliferative Activity in Laser Injured Retina

The stem cells are reported to exert paracrine effects by releasing neurotrophic factors upon transplantation. These neurotrophic factors act as a ligand and initiate cell signaling pathways. BDNF acts as a ligand for TrkB receptor and activates CREB transcriptional factor, further promoting cell survival and maintenance of neuronal cells[27]. Likewise, CNTF is known to initiate Jak-STAT pathway and provides antiapoptotic and proliferative effect[28]. Bcl2, a well-known antiapoptotic marker, acts as a downstream molecule of CNTF initiated pathway. We found significant upregulation

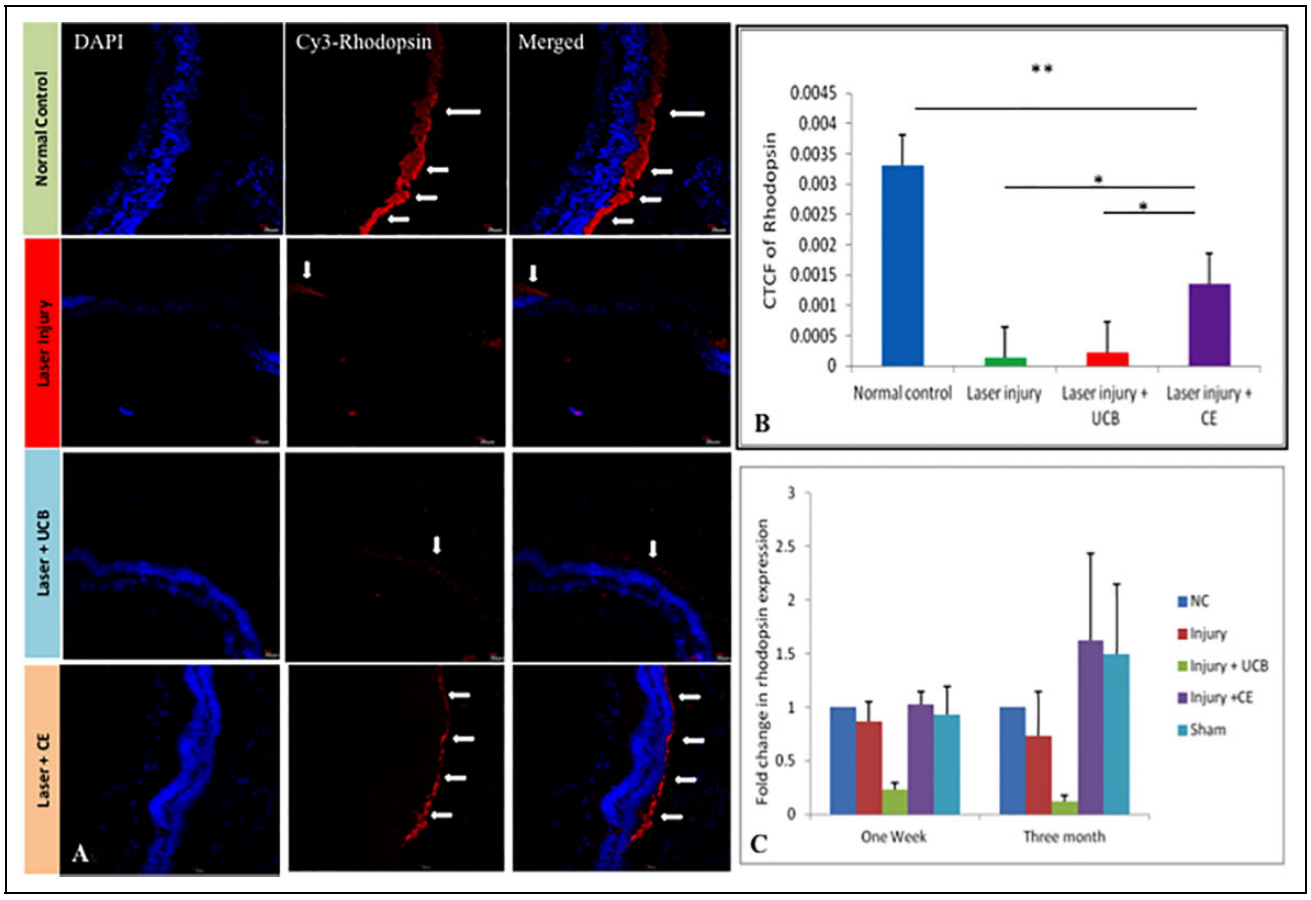

Figure 1. Differentiated CE cells transplantation enhances rod cells expression. (A) Immunohistochemistry of rhodopsin showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured retina mice, laser injured transplanted with Lin-ve SC mice, and laser injured transplanted with CE cells mice. (B) Quantitative protein expression of rhodopsin at 1-week time point measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of rhodopsin in all four groups was analyzed using real-time PCR at 1 week and 3 months after transplantation. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post-hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$ and **$P \leq 0.01$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

of Bcl2 mRNA expression after Lin-ve SC transplantation in laser injured mice in comparison to all the groups of 1 week and 3 months after transplantation. However, Bcl2 expression remained unaffected at 3 months after cells transplantation (Fig. 5A).

As proliferation markers are routinely analyzed in stem cell therapies, Ki67, a nuclear antigen of proliferation[29], was found highly upregulated upon transplantation of hUCB-derived Lin-ve SC (as compared to other groups at 1 week after transplantation). However, Ki67 expression was unaltered in all the groups at 3 months after transplantation (Fig. 5B).

Discussion

The series of degenerative changes in retina are due to diseased state with certain injuries resulting in irreversible damage. The currently used drugs have potential for symptomatic relief without halting the disease progression. Our data provide the comparative outcomes of transplantation of differentiated versus undifferentiated cells by utilizing a reproducible model of laser injured mouse retina. We wanted to examine the role of candidate markers of retinal repair and proliferation by transplantation of stem cells isolated from different origins. For that purpose, human eye from abortus fetuses was used to harvest the CE sourced stem cells. Second, we purified the Lin-ve SC from hUCB, a richly harvested source of undifferentiated stem cells.

Retina is an accessible and well-studied part of central nervous system. The retinal cellular structures have been studied in detail[30,31]. The existing studies provide evidence of the existence of stem cells in rodent retina. CE is one of the sources of stem cells shown as a rich source of cells with tremendous differentiation potential[15]. It has also been shown to possess the capacity to differentiate into specific retinal cell lineages for better

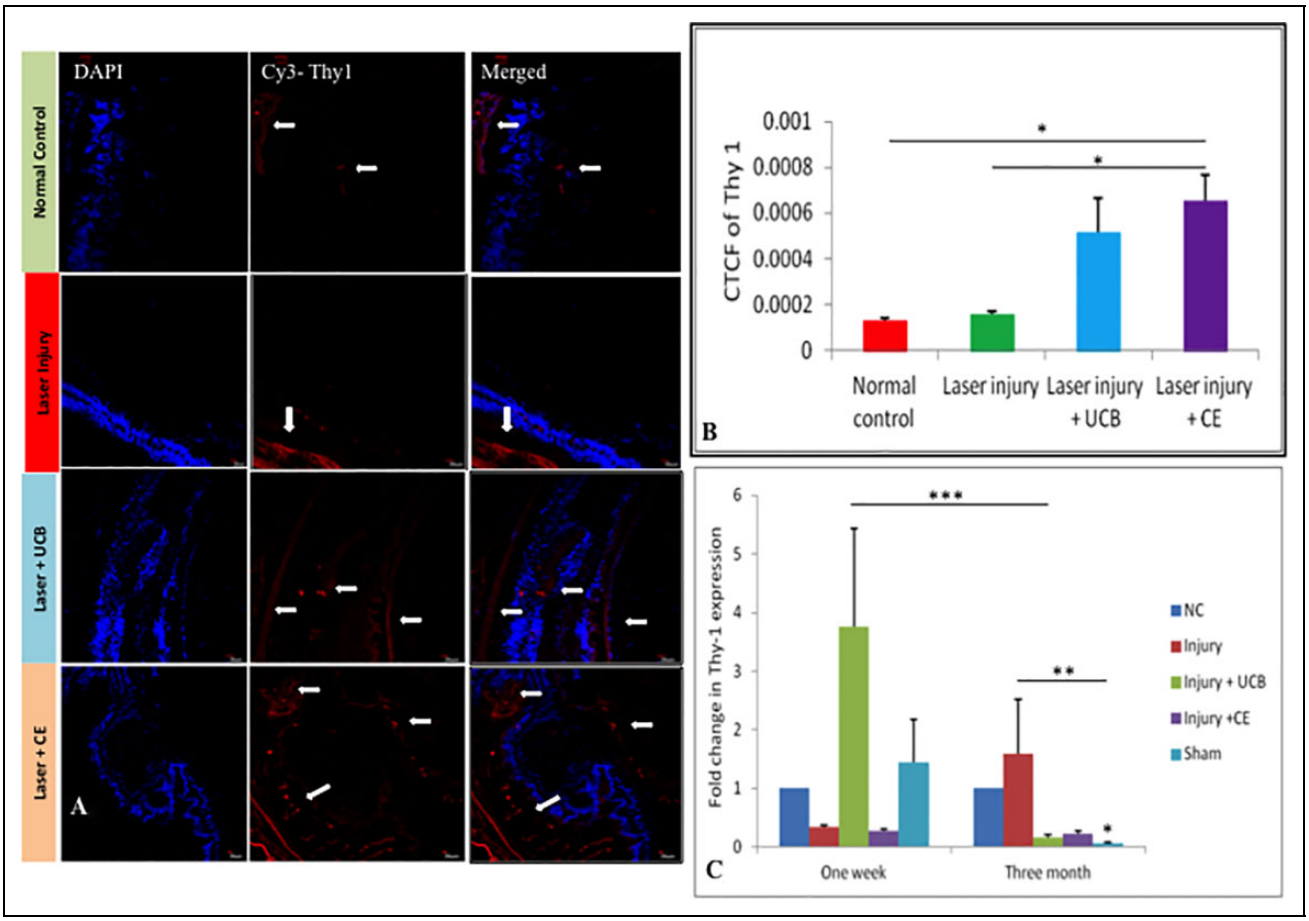

Figure 2. Differentiated CE cell transplantation enhances expression of retinal ganglion cells. (A) Immunohistochemistry of Thy1 showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control mice, laser injured retina mice, laser injured transplanted with Lin-ve SC mice, and laser injured transplanted with CE cells mice. (B) Quantitative protein expression of Thy1 at 1 week after transplantation was measured by corrected total cell fluorescence of immunohistochemistry images using Image J software (C). mRNA expression of Thy1 in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

integration into host tissue because of a similar niche. The pigmented CE has been shown to result in BrdU incorporation when provided with mitogens in vivo along with increase in cyclin D1 and Ki67 expression[32]. These cells, when stimulated with growth factors, rhEGF and bFGF, have been shown to form self-renewing colonies expressing retinal progenitor marker, i.e., Chx10[33]. Therefore, we used neurospheres that were differentiated from CE cells from human fetal eyes to examine how the stem cell markers are altered in laser-induced retinal injury in comparison to other cell types. We subretinally transplanted sixth day dissociated neurospheres into the laser-induced injury in retina of the mouse model. A significant expression of rhodopsin and Thy1 was reported in the mice retina after 1 week of transplantation (Figs. 1, 2). These data suggest that CE cells are involved in the rescue of retinal injury by activating the progenitor cells residing in the retina, which aid their differentiation into rods and RGCs. In comparison, CE cells are better positioned in the repair by activating the retinal markers and exerting neurotrophic effects superior to Lin-ve SC.

It has been shown that mesenchymal stem cells derived from hUCB exert neuroprotective effects by secreting several trophic factors including TGFbeta-1, NT-3, BDNF, and CNTF. These stem cells showed repair and regeneration of damaged neurons when transplanted in rat optic tract model[34]. Further, studies have shown transplantation of these cells intravenously in a neonatal hypoxic ischemia induced injury rat model could recover motor abilities mediated by neurotrophic factors, i.e., GDNF, BDNF, and NGF[35]. Endogenous cells secrete these neurotrophic factors mediated by paracrine effectors of transplanted cells. These

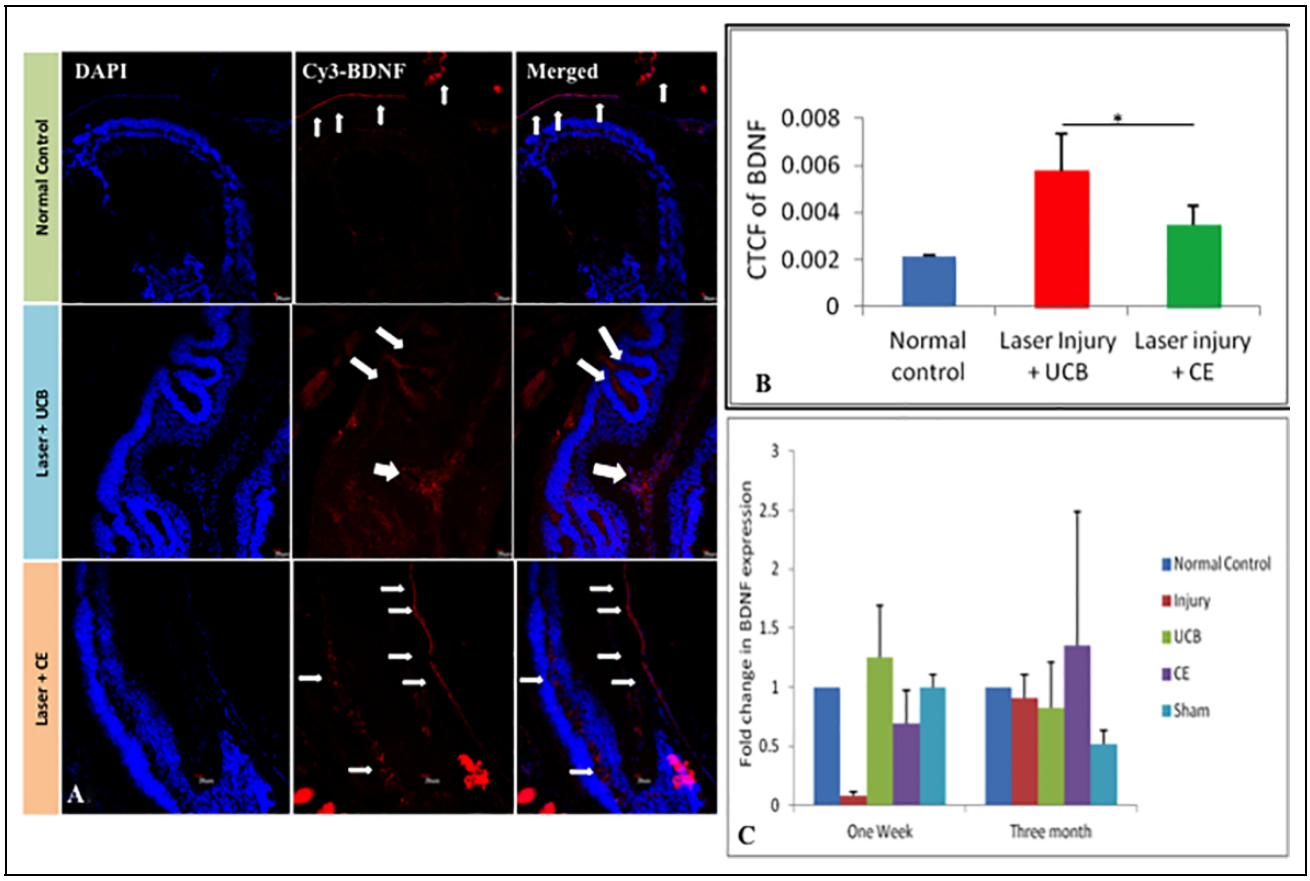

Figure 3. Neurotrophic factor modulation by the Lin-ve SC transplantation. (A) Immunohistochemistry of BDNF showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured mice transplanted with Lin-ve SC, and laser injured mice transplanted with CE cells. (B) Quantitative protein expression of BDNF at 1-week time point measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of BDNF in all four groups was analyzed using real-time PCR at 1-week as well as 3-month time point. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$ was regarded as statistically significant. ANOVA: analysis of variance; BDNF: brain-derived neurotrophic factor; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

trophic factors generally play an active role in neuronal development and survival by promoting proliferation, regeneration, and maturation of neurons[32]. The administration of trophic factors, e.g., CNTF, BDNF, and NT-4 has resulted in prevention of ON-injury-induced RGC death[36]. Other studies have described the effect of trophic factor administration (CNTF, BDNF, or PEDF) via intravitreal route on prevention of phototoxic-induced degeneration of photoreceptors cells[37]. Hence, such studies provide the necessary basis of studying the levels of these paracrine factors in transplantation studies. Our results that there is upregulation of BDNF after Lin-ve SC transplantation are consistent with other studies (Fig. 3).

Our results showed that there was significant enhancement in the expression of CNTF in the Lin-ve SC transplanted retina (when compared to the laser injured and CE cells transplanted group). CNTF is known to regulate via JAK/STAT pathway[28]. Bcl2 comprises the downstream effector molecule[38] and hence the upregulation of Bcl2 and CNTF mRNA (Figs. 4C, 5A). However, immunohistochemistry shows the shoot in the level of neurotropic factors in injured group, which are maintained sustainably by the CE injected group while there is not the similar sustenance in the UCB injected group. The dichotomy of CNTF mRNA and protein expression can be ascribed to post-translational differences in both the experimental and intervention groups. This can be further examined by using specific inhibitors of CNTF.

Ki67 is the universal molecule for investigating proliferation. It was found to have increased expression upon Lin-ve SC transplantation (Fig. 5B). The activity of the enhanced endogenous proliferation needs further elaboration. Therefore, our results unanimously reveal that hUCB-derived Lin-ve SC, when transplanted at the site of retinal injury, induce expression of neurotrophic factors, which may exert neuroprotective effect mediated by the antiapoptotic and

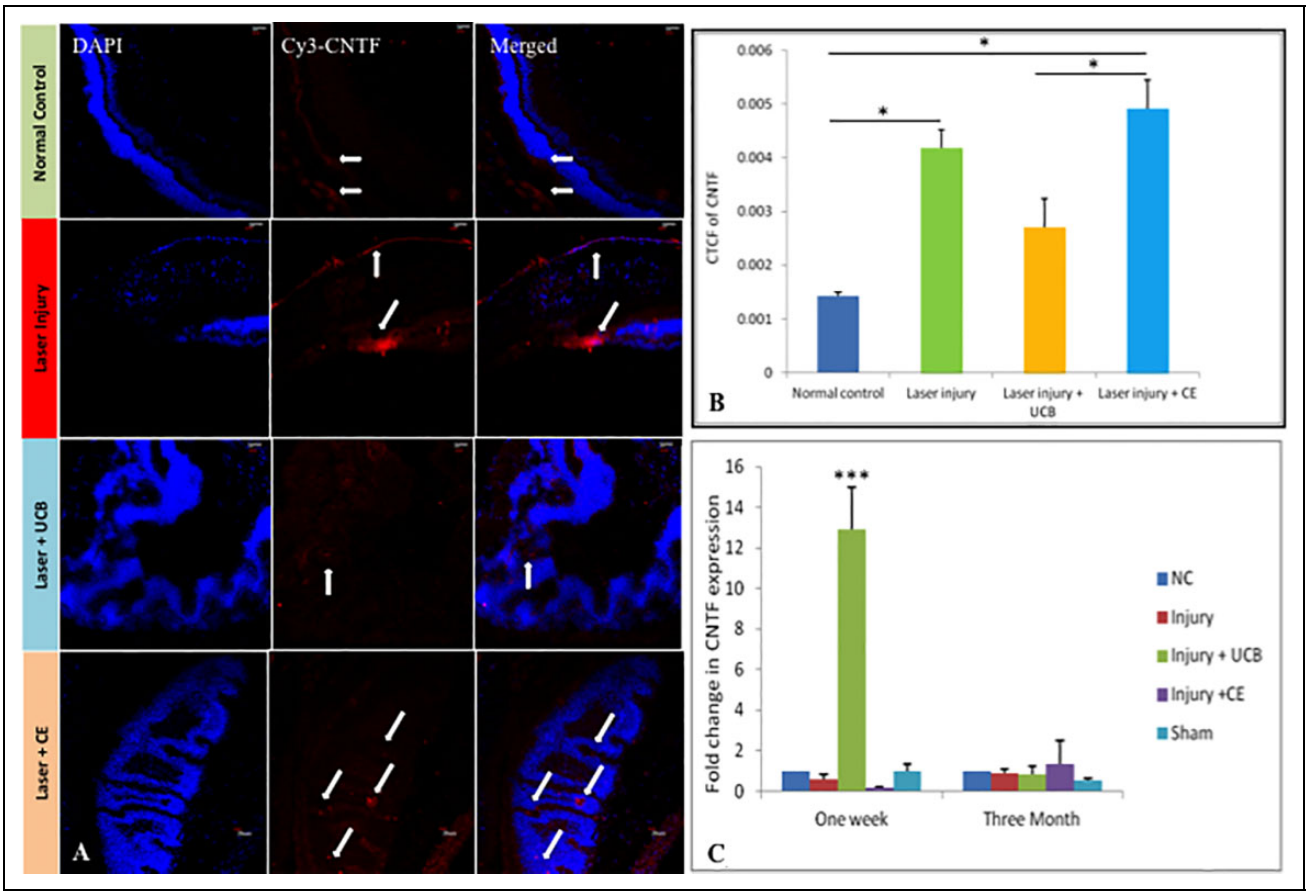

Figure 4. CE cell transplantation upregulated ciliary neurotrophic factor in the laser injured retina. (A) Immunohistochemistry of CNTF showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured mice, laser injured mice transplanted with Lin-ve SC, and laser injured mice transplanted with CE cells. (B) Quantitative protein expression of CNTF at 1-week time after transplantation measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of CNTF in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; CNTF: ciliary neurotrophic factor; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

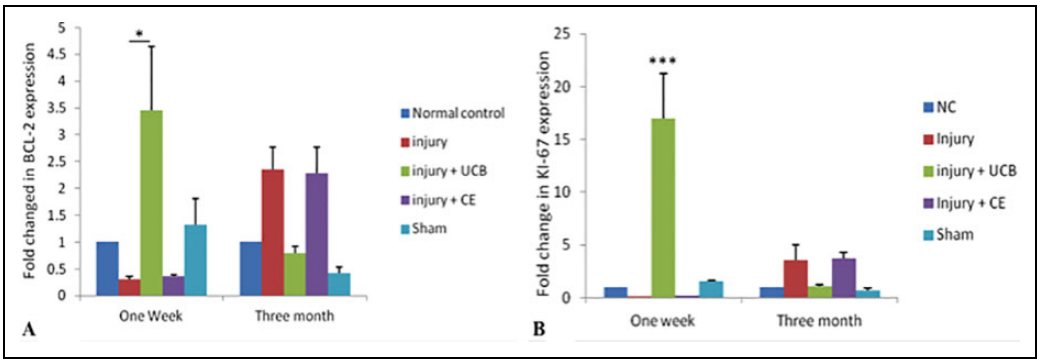

Figure 5. Antiapoptotic and proliferative activity enhanced by Lin-ve SC transplantation in laser injured retina. (A, B) mRNA expression of antiapoptotic marker Bcl2 and proliferative marker, i.e., Ki67 in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA for real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

proliferative mechanisms. Importantly, our study indicates the need for the multiple doses of Lin-ve SC transplantation instead of single dose as the paracrine effects noted in the study did not last for longer duration investigated.

Briefly, we explain the superior outcomes from transplantation of CE-related stem cells by ascribing it to same niche, i.e., retina providing conducive niche for rescue when compared to undifferentiated Lin-ve SC. In the context of noted paracrine effects, Lin-ve SC provide comparable outcomes but mediated by antiapoptotic and proliferative mechanisms. However, as Lin-ve SC effects lasted for a few weeks, it is suggested to examine the associated pathway at extended time frame using multiple doses of Lin-ve SC. Such studies will be helpful in utilizing the cord blood stored in the banks and for developing therapies for untreatable disorders of the retina. The main limitation of the study is that we were not able to perform immunohistochemistry of BDNF due to limited availability of eye samples.

Conclusion

The transplantation of CE cells showed superior outcomes than the undifferentiated Line-ve UCB-derived SC when tested in retinal injury mouse model. Based on the retinal marker analysis, we hypothesize that while the CE cells aid the formation of rods and ganglion cell layer, the Lin-ve SC transplantation promotes antiapoptotic and proliferative activity by neurotrophic modulation. We could not test the therapeutic outcome on the neuroprotective or paracrine effects from transplantation of multiple doses of Lin-ve SC or those at extended time points as the study was limited for a few weeks. Also, we have neither tested the per se effect of the transplantation of prominent markers on retinal repair nor evaluated the physiological changes. Hence, this study provides the rationale for more comprehensive analysis of the different sources of stem cells and their transplantation effects for extended duration. It is pertinent to note that clinical trial recently used RPE cells differentiated from autologous iPSCs and resulted in successful transplantation[39,40]. Our study thus provides preliminary data for future clinical application of the stem cell therapy for treating several retinal injuries.

Data Availability

All raw data are available with the first author of this manuscript.

Ethical Approval

Ethical approval for animals was obtained from Institutional Animal Ethics Committee (IAEC), Post Graduate Institute of Medical Education and Research, Chandigarh, India [71(69)/IAEC/423]. Human UCB samples were obtained after the approval from Institutional Committee for Stem Cell Research and Therapy, Post Graduate Institute of Medical Education and Research, Chandigarh, India (IC-SCRT: Approval No. PGI-ICSCRT-53-2014/1469).

Statement of Human and Animal Rights

All the experimental procedures were conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSEA) guidelines.

Statement of Informed Consent

The samples were obtained after filling the proper informed consent forms by participants after explaining the complete protocol.

Declaration of Conflicting Interests

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Neurotrophic Factors Mediated Activation of Astrocytes Ameliorate Memory Loss by Amyloid Clearance after Transplantation of Lineage Negative Stem Cells

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Abstract

Alzheimer's disease (AD) is one of the untreatable neurodegenerative disorders with associated societal burden. Current therapies only provide symptomatic relief without altering the rate of disease progression as reported by Lanctot et al. (Therapeutic Advances in Neurological Disorders 2 (3):163–180, 2009). The increased number of failed clinical trials in last two decades indicates the imperative need to explore alternative therapies for AD as reported by Tuszynski et al. (Nature Medicine 11 (5):551–555, 2005) and Liyanage et al. (Alzheimer's & Dementia 4:628–635, 2005). In this study, we aimed to decipher the role of neurotrophic factors in the reversal of memory loss by transplantation of lineage negative (Lin-ve) stem cells in a male mouse model of cognitive impairment induced by intrahippocampal injection of amyloid β-42 (Aβ-42). The efficacy of human umbilical cord blood (hUCB) derived Lin-ve stem cells were analyzed by neurobehavioral parameters, i.e., Morris water maze and passive avoidance after bilateral intra-hippocampal transplantation using stereotaxic surgery. Real-time PCR and immunohistochemistry was carried out in brain tissues in order to analyze the expression of neurotrophic factors, apoptotic, astrocytic, and other neuronal cell markers. The transplantation of Lin-ve stem cells led to reversal of memory loss associated with reduction of Aβ-42 deposition from the brains. The molecular analysis revealed increase in neurotrophic factors, i.e., glial derived neurotrophic factor (GDNF), ciliary derived neurotrophic factor (CNTF), and Brain-derived neurotrophic factor (BDNF) after transplantation. The administration of ANA-12, a TrkB inhibitor, reversed the behavioral and molecular effects of stem cell transplantation suggesting involvement of BDNF-TrkB pathway in the rescue of memory loss. We believe that the amyloid clearance results from activation of astrocytes and anti-apoptotic pathways added by neurotrophic factors.

Keywords Alzheimer's disease · Neurotrophic factor · BDNF · Umbilical cord blood · Lineage negative stem cells · Amyloid injury · Memory loss

Significance Statement
Umbilical cord blood (UCB) banking has increased manifold across the world without corresponding data generation for its preclinical testing and its efficacy. Since Alzheimer's disease (AD) is an untreatable neurological disorder and various drug trials have failed successively, this study unravels the preclinical efficacy of UCB-derived stem cells and underlying molecular mechanisms involved in rescue of amyloid pathology as well as memory loss. The data presented in this paper provides compelling evidence to show that UCB-derived lineage negative stem cells can upregulate BDNF which is the chief cause of rescue of memory loss caused due to artificially delivered Amyloid β in mouse model of brain injury. Hence, our study paves way for clinical trials for utilization of cord blood for the treatment of AD.

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Introduction

Alzheimer's disease (AD) is an untreatable neurodegenerative disorder which affects almost 29.8 million individuals worldwide [4]. It accounts for almost 60–70% cases of Dementia. Currently FDA-approved drugs, acetylcholinesterase inhibitors (donepezil) and NMDA receptor antagonists (memantine), provide symptomatic relief without relieving disease pathology [5–7]. In last decade, drugs that were found to be promising in pre-clinical studies by clearing amyloid load from AD mouse models, failed during clinical trials [8, 9]. Several factors which were argued to influence the negative outcomes in clinical trials, ranged from unavailability of suitable animal models to genetic and phylogenetic variabilities between human and rodents [10]. Hence, these failures call for reinvigorated efforts to test new biotherapeutic approaches in AD. Cellular therapies in pre-clinical studies have repeatedly shown potential disease modifying effect in AD models. For example, transplantation of various sources of stem cells has shown cognitive improvement in murine models of AD [11, 12]. Several reports including our previous studies have shown varying effects of stem cell transplantation. These therapeutic benefits could either include replacement or integration of degenerating neurons by stem cells aided by paracrine effects through release of neurotrophic factors, immunomodulation and migration [13–16]. However, there is a lack of clear understanding of underlying molecular mechanisms in the rescue of function effects of these cellular therapies.

In most of the recent findings, it is believed that transplanted stem cells exert non-redundant neuroprotective effects mediated by neurotrophic factors such as BDNF, GDNF, and CNTF [14]. Brain-derived neurotrophic factor (BDNF) is an essential neurotrophic factor which acts by BDNF-TrkB pathway for synaptic regulation [17]. In AD patients, lower levels of BDNF have been reported in postmortem brains [18] suggesting its critical role in the disease. Similarly, GDNF and BDNF levels have been found to be significantly reduced in serum of AD patients [19]. In transgenic (3xTg-AD) aged AD mice, transplantation of neural stem cells has been shown to result in improvement of cognitive dysfunction by increased hippocampal synaptic density mediated by BDNF [20].

The association of GDNF with AD is rarely studied; however, depletion of these factors appear to be linked to AD pathologies [21]. GDNF levels have been reported to be significantly low in serum of AD patients whereas in few studies, it has been concomitantly increased in the cerebrospinal fluid [22]. A dysregulated pattern of tissue expression of GDNF was found in postmortem middle temporal gyrus (MTG) of AD [23]. In 3xTg-AD mice, the downregulation of GDNF has been reported and this effect was ameliorated by 6-month voluntary exercise [24]. Furthermore, the overexpression of GDNF, using lentiviral vector for 6 months in 3xTg-AD mice, has been shown to improve learning and cognitive memory consolidation by upregulation of BDNF without alleviating amyloid pathology [25]. Interestingly, the interaction between BDNF induced TrkB signaling and GDNF-induced RET pathway has also been shown by inhibition of either TrkB receptor or RET using RNAi, which confirms their cross-talk [26].

Ciliary neurotrophic factor (CNTF) has also been shown to exert neuroprotective effect in the management of neurodegenerative disorders [27]. It is expressed in astrocytes of subventricular zone/dentate gyrus region in interface with neurogenic niche. Its receptors (CNTFRα) are present on neuronal progenitors as well as in hippocampal neurons. CNTF plays a fundamental role in hippocampal neurogenesis besides exerting its neurogenic effects in subventricular region [28, 29]. CNTF is known to regulate adult neurogenesis, enhance proliferation, and neuroblast formation mediated by dopaminergic activity [29]. The peripheral administration of peptide-6, which is an active region of CNTF in 3xTg-AD has shown enhanced neurogenesis in dentate gyrus and increased hippocampal neuronal plasticity which is possibly linked to cognitive improvement [30]. Expectedly, the localized delivery of recombinant cells secreting CNTF improved cognitive performance by stabilizing the synaptic proteins [31]. The various in vivo and in vitro studies suggest the dominant role of CNTF in astrocyte activation [32–36]. In this respect, it is pertinent to note that the insoluble Aβ plaque formation is one of the major symptoms of AD and astrocytes play a major role in its clearance from brain parenchyma into perivascular space [37]. Therefore, it is imperative that therapy targeting such putative neurotrophic factors may provide superior cognitive outcomes by alleviating AD pathology.

Our recent study showed that CD117 and CD34 stemness of hUCB Lin-ve stem cells reverse memory loss in mice intrahippocampally injected with aggregated Aβ-42 peptides [13]. We further aimed to investigate downstream molecular mechanisms underlying the therapeutic effect of hUCB Lin-ve stem cells transplanted in this mouse model. Our results reveal that the neurobehavioral improvement exerted by the Lin-ve stem cells was mediated by neurotrophic factors associated activation of anti-apoptotic pathway at the site of injury. This was evidenced by alteration of neurotrophic factors such as BDNF, GDNF, and CNTF in experimental mice after stem cell transplantation. Hence, we speculated that transplanted stem cells could trigger the release of endogenous neurotrophic factors and consequently alleviate Aβ-induced damage and perhaps delay apoptosis in AD brain causing neurobehavioral improvement.

Material and Methods

Study Design

The experimental procedures are supplementary to our previously published article [14]. Six to eight-week old Swiss albino male mice were used for experiment after approval from Institute Animal Ethical Committee (75/IAEC/473). Mice were anesthetized using intra-peritoneal injection of Xylazine Hydrochloride (50 mg/ml) and Ketamine Hydrochloride (50 mg/ml) in the ratio of 1:4. The memory loss was established by intra-hippocampal delivery of oligomeric form of Aβ-42. The Lin-ve SC were transplanted after 21 days of Aβ-42 induced injury. The memory was assessed by neurobehavioral analysis (Morris water maze and passive avoidance) 10-day post-transplantation. ANA-12 (1 mg/kg body weight) (Sigma) is an effective TrkB receptor inhibitor which causes cognitive impairment [38, 39]. It was intraperitoneally administered 1 h before the start of behavioral analysis daily from day 31 to day 41 in Aβ-42 injury + Lin-ve SC transplanted mice. Mice were sacrificed using high dose of xylazine and ketamine on day 41 after completion of all the behavioral analysis. The experimental timeline is shown in Fig. 1 (reused and modified from Parul Bali et al. 2018 with permission) [13].

Mouse Model of Amyloid Pathology and Memory Loss

Amyloid β (1-42) aggregates were prepared from 0.1 mg stock of Aβ-42 protein fragment (Sigma-Aldrich, USA) and incubated at 37 °C for 4 days and 4 °C for 6 h. Stereotaxic surgery was performed to inject 5 μL of amyloid solution into hippocampal region of mice brains. Mice were fixed over stereotaxis apparatus to restrict any head movement. Bilateral craniotomy was done using 26G needle after exposing the skull. Bregma zero was located and micro-syringe was placed at a specific axis for hippocampal delivery, i.e., anteroposterior (AP) + 2 mm, mediolateral (ML) ± 2 mm and dorsoventral (DV) − 2.5 mm. One micromolar of Aβ-42 was injected at a controlled speed of 1–2 μL/min and the needle was retained for 5–7 min for proper diffusion. The skin was sutured back and neosporin was applied for antiseptic. Mice were kept into a single cage and observed until they recovered. Similarly, 1xPBS was injected bilaterally for vehicle control.

Isolation and Purification of hUCB Lin-ve Stem Cells

hUCB Collection

hUCB collection was done in accordance with ethical guidelines as approved by Institutional Committee on Stem Cell Research (IC-SCR) (Approval no. PGI-IC-SCR-67-2015/1654). The blood was collected from umbilical cord with placental tissue of newborn deliveries (aged between 20 and 35 years) at gestation period of ≥ 28 weeks after obtaining patient's consent. The donors were screened and following conditions were excluded, i.e., hepatitis B infection, HIV infection, syphilis infection, untreated urinary tract infection, acute infection, unclean vaginal examination, fever, prolonged rupture of membrane (> 24 h), foul smelling amniotic fluid, and major congenital malformation in the new born.

The cord was clamped using mosquito forceps to prevent further blood loss. The vein of cord was located and blood was withdrawn using 21G needle with 50-ml syringe. Blood was immediately transferred into EDTA (anticoagulant)-

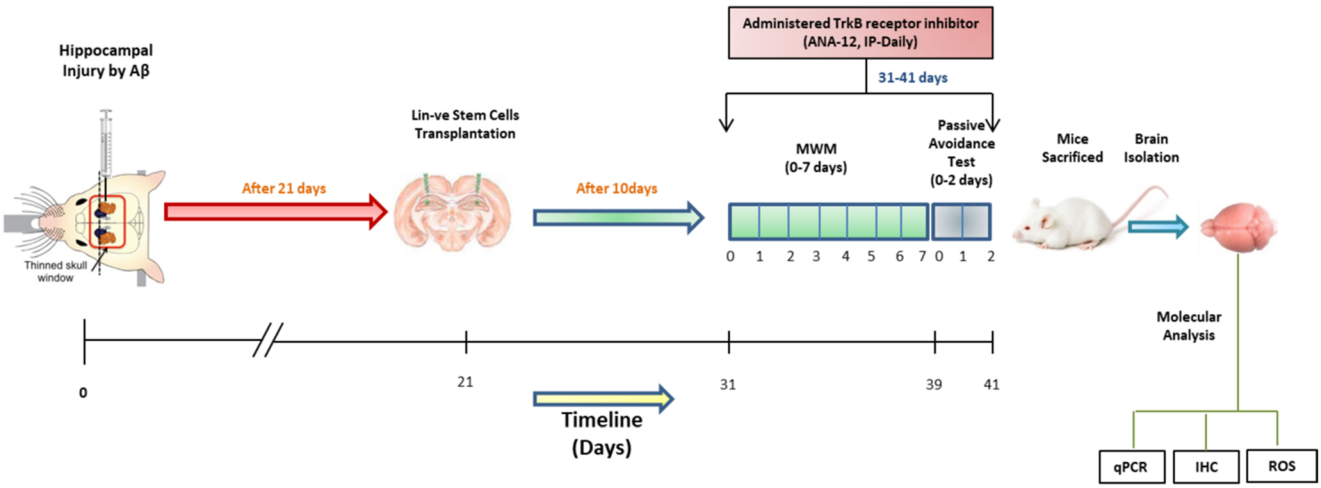

Fig. 1 Schematic representation of in vivo study design and timeline. The timeline of experiments starting at 0 day point with amyloid injury by stereotaxic surgery ending at the 41st day by sacrificing the mice for end-point analysis has been represented. After 21 days of amyloid injury, Lin-ve stem cells were transplanted in a treatment group and neurobehavioral analysis was carried out at 10-day post-transplantation. In another group, ANA-12 (TrkB Inhibitor) was administered during neurobehavioral analysis. The end-point analysis was done by real-time PCR, immunohistochemistry and ROS analysis

containing vials and transferred in ice for further processing for Lin-ve stem cell isolation.

Lin-ve Stem Cell Isolation

The hUCB was layered over the Ficoll Histopaque (Sigma-Aldrich, USA) in 1:1 ratio and subjected to density gradient centrifugation in a swing bucket centrifuge (REMI Lab. Instruments, India) at 1500 rpm for 30 mins for lymphocyte separation. The buffy coat containing lymphocytes appeared at ficoll plasma interface after centrifugation and was subsequently collected. It was mixed and washed with equal volume of MACS-BSA (1:50) buffer. The mononucleated cells (MNCs) were subjected to magnetic activated cell sorting (MACS) for Lin-ve cell enrichment using human Lin-ve isolation kit (Miltenyi Biotech, Germany). Lineage depletion kit contained biotinylated monoclonal antibodies for markers viz. CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A). MNCs were incubated with the antibodies cocktail and then to streptavidin coated magnetic microbeads. The cells were then subjected to magnetic separation by passing it through column under strong magnetic field. Lineage positive cells, i.e., committed to specific cell type remained in the column because of magnetic beads, whereas unbound Lin-ve SC passed through it. The enriched Lin-ve stem cells were then kept at 4 °C until transplanted into mouse model.

Labeling and Transplantation of Lin-ve SC

Lin-ve stem cells were labeled using CFDA-SE (Vybrant CFDA cell tracer kit, Invitrogen, USA) dye, which diffuses passively into the cells. It is a colorless dye which gives green fluorescence after the action of cellular esterase. Cells were incubated in 5–10 μM of CFDA-SE solution at 37 °C for 15 min. Cells were then washed with PBS to exclude excess dye from the cell suspension. CFDA-SE labeled 50,000 cells were transplanted in the intra-hippocampal region bilaterally using stereotaxic surgery. The recruited cells (CFDA tagged) were tracked under fluorescence microscope using FITC filter.

Behavioral Analysis

Morris Water Maze

The long-term spatial memory loss was assessed by Morris water maze (MWM) assay. MWM consisted of a circular water tank, artificially divided into four quadrants, with the target platform kept hidden in the quadrant. The protocol was executed for 7 days. It consisted of 6 acquisition days and one retrieval day (7th day). The mice were subjected to each of four quadrants and the time taken to locate the hidden platform was recorded. The mice behavior was assessed and recorded by video camera kept over the tank, which was connected to ANY-maze software. The escape latency of mice was estimated for six acquisition days and compared to amyloid injury group, stem cell-transplanted group and TrkB inhibitor injected group. On retrieval day (day7), the time spent by mice in each quadrant and the mean distance from the platform (i.e., search error) was measured.

The experimental design for MWM varies depending on the question we ask. In some studies, the animals are exposed to MWM after amyloid injection followed by therapeutic treatment in the same cohort [40]. Others train the mice first before injury. We used the first paradigm in order to examine the effect of stem cell transplantation on acquisition and retrieval in the Amyloid injured mice. We have used this well-standardized protocol in our previous studies.

Passive Avoidance Test

Passive avoidance is a fear motivated test used for short term memory assessment. Mice neurobehavior was contrary to their innate tendencies for disposition to dark chamber. In conditioning phase, mice were kept in light chamber and received mild foot shock of 20 mA for 2–3 s, as soon it entered the dark chamber. In the test phase, i.e., after 24 h, the latency to cross the door was calculated.

Molecular Analysis

Real-time PCR

cDNA was prepared from RNA extracted from test and control brains using RNeasy Lipid Tissue Mini kit (Qiagen). Real-time PCR was performed to estimate the expression of genes using primers (Sigma) for BDNF, CNTF, GDNF, GFAP, Bcl2, Caspase3, SV2A, Ki67, and VEGF in 96-well plate using StepOnePlus system (Applied Biosystems, USA). The expression and fold changes were analyzed using (Step one, Applied Biosystems) software and each sample was normalized to its β-actin expression. The details of primer sequences are listed in Table 1.

The list of markers analyzed in the study and their primer sequences used in real-time PCR.

Immunohistochemistry

The immunohistochemistry (IHC) of hippocampal region was carried out in the 6–7-μm-thick brain sections obtained from Cryostat (Leica CM 1510S) at − 24 °C. The sections for IHC were fixed using Histochoice (Sigma) and the blocking was done using 5% BSA (Sigma). Sections were incubated with anti-mouse primary antibodies in 1:100 dilutions at 4 °C, overnight. Sections were then incubated with rabbit or goat raised secondary antibodies in 1:200 dilution at room

Table 1 List of forward and reverse primers used for qPCR analysis

Sr. no.	Gene	Primer sequence	
1	GDNF	Forward	5′-TGGGCTATGAAACCAAGG-3′
		Reverse	5′-CAACATGCCTGGCCTACT-3′
2	GFAP	Forward	5′-ACAGACTTTCTCCAACCTCCAG-3′
		Reverse	5′-CCTTCTGACACGGATTTGGT-3′
3	SV2A	Forward	5′-GTCTTTGTGGTGGGCTTTGT-3′
		Reverse	5′-CGAAGACGCTGTTGACTGAG-3′
4	VEGF	Forward	5′-CTACTGCCGTCCGATTGAGAC-3′
		Reverse	5′-GGCTTGTCACATCTGCAAGTAC -3′
5	BDNF	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
6	CNTF	Forward	5′-GCGAGCGAGTCGAGTGGTTGTCTG-3′
		Reverse	5′-TTAGCTTTCGGCCACCAGAGTGGAGA ATTC-3′
7	Ki67	Forward	5′-CAGTACTCGGAATGCAGCAA −3′
		Reverse	5′- CAGTCTTCAGGGGCTCTGTC-3′
8	Bcl-2	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
9	Caspase3	Forward	5′-ATTCAGGCCTGCCGGGGTAC-3′
		Reverse	5′-AGTTCTTTCGTGAGCATGGA-3′

temperature for 30 mins. Nuclei were counterstained with fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) and slides were mounted with fluorosave reagent (Merck). The details of all the primary and secondary antibodies are listed in Table 2.

The details of primary antibodies used viz. BDNF, GDNF, CNTF, GFAP, Aβ42, and Caspase3 is mentioned which includes host, specificity, make, catalog no., and dilution.

Reactive Oxygen Species

Biochemical assay was done to estimate the levels of Reactive oxygen species. The hippocampus was dissected out from mouse brain and homogenized with 1×PBS. The homogenate was mixed with Dichlorodihydrofluorescein diacetate dye (DCFDA) from 3.05 mg stock prepared in 5 ml methane. By the action of cellular esterases and upon oxidation, it turns to fluorescent 2′,7′-Dichlorodihydrofluorescein. The readings were obtained in duplicates. The fluorescent intensity was measured at 488-nm excitation and 525-nm emission filters using Fluorimetry (BiotecK).

Statistical Analysis

The results were represented as mean ± S.E.M. Data was analyzed using GraphPad Prism 7.04. The normality of data was checked using 1-KS sampling. In MWM, the acquisition and retrieval data was analyzed by two-way ANOVA. Further, Tukey's test was used for post-hoc analysis in order to compare mean between days and groups. For passive avoidance and real-time PCR, data was analyzed by one-way ANOVA with Tukey's multiple comparisons test for post-hoc. For amyloid fluorescent intensity analysis, student *t* test was applied whereas for all the marker analysis by IHC and real-time PCR, one-way ANOVA with Tukey's test was applied. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$ were regarded as statistically significant.

Results

Aβ-42-induced Spatial Memory Loss Was Ameliorated by Transplantation of hUCB-derived Lin-ve Stem Cells

We used the Morris water maze (MWM) data from our recently published article (with permission from Bali et al. 2017) [13] in order to compare the effects of TrkB inhibitor on the effect of Lin-ve stem cell transplantation in amyloid β-42 injured mice. The injected oligomeric form of 1uM amyloid β-42 in the hippocampal region resulted in memory loss in Aβ-injected mice, as assessed by escape latency time (ELT) in MWM. ELT was significantly higher in amyloid β-42-injured mice when compared to sham control. The injured mice when transplanted with 50,000 Lin-ve stem cells, showing significant improvement in day-wise (acquisition days 1–6) escape latency and found to be comparable to the sham control mice. In our previous study, we have shown the effect of lineage negative stem cells after 10 and 60 days with cell doses of

Table 2 List of details of primary and secondary antibodies

Sr. no.	Primary antibodies	Specificity	Make	Catalog no.	Dilution
1.	BDNF	Mouse monoclonal IgG1	Santa Cruz Biotechnology	sc-65514	1:100
2.	GDNF	Mouse monoclonal IgG1 (kappa light chain)	Santa Cruz Biotechnology	sc-13147	1:100
3.	CNTF	Rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-13996	1:100
4.	GFAP	Polyclonal rabbit anti-mouse	Sigma	HPA056030-100UL	1:100
5.	Aβ42	Rabbit polyclonal mouse	Elabscience	E-AB 40038	1:100
6.	Caspase3	Mouse monoclonal IgG1	Santa Cruz Biotechnology	sc-271759	1:100

50,000 as well as 100,000 [15]. We had described a dose-dependent recovery of cognitive impairment in amyloid-injured mice using 50,000 cells at 60 day. In our comparative analysis, results showed effective rescue by 50,000 Lin-ve stem cells when used at 10-day time-point with 1 μM concentration induced Aβ injury [13]. Hence, we used this dose and time-point for further investigation.

This improvement in ELT in the hUCB stem cell transplanted mice was noticed after they were injected with TrkB inhibitor (ANA-12) daily before acquisition and retrieval trials (Fig. 2a). On retrieval day (7th day), the time spent in each quadrant was measured. The amyloid-injected mice did not spend more time in the target quadrant (Q1) as seen in the sham control group. However, the time spent by the mice with Lin-ve stem cell transplantation was marginally increased when compared to amyloid injury mice. The inhibitory effect of TrkB on the retrieval trials was found to be comparable to amyloid injury mice injected with ANA-12 (Fig. 2b). On retrieval day (day 7), the mean distance from the platform was calculated and found increased significantly in mice injected with amyloid β when compared to control groups. The distance was also found to be reduced in mice transplanted with stem cells. Although insignificant, this distance was further increased in ANA-12-injected mice, suggesting the crucial role of TrkB signaling in the effect of Lin-ve stem cell-mediated cognitive improvement in the amyloid β injured mice (Fig. 2c).

Improvement of Fear Conditioning Memory after hUCB Stem Cell Transplantation

To further confirm the learning and memory quotient in mice of various groups, we performed another behavioral test, i.e., passive avoidance. The learning and memory is estimated by the time taken by mice to avoid aversive stimuli (i.e., electric shock). Memory is positively correlated with the time taken by mice to move from light to dark chamber. The increase in latency signifies improved retrieval of fear-associated memory. The results showed significant reduction in latency time in amyloid-injected mice when compared to healthy control ($p \leq 0.0001$), suggesting worsening of associative memory in amyloid-injected group. The intra-hippocampal transplantation of human Lin-ve stem cells resulted in significant increase ($p < 0.01$) in latency as compared to the amyloid injury group. This improvement in latency was found to be reversed in the mice injected with TrkB inhibitor ($p < 0.001$) (Fig. 2d).

Swimming Track Plots Depict Behavioral Pattern

During MWM experiment, all the trials were analyzed using Anymaze software in order to analyze swimming pattern of mice. The track plots from acquisition days (days 1–6) were assessed for sham control, amyloid injury, stem cell transplantation, and TrkB inhibitor-injected mice. The reduction in swimming path and movement around the platform was observed in mice of sham control groups as the acquisition days progressed. Amyloid injury group showed circular movement around the periphery of tank with complex swimming track pattern. However, the hUCB Lin-ve cell transplanted mice showed better swimming performance and concomitant reduction in path, unlike injured mice. This improvement was further reversed when the mice were injected with TrkB inhibitor, showing similar pattern of swimming track as of amyloid injured mice (Fig. 3).

hUCB Lin-ve Stem Cell Transplantation Reduces Amyloid Load in Mouse Brain

The improvement in behavioral tests was found associated with amyloid staining in the mouse brains transplanted with Lin-ve stem cells. The immunohistochemical analysis for Cy3-Aβ antibody revealed deposition of amyloid aggregates around the site of injection (Fig. 4a). These aggregates were found to be significantly reduced in the mice brains after 20 days of hUCB Lin-ve stem cell transplantation (Fig. 4b). The fluorescent intensity measured by ImageJ showed significant reduction of corrected total cell fluorescence (CTCF) in the hUCB Lin-ve stem cell-transplanted brain sections as compared to amyloid injured brains (Fig. 4c).

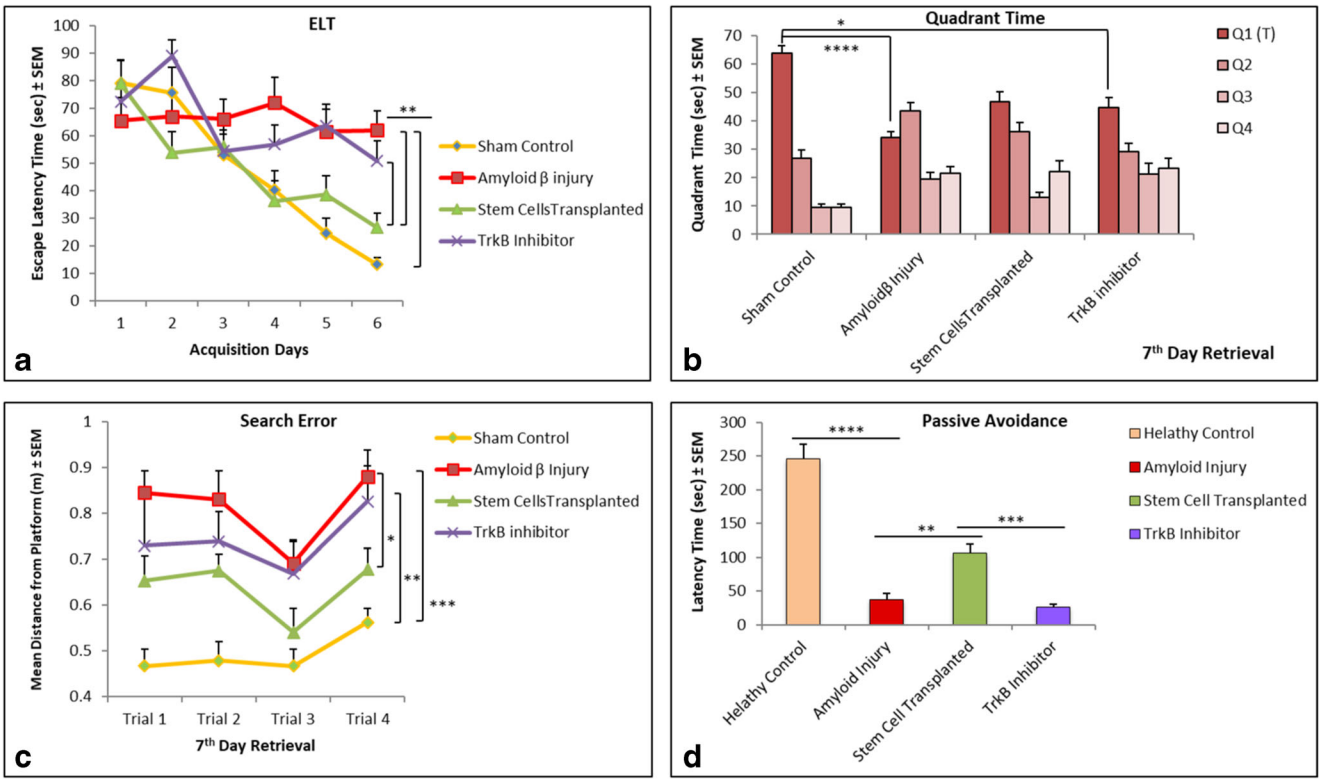

Fig. 2 Neurobehavioral analysis suggesting involvement of TrkB pathway in amelioration of memory loss by Lin-ve stem cells. **a** The graph depicts day-wise escape latency time during acquisition days of mice in sham control ($N = 7$), amyloid β injury ($N = 7$), stem cell transplantation ($N = 8$), and TrkB inhibitor ($N = 10$). The ELT was found to be more in mice with Aβ injury as compared to the sham control group whereas; stem cell transplantation ameliorated the memory loss depicted by reduced ELT. The mice were administered with TrkB inhibitor resulted in significant increase of ELT along the acquisition days. **b** The graph showing time spent by mice of different groups in MWM quadrants Q1, Q2, Q3, and Q4 on retrieval day (7th day). Mice with Aβ injury spent less time in target quadrant (Q1) in comparison to sham control and stem cell-transplanted group. **c** The search error graph showing mean distance from hidden platform traveled by mice. The mice with Aβ injury traveled at significantly more distant from the platform in comparison to sham control and stem cell-transplanted groups. The mice administered with TrkB inhibitor after stem cell transplantation in mice with Aβ injury resulted in more mean distance from platform comparable to Aβ injury group. **d** Passive avoidance graph depicts the role of TrkB pathway in amelioration of memory loss. The stem cell transplanted with Aβ injury mice showed significant increase in latency time (in sec). The administration of TrkB inhibitor (ANA12) significantly reduced the latency time suggesting involvement of TrkB pathway in rescue of memory loss. For MWM, data was analyzed by 2-way ANOVA with acquisition days and retrieval trials as a measure of repeated observations. Further, Tukey's test was used for post-hoc analysis to compare mean between days and groups. For passive avoidance, one-way ANOVA with Tukey's multiple comparisons test for post-hoc was applied. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ were regarded as statistically significant

hUCB Lin-ve Stem Cells Modulate Neurotrophic Secretion

The mRNA and protein expression of neurotrophic factors was analyzed by real time PCR and immunohistochemistry, respectively. We report significant reduction of BDNF expression in mRNA as well as protein levels in the amyloid injury group when compared to controls whereas hUCB Lin-ve stem cell transplantation upregulated its corresponding protein expression. Although TrkB inhibitor could not alter the mRNA levels in the mice brain (Fig. 5a–e), the expression of GDNF mRNA and protein was found to be reduced in the amyloid injury group and after transplantation of hUCB Lin-ve stem cells, it was significantly increased as compared to injured brain. Further, GDNF mRNA expression was significantly reduced when mice were injected with ANA-12 (TrkB antagonist) (Fig. 5f–j). Although non-significant, CNTF expression was also found to be increased after transplantation of hUCB Lin-ve stem cells when compared to amyloid-injured brains. It remained undetermined while the endogenous expression of CNTF was very low in healthy control mice brains. This expression was further abolished in mice in which ANA-12 was administered (Fig. 5k–o). Glial fibrillary acidic protein (GFAP) expression was also found to be significantly decreased in amyloid injury brains and when transplanted with hUCB Lin-ve stem cells, their expression was significantly upregulated. TrkB inhibitor did not alter mRNA expression of this protein when compared to either amyloid injury or hUCB Lin-ve stem cell-transplanted brains (Fig. 5p–t).

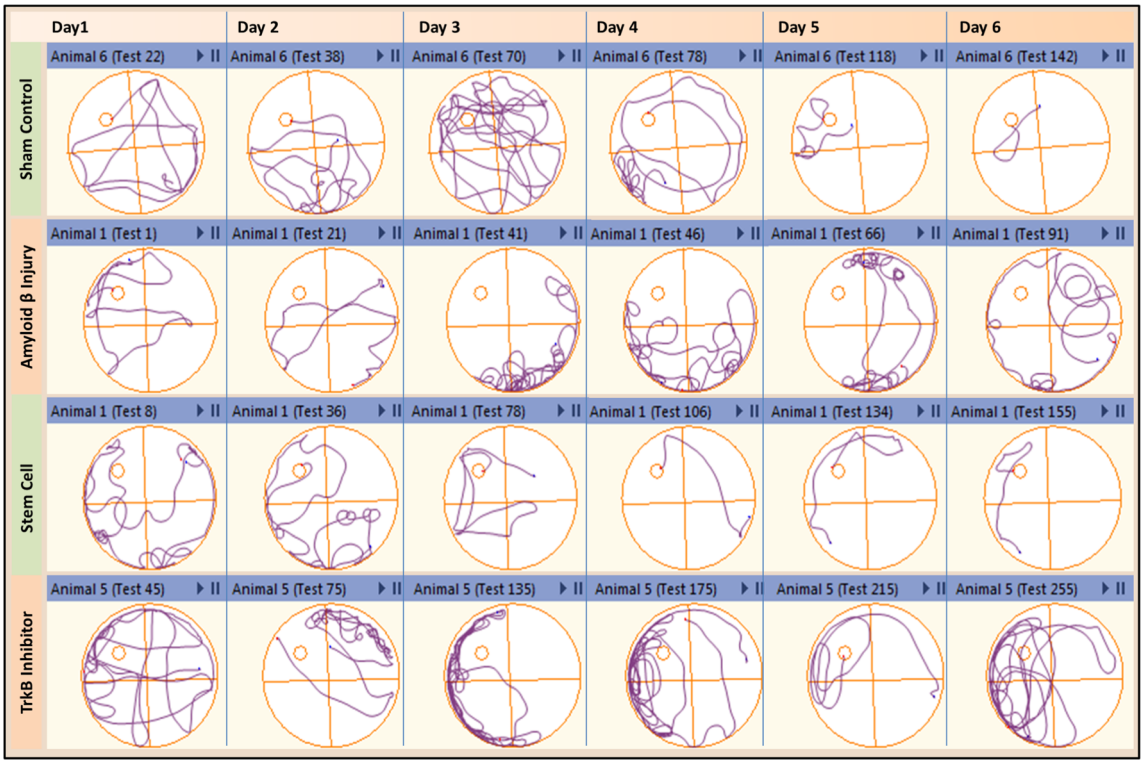

Fig. 3 MWM track plot representative swimming track plots as an index of learning from acquisition days 2, 4, 5, and 6 of different groups. The track plots depict that Aβ injury mice could not reduce their swimming path and moved towards periphery of MWM tank. The stem cell transplantation in Aβ injury mice resulted in shortening of path and movement more towards the hidden platform along the acquisition days, which is comparable to sham control group. The TrkB-administered mice could not reduce their path and moved more towards periphery region depicting memory loss

hUCB Lin-ve Stem Cells Prevent Cellular Apoptosis and Enhances Proliferation

The amyloid β is known to induce series of apoptotic cascade which eventually leads to neuronal cell death. We wanted to estimate the levels of apoptotic and proliferative markers in brains after hUCB Lin-ve stem cell transplantation in order to evaluate the mechanism through which these cells exert therapeutic effects in amyloid-injured mice brains. The protein level of Caspase3, an apoptotic marker, was found to be significantly upregulated in amyloid injury brains. This was found to be ameliorated by hUCB Lin-ve stem cell transplantation at the site of injury. The mRNA levels of Bcl-2, an anti-apoptotic marker (Fig. 6a–c) were significantly reduced by amyloid β aggregation in comparison to healthy controls. This reversed significantly after the transplantation of hUCB Lin-ve stem cells. This increase in expression was blunted by TrkB inhibitor in these brains (Fig. 6d). Further, Ki-67, a marker for cellular proliferation [41] expressed during the active phases of cell cycle [42], was found to be significantly reduced in amyloid injury group in comparison to control brains. This was partly rescued by hUCB Lin-ve stem cell transplantation. This induced Ki-67 expression was reversed when mice were administered with ANA-12 (Fig. 6e).

The levels of hydroxyl, peroxyl, and other reactive oxygen species (ROS) activity were also assessed using 2′,7′–dichlorofluorescindiacetate (DCFDA) in hippocampus. The levels of ROS were measured by the fluorescent intensity of 2′,7′–dichlorofluorescein (DCF) compound released from DCFDA by the action of cellular esterase. Our data suggests that there were no changes in the ROS activity in the hippocampus from all the groups (Fig. 6f).

Discussion

Our data provides the fundamental framework for therapeutic efficacy of hUCB-derived Lin-ve stem cells in the mouse model of Aβ-42 injury. The transplantation of hUCB-derived Lin-ve stem cells after amyloid injury resulted in decrease of day-wise escape latency time in MWM test, comparable to control groups (Fig. 2). Similarly, in track plot analysis, the mice with transplanted Lin-ve stem cells took lesser time to locate the platform in comparison to Aβ-42 injury Lin-ve mice (Fig. 3). Hence, shortening of swimming path suggests that hUCB Lin-ve stem cells have potential to rescue the spatial memory loss induced by Aβ-42. We also subjected these mice to passive avoidance analysis in which mice

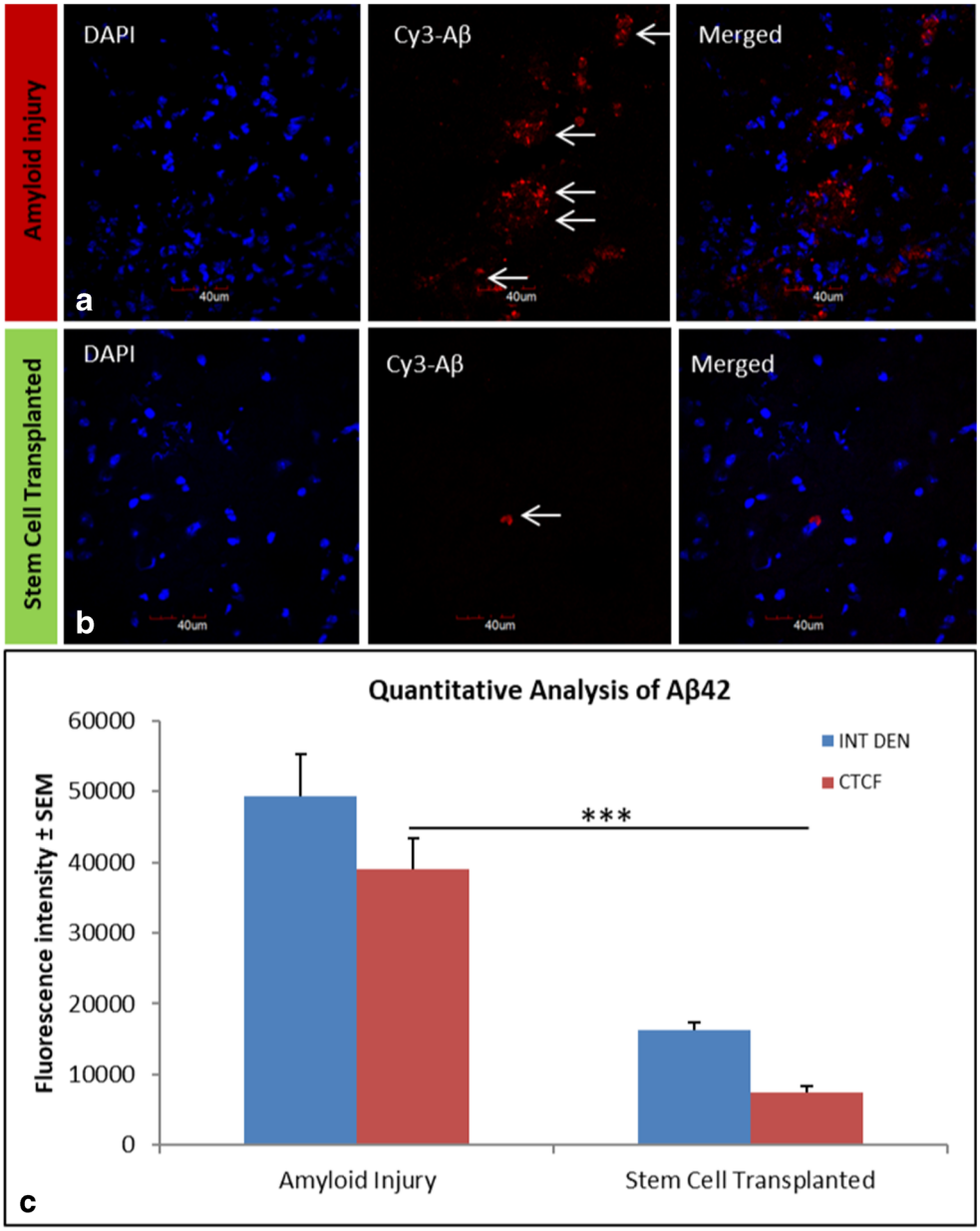

Fig. 4 Amyloid deposition and its clearance by Lin-ve stem cells. **a** Immunohistochemistry analysis using primary antibody (1:100 dilutions) of Aβ-42 and secondary antibody (1:200 dilutions) showed deposition of amyloid aggregates near hippocampal region in Aβ injury group at × 10 and × 60 magnification. However, we found significant reduction in amyloid aggregates in mice transplanted with Lin-ve stem cells as visualized under confocal microscope at × 20 and × 60 magnification. **b** Quantification of the hippocampal brain sections based on florescence intensity using ImageJ software Data was statistically analyzed using GraphPad Prism 7.04. Student *t* test was applied between the groups. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ were regarded as statistically significant

transplanted with hUCB Lin-ve stem cells showed better memory retrieval with increased latency to stimuli (Fig. 2).

Our previous study had shown that this improvement is mediated by the upregulation of two putative neurotrophic factors, i.e., BDNF and CREB (cAMP response element-binding) [15]. It is also well known that BDNF binds to tropomyosin receptor kinase B (TrkB) which dimerizes to initiate three pathways, i.e., PKC, PI3, and Ras/MAPK pathways [43]. These pathways eventually lead to the activation of transcription factor CREB which further activates the genes involved in synaptic plasticity [43]. Therefore, we targeted the TrkB pathway using ANA12, a non-competitive antagonist of TrkB receptor, because it has been shown to successfully block the action of BDNF [44]. The mice administrated with ANA12 showed reversal of behavioral outcomes by hUCB Lin-ve stem cell transplantation, as confirmed by both MWM (Fig. 3a–c) and passive avoidance test (Fig. 3d). This provides a compelling argument to suggest that the hUCB Lin-ve stem cells rescue memory loss via BDNF-TrkB pathway.

We further sought to determine if the hUCB Lin-ve stem cells could reduce the artificially deposited amyloid load from the mouse brain. In order to evaluate this, we examined the deposits of Aβ-42 in the hippocampus by immunohistochemistry. We found significant reduction of Aβ deposits in hUCB Lin-ve stem cell-transplanted group as compared to the amyloid injury group (Fig. 4). We found that the clearance of Aβ from the brain was associated with activation of astrocytes

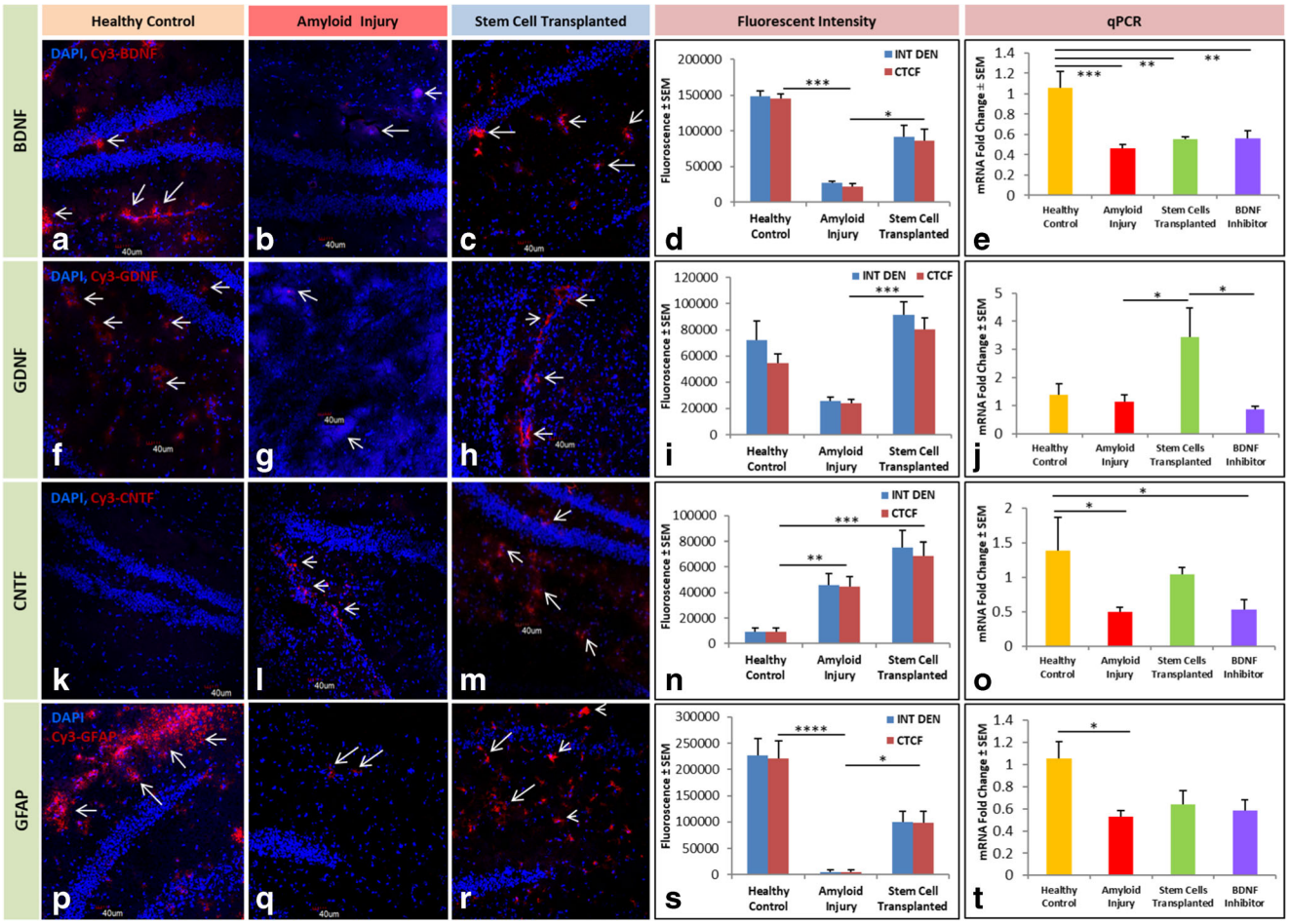

Fig. 5 Modulation of neurotrophic factors and activation of astrocytes by Lin-ve SC. **a**–**c** Immunohistochemistry showing cy3 expression (red) bound to primary antibody specific for BDNF at 20× in healthy control, amyloid injury and stem cell-transplanted group and counterstained with DAPI. **d** BDNF IHC images were quantified based on florescence intensity using ImageJ software. **e** The mRNA expression of BDNF analyzed by real-time PCR showed significant reduction in amyloid injury group ($p = 0.0005$) as well as in the TrkB inhibitor group ($p = 0.0017$) in comparison to healthy control. **f**–**h** GDNF expression (cy3-red) at 20× was analyzed in immunohistochemistry using coronal section of hippocampus merged with DAPI staining. **i** GDNF IHC images were quantified based on florescence intensity using ImageJ software. **j** The mRNA expression of GDNF analyzed by real-time PCR showed significant increase in stem cell-transplanted group in comparison to amyloid injury group ($p = 0.0447$). Also, expression was significantly reduced after administration of TrkB inhibitor (ANA12) ($p = 0.0207$). **k**–**m** Similarly, CNTF expression was analyzed using immunohistochemistry in all three groups at 20×. **n** IHC of CNTF images were quantified based on florescence intensity using ImageJ software. **o** The mRNA expression of CNTF analyzed by real-time PCR showed significant reduction in amyloid injury group ($p = 0.0405$) as well as in the TrkB inhibitor group ($p = 0.0485$) in comparison to healthy control. **p**–**r** GFAP expression was similarly visualized in immunohistochemistry merged with DAPI at 20×. **s** Quantification of the hippocampal brain sections based on florescence intensity using ImageJ software. All sections were analyzed under confocal microscope. **t** mRNA expression of GFAP was found to be significantly reduced by Aβ injury as compared to the healthy control group ($p = 0.0139$). For all the marker analysis by IHC and real-time PCR, one-way ANOVA with Tukey for post-hoc analysis was applied. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ were regarded as statistically significant

(increased level of GFAP), as suggestive from previous data showing astrocytes' role in both Aβ clearance as well as in modulation of neuroinflammation [45]. The astrocytes take up the Aβ-ApoE complexes from the extracellular matrix and degrade by neprilysin, insulin-degrading enzyme, or matrix metalloproteinase-9 (MMP-9) (Wyss-Coray). The accumulation of Aβ is often suggestive of the failure of its clearance by astrocytes, resulting in astroglial pathology. We showed that the Aβ-induced injury group showed significant reduction in its mRNA expression besides corresponding changes in the protein expression. The astrocyte expression after hUCB Lin-ve stem cell transplantation was found significantly increased when analyzed by immunohistochemistry of mouse hippocampus; however, no change was observed in the associated mRNA expression (Fig. 5p–t). These results indicate that hUCB Lin-ve stem cells facilitate scavenging of Aβ by the activation of hippocampal astrocytes.

As Aβ is the major contributor for amyloid plaque formation, it initiates the pathological events inducing formation of neurofibrillary tangles (NFT) and neuronal cell death [46, 47].

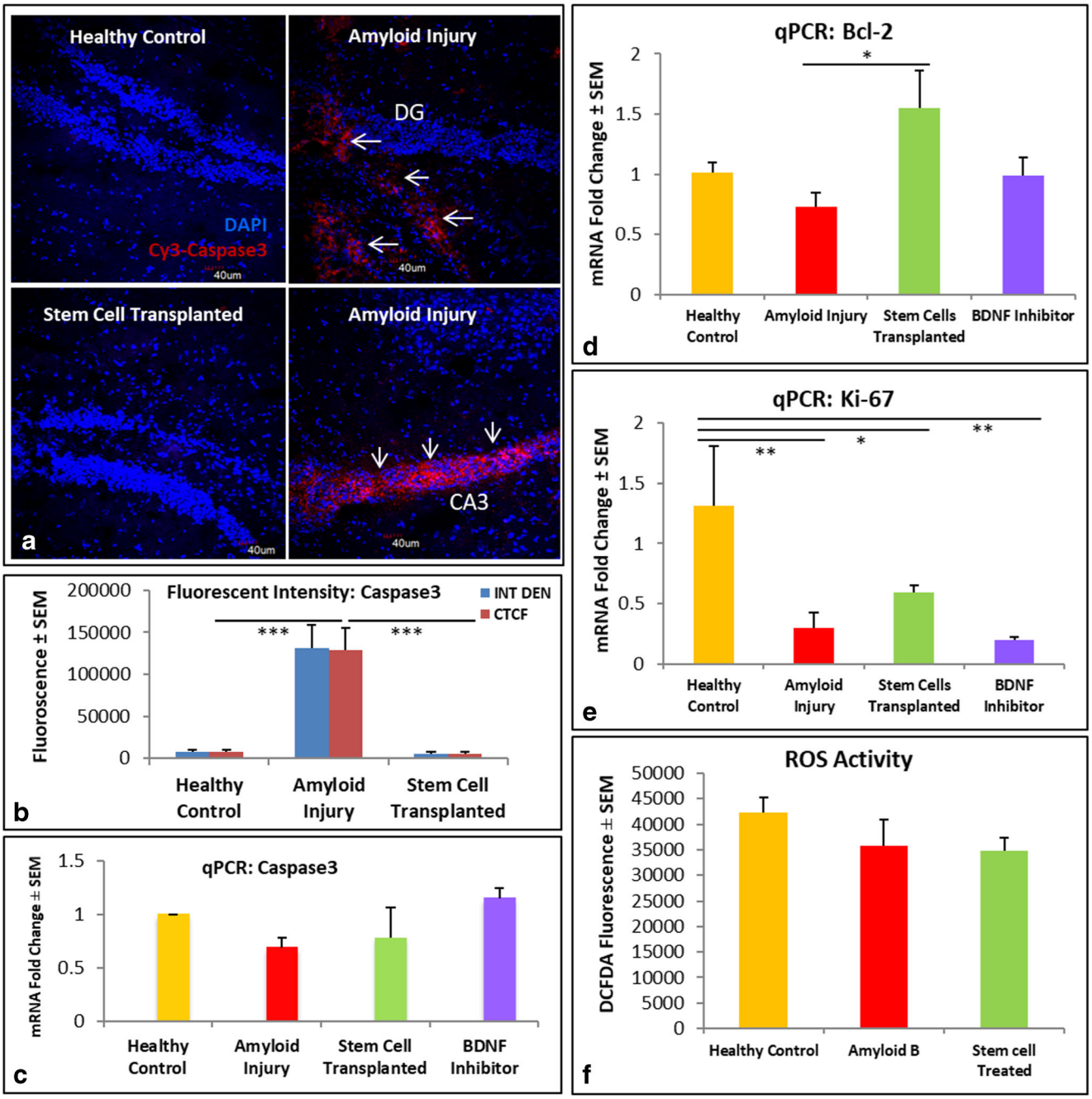

Fig. 6 Lin-ve SC exerts anti-apoptotic activity. **a** Immunohistochemistry of caspase3 was performed in mouse brain hippocampal sections of healthy control, Aβ injury, and stem cell-transplanted group. The upregulated expression of caspase3 in Aβ injury mice was observed in hippocampus especially dentate gyrus region as well as in CA3 region and reduced in stem cell-transplanted group. **b** Caspase3 IHC images were quantified based on florescence intensity using ImageJ software. **c** mRNA expression of Caspase3 was found to be changed non-significantly analyzed by qPCR. **d** mRNA expression of Bcl2 was found to be significantly higher in stem cell-transplanted group in comparison to Aβ injury group analyzed by real-time PCR ($p = 0.0245$). **e** mRNA expression of Ki67 was significantly reduced by the Aβ injury group compared to healthy control ($p = 0.0026$), however, was found to be increased in stem cell-transplanted group compared to injury group, but non-significant ($p = 0.5135$). TrkB inhibitor-injected group showed significant reduction of Ki-67 compared to healthy control ($p = 0.001$). **f** The reactive oxygen species levels was analyzed using DCFDA fluorogenic dye and levels were estimated in healthy control, Aβ injury, and stem cell-transplanted group using fluorimeter. There was no difference recorded among the groups. One-way ANOVA with Tukey for post-hoc analysis was applied. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ were regarded as statistically significant

The Aβ-associated apoptosis studies have already uncovered the underlying mechanisms [47]. It is for this reason that we wanted to analyze the effect of Aβ injection on neuronal cell death and consequent memory loss. Caspase3, which is a hallmark of apoptosis, was found to be significantly upregulated in the hippocampus (especially dentate gyrus and CA3

region), when analyzed by immunohistochemistry. However, apoptosis was significantly reduced when hUCB Lin-ve stem cells were transplanted. The corresponding mRNA expression was, however, not significant (Fig. 6a–c). Similarly, Bcl2, which is a marker for anti-apoptotic activity, was analyzed by real time PCR and found to be unaltered in the amyloid injury group, whereas a significant increase was noted after hUCB Lin-ve stem cell transplantation. The administration of TrkB pathway inhibitor showed comparable results as reminiscent of amyloid injury.

The oligomers of Aβ along with tau protein are considered as causative of neuroinflammation, cholinergic denervation, and synaptic loss via oxidative stress [48]. It is also widely believed that the mitochondrial dysfunction causes neurodegeneration in AD and is associated with ROS generation [49]. When ROS was analyzed in the Aβ-injected hippocampus and the hUCB Lin-ve stem cell transplantation group, no changes was noticed (Fig. 6f) indicating the dominant role of other processes described in this paper. Hence, it can be concluded that BDNF, Caspase3 and Bcl-2 play dominant role in the rescue of memory loss than ROS. In order to further probe whether increase in neurotrophic factors and reduction in apoptosis was associated with neuronal cell proliferation, we examined proliferating cell nuclear antigen, i.e., ki67, used to identify actively dividing cells. Expectedly, the ki67 was significantly downregulated in the amyloid injury group and marginally increased after hUCB Lin-ve stem cell transplantation (Fig. 6e). The expression of ki67 can be associated with the GDNF expression as it has a significant role in proliferation [50]. It is thus compelling to mention that Aβ-42-induced memory loss was prevented by transplantation of hUCB Lin-ve stem cell through modulation of neuroinflammation and apoptotic pathways.

Further, CNTF expression was also found to be significantly reduced in the injury group and increased in the hUCB Lin-ve stem cell-transplanted group. [51]. The most important downstream molecule of this pathway is Bcl-2 [52], a well-identified anti-apoptotic marker, which was found to be significantly upregulated in hUCB Lin-ve stem cell-transplanted mice brains. It can thus be speculated that hUCB Lin-ve stem cells induced amelioration of memory loss is partly due to activation of anti-apoptotic machinery via Bcl-2. It needs

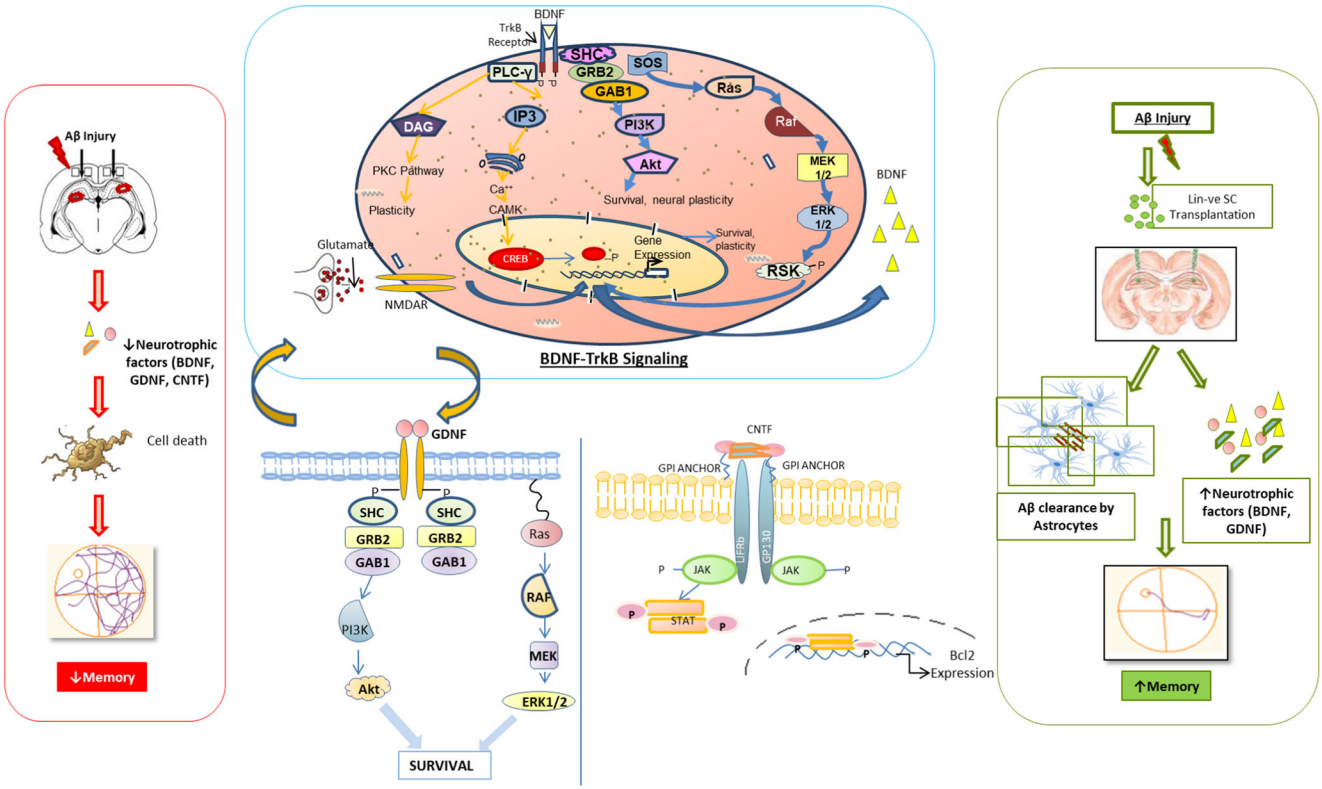

Fig. 7 Schematic of overall molecular mechanism initiated by Lin-ve stem cells. The schematic showing possible mechanisms involved by the transplantation of UCB derived Lin-ve stem cells. Our study showed that amyloid injury reduces neurotrophic factors which would affect neurons by cell death. The transplantation of Lin-ve exerted paracrine effects by modulating or increasing the production of neurotrophic factors, i.e., GDNF, BDNF, and CNTF. This might have activated astrocytes which possibly involved in the clearance of Aβ deposits and hence alleviating memory loss. The Lin-ve stem cells exerted its paracrine effects by the involvement of TrkB pathway. This study also suggested that there might be the involvement of RET pathway as indicated by upregulation of GDNF and cross-talk which exists with TrkB pathway [26]. Similarly, upregulation of CNTF and Bcl2 also indicated the probable role of Jak-STAT pathway mediated by these transplanted cells bringing out the therapeutic outcome in this study. Hence, our study suggests that Lin-ve stem cells have the potential to ameliorate cognitive impairment by initiating complex molecular mechanisms by its paracrine effects

further investigation to determine if it is directly mediated by CNTF. However, a study has previously linked the role of CNTF in the activation of astrocytes [53]. In order to test whether hUCB Lin-ve stem cells exerted paracrine effects via the CNTF secretion, mediated by astrocytes or anti-apoptotic activity via JAK-STAT pathway, additional experiments are imperative.

GDNF is another putative neurotrophic factor, which is also implicated in AD and was decreased in MTG of AD postmortem brains [23]. Its overexpression has been also shown to improve the learning and memory by concomitant upregulation of BDNF. Our results showed decrease in the mRNA expression of GDNF in the amyloid injury group whereas there was significant increase in the hUCB Lin-ve stem cell-transplanted group. This suggests that GDNF plays active role in reversal of memory loss by hUCB Lin-ve stem cell transplantation. Furthermore, the mRNA expression of GDNF was significantly reduced after ANA12 administration in comparison to the hUCB Lin-ve stem cell-transplanted group. This suggests that BDNF-TrkB pathway may be associated with GDNF expression. The therapeutic outcome in our study could be possibly due to the cross-talk between BDNF induced TrkB signaling and GDNF induced RET pathway (15).

ANA-12 is a non-competitive antagonist of TrkB receptor, chiefly blocking the action of BDNF [44]. ANA-12 was administered intra-peritoneally before behavioral performance. The mice administered with TrkB receptor inhibitor resulted in significant increase in escape latency time as well as the search error, i.e., mean distance from platform, which was found to be comparable to amyloid injury mice (Fig. 2a–c). This was further confirmed by fear conditioning memory using passive avoidance test. On test day, the latency to cross from light to dark compartment was significantly increased in Lin-ve stem cell-transplanted group compared to the amyloid injury group (Fig. 2d). By inhibition of TrkB receptors, the complete reversal of the neurobehavioral outcomes from transplanted hUCB Lin-ve stem cells was noted. This data suggests that hUCB Lin-ve stem cells could effectively rescue memory loss via TrkB signaling.

It can be further speculated whether the restoration of neurotrophic factors such as BDNF, GDNF, and CNTF in our study is released by transplanted Lin-ve stem cells or these cells stimulated endogenous neurons or astrocytes to secret these factors? We analyzed the expression of these neurotrophic factors using mouse primers as well as antibodies utilizing real-time PCR and immunohistochemistry respectively. The source of BDNF is from endogenous neurons; GDNF is secreted by glial cells while CNTF is secreted by astrocytes. This can be further tested using co-culture of stem cells with neurons/astrocytes. Recent study by Joseph Park et al. developed unique 3D triculture system, which is found to be effective for conducting such study of co-culture of stem cells with neurons/astrocytes, in order to decipher the paracrine role of Lin-ve stem cells on neurons/astrocytes. This model has successfully recapitulated AD pathology because it mimics the in vivo extracellular matrix as in AD brains and 3D environment provides a large surface area for growth and differentiation of neurons/astrocytes [54].

As Lin-ve stem cells mediate its effect via release of neurotrophic factors, therefore, we further carried out the comparative analysis by injecting BDNF and compared it with Lin-ve cell transplanted group (data not published). This study is currently ongoing and further experiments are required before we test the hypothesis of BDNF-mediated effects. Although, the aim of the paper was to develop understanding of the effect of Lin-ve stem cells transplantation on neurotrophic factors, future studies can also examine the role of diet, circadian rhythmicity, gender, etc. on stem cell-mediated reversal of memory loss.

Conclusions

Our results reveal that transplantation of hUCB Lin-ve stem cell can rescue the memory loss by clearance of Aβ mediated by activation of astrocytes. hUCB Lin-ve stem cells exert paracrine effects by modulating the hippocampus neurochemistry as noted by escalation of neurotrophic factors. The increase in neurotrophic factors by hUCB Lin-ve stem cell transplantation may also exert anti-apoptotic effects and may activate complex molecular pathways involving TrkB, RET and Jak-STAT (Fig. 7). It will be of interest to dissect the putative molecular pathway in future studies for further insights.

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Author Contribution PB conducted all the experiments, acquisition of the data, and writing of manuscript. AB was involved in manuscript writing/editing and data/statistical analysis. BN was first author's PhD supervisor and edited the manuscript. AA conceptualized the study, secured research grant, and edited the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations Lin-ve, Lineage negative; SC, Stem cells; UCB, Umbilical cord blood; BDNF, Brain-derived neurotrophic factor; GDNF, Glial-derived neurotrophic factor; CNTF, Ciliary neurotrophic factor; TrkB, Tyrosine receptor kinase B; Bcl2, B cell lymphoma 2;

JAK, Janus kinases (JAKs); STAT, Signal transducer and activator of transcription proteins; DG, Dentate gyrus; CA, Cornu Ammonis; Aβ, Amyloid β

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RESEARCH ARTICLE

Human Fetal Pigmented Ciliary Epithelium Stem Cells have Regenerative Capacity in the Murine Retinal Degeneration Model of Laser Injury

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Abstract: ***Background*:** Retinal degeneration and related eye disorders have limited treatment interventions. Since stem cell therapy has shown promising results, ciliary epithelium (CE) derived stem cells could be a better choice given the fact that cells from eye niche can better integrate with the degenerating retina, rewiring the synaptic damage.

***Objective*:** To test the effect of human fetal pigmented ciliary epithelium-derived neurospheres in the mouse model of laser-induced retinal degeneration

***Methods*:** C57 male mice were subjected to retinal injury by Laser photocoagulation. Human fetal pigmented ciliary epithelium was obtained from post-aborted human eyeballs and cultured with epidermal growth factor (rhEGF) and fibroblast growth factor (rhFGF). The six day neurospheres were isolated, dissociated and transplanted into the subretinal space of the laser injured mice at the closest proximity to Laser shots. Mice were analyzed for functional vision through electroretinogram (ERG) and sacrificed at 1 week and 12 week time points. Retinal, Neurotropic, Apoptotic and proliferation markers were analysed using real-time polymerase chain reaction (PCR).

***Results*:** The CE neurospheres showed an increase in the expression of candidate genes analyzed in the study at 1 week time point, which sustained for longer time point of 12 weeks.

***Conclusion*:** We showed the efficacy of human CE cells in the regeneration of retinal degeneration in murine model for the first time. CE cells need to be explored comprehensively both in disease and degeneration.

Keywords: Ciliary epithelium, laser injury, retinal degeneration, subretinal, transplantation, Age-related macular degeneration (AMD).

1. INTRODUCTION

A number of degenerative conditions of retina-like age-related macular degeneration (AMD), glaucoma and Diabetic retinopathy (DR), lead to partial or complete vision loss. Laser injuries are also responsible for severe retinal damage. Occupational injuries due to widespread use of Laser in industries, medicine and military also lead to increased number of patients with severe vision loss [1]. Cell-based interventions have shown promise and may provide treatment avenues for such injuries and degenerative disorders. Ongoing stem cell research findings have also raised hopes of people suffering from central nervous system (CNS) disorders. Differentiation potential of stem cells isolated from various sources such as hematopoietic [2], mesenchymal [3], embryonic [4], neural stem cells [5], retinal progenitors [6], has been studied. These cells have been reported to successfully differentiate into different retinal cell lineages (neuronal and glial). Retinal cells derived from human embryonic stem cells (hESC) have been shown to be safe for human trials [7]. Similar applications of mesenchymal stem cells in retinal degenerative diseases have also been investigated [8]. Besides these stem cells, an effort is being made to use Muller glia cells to differentiate into different neuronal lineage to avoid the chances of immune rejection [9]. Due to the huge differentiation potential of stem cells into defined lineages, transplantation therapy is being exploited for their use in chronic diseases of ageing. However, most of the studies lack the rationale for the use of a specific population of stem cell to be used based on its potential for homing and therapy.

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In lower vertebrates including fish, frogs and birds, increased regenerating capacity for retinal tissue has been described. This is attributed to the presence of ciliary marginal zone (CMZ) [10, 11]. CMZ is present at the periphery of the retina in these vertebrates and believed to have resident retinal stem cells. Due to stem cell properties of cells present in CMZ, they can proliferate during tissue injury and take part in the regeneration process [12, 13]. With evolution, higher vertebrates derived ciliary marginal zone has restricted limited zone. Therefore, their regenerating capacity is highly diminished. However, there exists a quiescent population of stem cells lining the ciliary body in higher vertebrates, known as pigmented ciliary epithelium [14-16]. Factors that are regulating the quiescence, localization and activation of these ciliary epithelium stem cells have been studied [17]. Various retinal stem cells and their respective efficacy in the regeneration of diseased/degenerated retina both *in vitro* [18] and *in vivo* have been shown. Since the identification of the CE stem cells by Tropepe *et al.* [19] and Ahmad *et al.*, [20] these cells have been manipulated into different cell lineages [21]. There has been an equal or a smaller number of studies contradicting the efficacy of these cells as possible neurological precursors [22]. These cells have been studied extensively *in vitro*, it is imperative to conduct *in-vivo* studies as well. The response of ciliary epithelial stem cells upon transplantation in retinal injury models has also been examined [23]. There are a number of factors which govern the fate of stem cells present in a tissue or population. The intrinsic and extrinsic factors which can influence the growth, proliferation and differentiation of these cells both *in-situ* and *ex-situ* need to be characterized and regulated to obtain desired results for therapy. Apart from manipulating the factors, it is well understood that the niche or cell microenvironment is crucial for expression of cell characteristics and fate regulation. Though significant work has been done in this area *in vitro*, many aspects involving understanding the transplantation efficacy of these stem cells are still in its infancy. Our study is the maiden attempt to describe the recruitment of differentiated human fetal ciliary epithelium-derived neurospheres delivered in the subretinal space of the mouse.

2. MATERIALS AND METHODS

2.1. Animals

C57BL/6J mice were used in the study as per the guidelines and approval of the Institutional Animal Ethics Committee (IAEC), PGIMER, Chandigarh, INDIA. Animals were on standard chow diet, clean drinking water and were maintained at 12 hr dark/light cycle.

2.2. Laser-induced Injury Model of Mouse Retina

For laser injury, the animals were anesthetized with a cocktail of xylazine:ketamine (1:10). Pupils were dilated (1% Tropicamide) and topical anesthesia (2% Lignocaine) was applied to the cornea. A fundus shot was taken through a slit lamp connected to the Laser photocoagulator. 8 laser spots per eye were shot around the optic disc. The laser spot size was 100 microns; the duration of exposure to laser pulse was kept 100 msec with a power of 200mW.

2.3. Fundus Fluorescein Angiography (FFA)

The animal was maintained in anesthesia after Laser shots and the pupils were dilated (1% Tropicamide). Fluorescein was injected through tail vein and fundus was visualized using Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany).

2.4. Isolation and Culture of Ciliary Epithelial Cells from human Fetal Eyes

Eyes from aborted fetus were enucleated and immediately transported in ice-cold Hanks' Balanced Salt Solution (HBSS) under sterile conditions. Enucleated eyes were dissected through anterior edge of the pars plana under stereo zoom microscope in order to obtain ciliary rings. Ciliary rings were washed with HBSS (GIBCO, USA) and the pigmented CE was carefully stripped off avoiding any contamination with underlying non-pigmented epithelium, retinal pigment epithelium (RPE), iris and retina. CE tissue was cut into small pieces and was dissociated by treatment with 0.25% Trypsin with ethylene diamine tetra-acetic acid (EDTA, GIBCO, USA) at 37ºC (shaking after every 5 min) followed by neutralization with equal quantities of ice-cold Dulbecco's Modified Eagle media (DMEM/F12, GIBCO, USA). The resultant cell suspension was filtered using 0.70μm filter (BD Biosciences) and centrifuged for 10 minutes at 800g. The pellet was re-suspended in DMEM/F12 and washed thrice by centrifuging at 800g for 10min each time. The final cell pellet was suspended in medium containing DMEM/F12, N2 supplement, 2mM L-glutamine along with antibiotics (penicillin-streptomycin) and Fungizone. Pigmented ciliary cells were counted manually using hemocytometer and validated by an automatic cell counter (Millipore, USA). 3,000 cells/well were then plated in 96 well culture plate. Cells in different wells were grown in medium containing the rhEGF (20ng/ml; R&D systems, USA) and rhFGF basic (20ng/ml; R&D systems, USA) along with the proliferation medium in CO_2 incubator at 37ºC. The shape, size and the number of neurospheres were determined on various time points and cells from the 6^{th} day of culture were harvested and used for transplantation.

2.5. Transplantation of hCE Derived Neurospheres

To track the transplanted cells in host retinal tissue, they were marked with fluorescent dye Carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE) prior to transplantation. Cells were counted and suspended in PBS for all transplantation experiments. 24 h after laser injury, the animals were subretinal injected CFDA labeled CE cells (50,000 cells in 3μl of PBS). Subretinal delivery was done *via* transcorneal route. A prick was made in the anterior chamber using a beveled (25G) needle to release the pressure while simultaneously preventing any rise in post-operative intra ocular pressure (IOP). Precautions were taken not to injure the cornea. For injecting cells, through cornea-scleral junction, a 33G blunt needle was introduced and was carefully moved along the edges and behind the lens into the retina. The retina/RPE junction was assessed (by the back pressure on the needle) and injected with cells into space without exerting pressure on the underlying RPE. For the slow release of cells, the needle was attached to a microsyringe (EX-

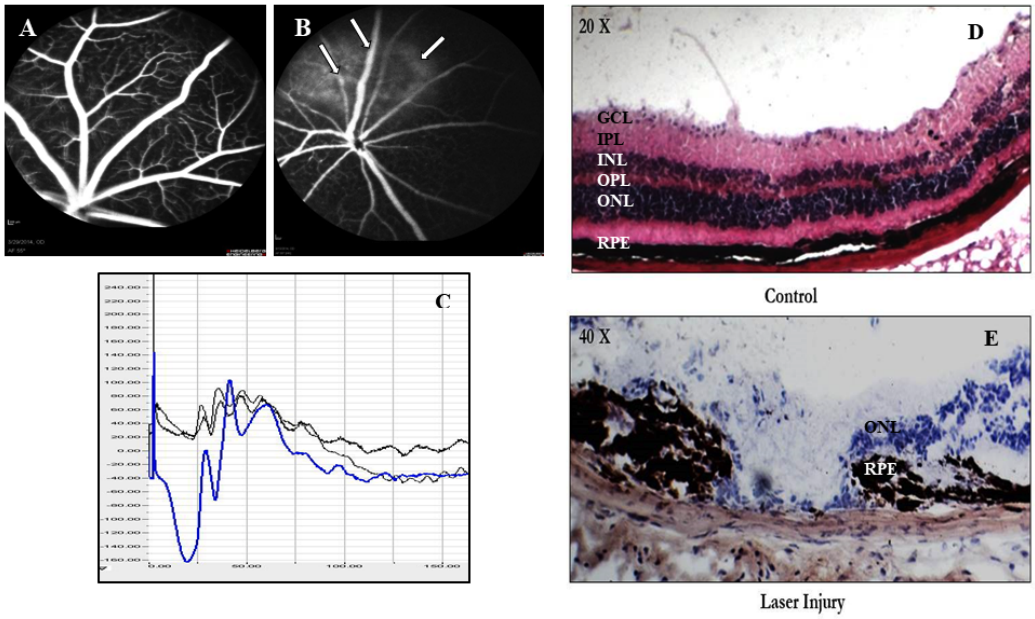

Fig. (1). Establishment of the mouse model of retinal degeneration by Laser injury. Mice were injured with laser shots around the optic disc, for injuring the RPE and checked by Fundus Fluorescein Angiography (FFA). FFA shows no leakage of fluoresce in dye in the control retina of the left eye, (**A**) The laser injured retina of the right eye shows leakage at injury sites (whitearrows) (**B**). Scotopic electoretinography (ERG) was performed on dark adapted mice, to assess them for functional vision. ERG wave form of the comparison between uninjured (blue wave) and injured retina (black wave) (**C**). The animals were euthanized at 1week and12 week and eyeballs enucleated and sectioned for histology. Hematoxylin and Eosin staining of retinal sections from control eye (**D**) and post Laser photocoagulation (**E**). (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper*).

MIRE, Japan). Successful injections were validated by fundus shots post-surgery. Bleb, as a result of retinal detachment, signifies successful transplantation into subretinal space. These cells were allowed to integrate into host retina and the effect of transplanted cells was determined at 1 week and 12 weeks after-transplantation.

2.6. Electroretinography

Functional analysis of retina was carried out using electroretinography (ERG). The mice were provided dark adaptation 12 h prior to ERG recordings. All the experiments were performed in red light illumination. For recordings, mice were anesthetized and body temperature was maintained throughout by placing the animal on a heating pad. The pupils were dilated with tropicamide drops and in order to prevent corneal injury due to dryness or electrode, the eye was moistened using sodium carboxymethylcellulose. Response to continuous white flashes of light was recorded using gold corneal electrode. To complete the circuit, the ground electrode was placed in the tail while the reference electrode was placed subcutaneously between the ears. The signals from retina were amplified and analyzed using labscribe software (iWorx Systems, Dover, NH, USA). Electroretinogram was analyzed for four parameters: a-wave amplitude, b-wave amplitude, implicit time to a-wave and implicit time to b-wave.

2.7. Real-time PCR

Gene expression for various retinal cell markers and neurotrophic factors was carried out by quantitative real-time PCR. The whole retina was dissected from the enucleated eye and it was homogenized. Ribo-nucleic acid (RNA) isolation was carried out from homogenized retina using RNA isolation kit as per the protocol provided from manufacturers (Qiagen, Netherlands). Immediately after isolation, the RNA was used as a template for complementary deoxyribonucleic acid (cDNA) synthesis using a commercially available kit (Thermo Scientific, USA). cDNA was amplified (StepOne, Applied Biosystems, USA) using specific primers for markers under study. The reaction was set up in triplicates or duplicates. Data were normalized to the expression levels of β-actin gene which was used as endogenous control and quantified by calculating fold change with respect to control.

3. RESULTS

3.1. The Laser Injury Caused RPE Aberration and Electrophysiological Deficits

Retinal injury in the mouse model subjected to Argon Green Laser injury was assessed through Fundus Fluorescein Angiography, and found to have leakage of the dye in the Fundus, showing disruption of the blood retinal barrier (BRB). The control eye/retina showed no leakage (Fig. **1A**) when compared to the injured retina, diffusing the dye at the site of injury (white arrows) (Fig. **1B**). On analyzing the physiological vision of the mice with ERG, the injured eye showed a stunted waveform (black wave, Fig. **1C**) while the uninjured control showed robust waveform with defined a & b peaks in the ERG wave (Blue wave. Fig. **1C**). The anatomy of the control retina showed evenly distributed and intact retinal layers in its sections stained for H&E (Fig. **1D**). On the other hand, the Laser interacted in a deleterious way with the RPE and adjacent layers and caused retinal holes resulting in the disruption of all the layers (Fig. **1E**).

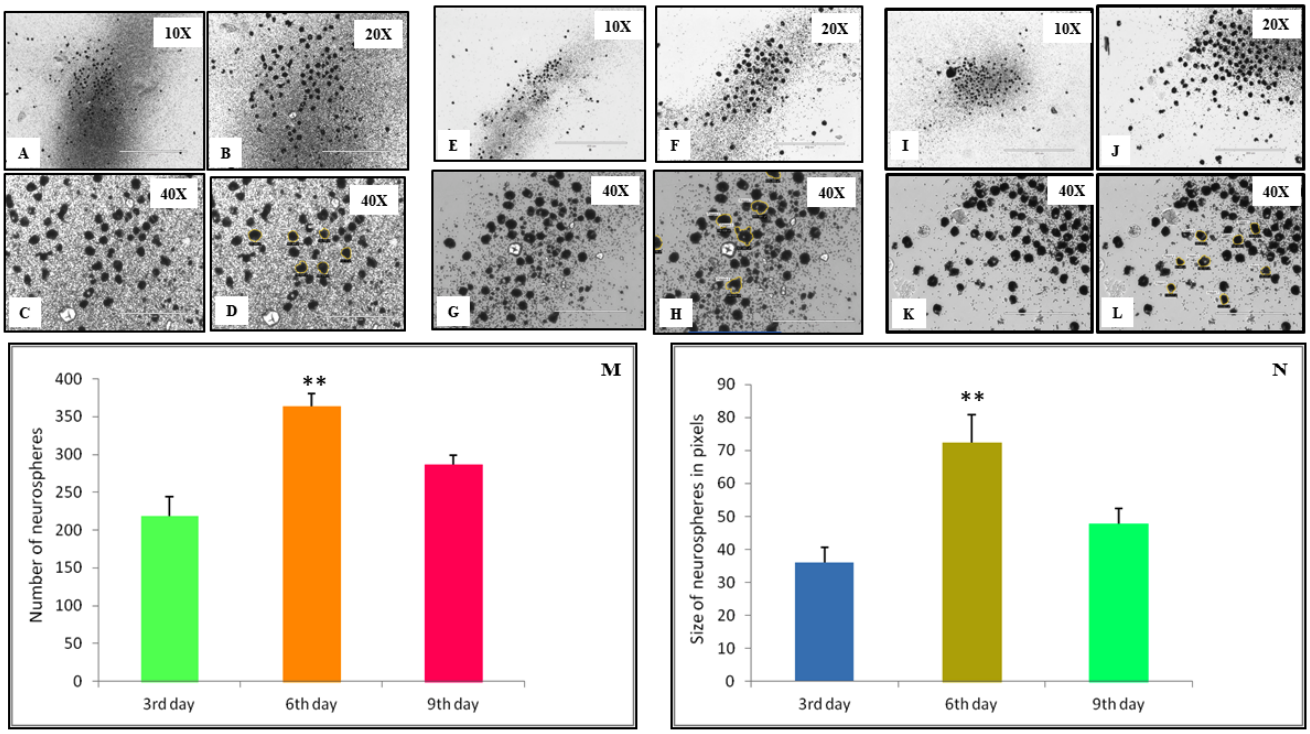

Fig. (2). Characterization of the pigmented Ciliary Epithelium neurospheres.
Pigmented ciliary epithelium was isolated from the enucleated eyeballs of the aborted fetuses and the cells were cultured in media containing rhEGF and rh FGF. The neurospheres were characterized based on size and number for a period of 9 days. Bright field microscopy of the ciliary epithelium cultures for a period of 3 days (**A, B, C & D**), 6 days (**E, F, G & H**) and 9 days (**I, J, K & L**). Graphs representing the number (**M**) and size of neurospheres (**N**) at 3, 6 & 9 days respectively.

3.2. Ciliary Epithelial Cells Grown in the Combination of Epidermal Growth Factor and Fibroblast Growth Factor, Show a Maximum Yield of Healthy Neurospheres

The ciliary epithelium cells, cultured for over a period of 9 days, in a combination of rhEGF + rhFGF with the retinal culture medium, were analyzed for their shape, size and number. The area and perimeter of the Neurospheres were measured by the EVOS microscope (life Technologies). The number of neurospheres was counted in the entire well in triplicates and then plotted against the average of size and number by the increasing days. We found that the size and number of neurospheres were significantly higher in the case of the 6th day compared to the 3rd and 9th day (Fig. **2**). In order to analyze the ultrastructure of the ciliary epithelial cells, we analyzed the neurospheres of the sixth day by using Scanning electron Microscopy. We found intact morphology of the cells in the aggregated rosettes/neurospheres as well as in the dissociated cells for transplantation.

3.3. Ciliary Epithelial Cells in Culture Differentiate Over a Period of Time, Losing Epithelial Characteristics and Forming Neurospheres

The ciliary epithelial cells start migrating across the plate *in-vitro* and assimilate to dense structures (Fig. **3A** and **B**). These dense structures visibly start losing melanin over a period of time, as observed at different time intervals. To rule out the possibility that these structures are not just cell aggregates, Scanning Electron microscopy based images were captured. We found viable and large neurospheres at the 6-day time point (Fig. **3C**). These neurospheres were dissociated after characterization and transplanted subretinally in the laser injured mouse models.

3.4. The Transplanated Ciliary Epithelial Derived Neurospheres Show a Fold Change in the mRNA Expression of the Injured Retina Over Prolonged Periods of Time

Fold change in mRNA expression of the different markers *i.e.* Rhodopsin, Thy 1 (Thy1-cell surface antigen), SV2A (Synaptic vesicle glycoprotein 2A), Rpe 65 (Retinal pigment epithelium specific-65), BDNF (Brain derived neurotrophic factor), CNTF (Cliliary neurotrophic factor), VEGF (Vascular endothelial growth factor) and Ki67was estimated through the ΔC_T values quantitated by the Real-time PCR. There was an increased expression in the markers Rhodopsin and Rpe65, specific for Rod photoreceptors and Retinal pigment epithelium, respectively (Fig. **4A** and **C**). There was a significant change in the expression of Thy1 and SV2A at 1week time point but was gradually downregulated at 3 months (Fig. **4B** and **D**). The endogenous neurotrophic factors BDNF and CNTF showed upregulation at the 3 months indicating a neurotropic response (Fig. **4E** and **F**). There was a downregulation of VEGF, an inflammatory marker identified in retinal disorders (Fig. **4G**). There was a marked increase in the expression of the proliferative marker Ki67 (Fig. **4H**) depicting proliferation at a longer time point. The fold change expression was normalized against normal healthy controls without injury. mRNA expression of the candidate genes for individual samples was normalized against endogenous controls β-Actin, n = 3, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq .001$.

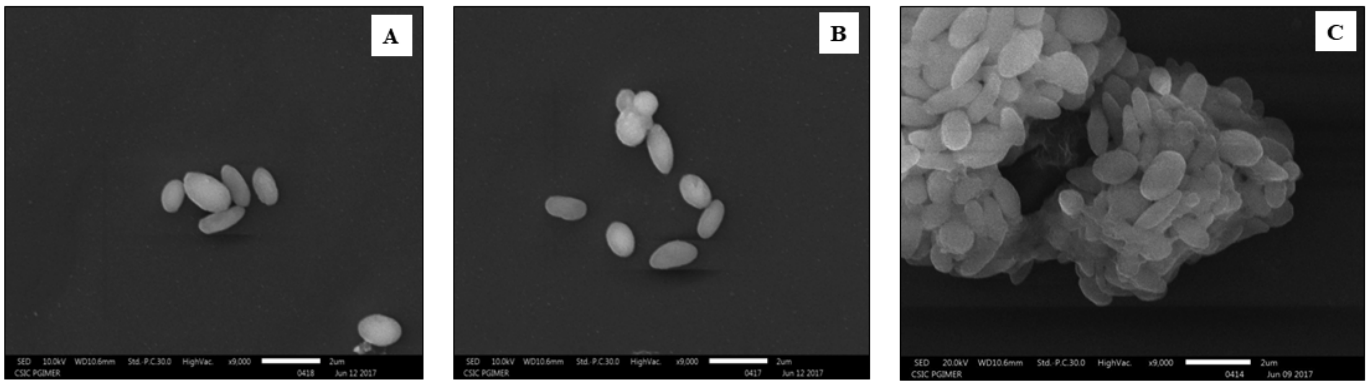

Fig. (3). Morphological characterization of the pigmented ciliary epithelial cells *in-vitro*. The cultured ciliary epithelium cells were characterized for the homogeneity and aggregation of the neurospheres. Scanning electron microscopy (SEM) on glutaraldehyde fixed cultures coated with platinum reveal Ciliary epithelium cells clumped together in 1 & 3 days of culture (**A** & **B**). SEM images for the aggregation of neurospheres and the neurosphere at the 6th Day (**C**).

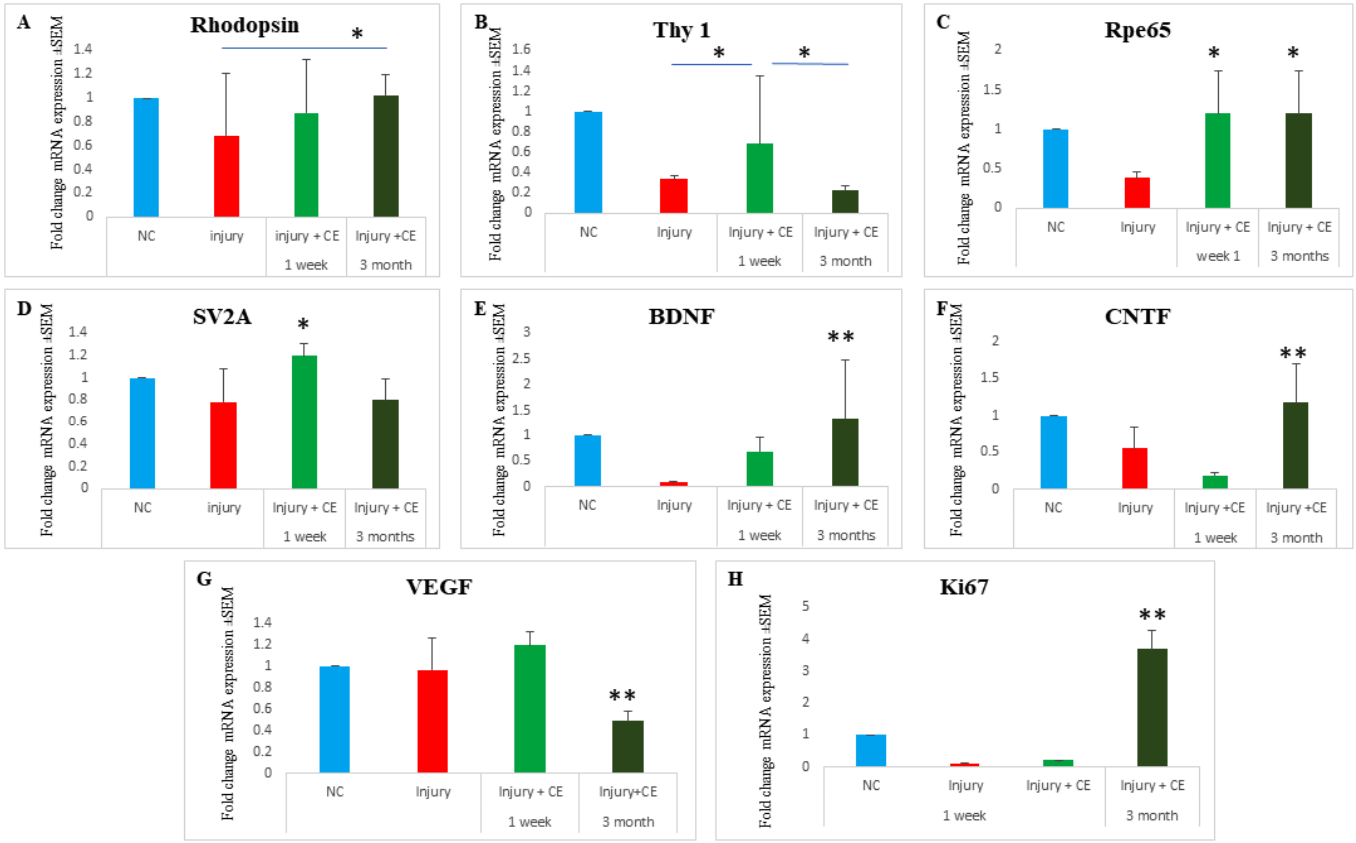

Fig. (4). (A-H) Fold change in mRNA expression of the candidate genes was analyzed by Real time PCR. The expression of the different markers for cell types, survival and neurotropic factors viz. Rhodopsin, Thy 1, SV2A, Rpe 65, BDNF, CNTF, VEGF and Ki67 was calculated through the ΔC_T values quantitated by the Real-time PCR. The fold change expression was normalized against the normal healthy controls without injury. mRNA expression of the genes for individual samples was normalized against endogenous controls β-Actin, n = 3, * $p \leq 0.05$, ** $p \leq 0.01$.

4. DISCUSSION

The retina is a well-characterized central nervous system derivative. The cellular specification in the vertebrate retina has been studied extensively for more than a century. With the growing evidence of the presence of stem cells in adult central nervous system, these cells are believed to have a more differentiating capacity for these specific tissue cell lineages and better integration into host tissue as they share the same niche. The presence of CMZ in lower vertebrates and its homologue ciliary epithelium in higher vertebrates has provided new hope to the regenerative therapeutics for retinal degeneration. CMZ has high proliferative capacity as it contains retinal progenitor cells that continuously add neurons and glial cells throughout life [24-26]. On the other hand, the mammals lack ciliary marginal zone (CMZ) and hence do not appear to possess a regenerative capacity.

However, it has been shown that a region which is similar to CMZ *i.e.* ciliary epithelium in mammals harbors a quiescent population of stem cells. When mitogens are given, incorporation of BrdU was reported in pigmented ciliary epithelium *in vivo*. Besides BrdU incorporation, the expression of cell cycle markers like cyclin D1 and Ki67 have also been observed in ciliary epithelial cells when induced with growth factors [27]. Pigmented CE cells, when cultured in the presence of rhEGF and bFGF, have shown to form colonies which are capable of self-renewal and express cell markers specific to retinal progenitor cells such as Chx10 [20]. Upon stimulation, these progenitor cells have the potential to differentiate into almost all types of retinal cells whether neurons (photoreceptors, bipolar, *etc.*) or supporting cells such as Müller glia cells [28].

The laser-induced photocoagulation induces an injury response resulting in Choroidal neovascularization (CNV) because of the upregulation of VEGF. CNV induces infiltration of blood-derived macrophages which results in the activation of Microglia. Macrophage infiltration is followed by histological changes in the retina [29]. Laser injured models have been shown to exhibit glutamate toxicity which is similar to what is found in other parts of the CNS. The photoreceptors in normal conditions do not exhibit glutamate toxicity while they do so in photocytotoxicity. The disruption of the adjacent cells after the photocoagulation results in the hypertrophy and retinal burns.

We cultured the ciliary epithelial cells in the presence of bFGF and EGF and characterized them for shape, size and number. We found that the 6 day neurospheres showed maximum growth and number. The number and size either vanished or showed a drop by the 7, 8 and 9 days. This could be because of the increase in the size of the cell aggregates, preventing nutrients and gaseous exchange to the central region. The CE cultures were also characterized for their morphology by Scanning electron microscopy. We found aggregates and well-formed rosettes in the micrographs. Our previous studies have shown Ciliary epithelial neurospheres to downregulate the epithelial characteristics and an upregulation of the neuronal characteristics through a Notch, Jagged and β-catenin pathway [16].

The retinal samples were analyzed for gene expression through Real-time PCR for retinal pigment epithelial (RPE), retinal, neurotropic and apoptotic markers. The animals transplanted with CE derived neurospheres exhibited better cell survival and functional recovery, consistent from the shorter to the longer time points. There was a sustained expression pattern in the Rhodopsin and RPE65 markers. At the 3 month time points, the CE transplanted group showed an increased but optimum expression of BDNF in tandem with the RPE65 and Rhodopsin, while the expression of inflammation marker *i.e.* VEGF decreased. There have been studies which demonstrate that CE stem cells display the potential to generate retinal neurons, where the *in-vitro* characterization of these cells exhibits better proportion of markers related to rods and bipolar cells when compared to RGCs [30]. Based on this data, we propose that the CE cells tend to get localized in the RPE and the BDNF plays a redundant role in the protection and survival of non-neuronal cells as well. We could not demonstrate any structural localization or differentiation in-situ, which needs further attention and future studies.

CONCLUSION

There have been various studies on the ciliary epithelial cells conducted *in-vitro*. Cicero *et al*. [18] demonstrated that the ciliary epithelial cells post-mortem, when cultured under mitogens, over a period of three months, and co-cultured with rat neurons, showed synapse formation. At the same time, investigators have shown that the CE cells fail to regenerate as neurons. In the light of this conflicting evidence, we show the transplantation efficacy of human fetal pigmented ciliary epithelial (CE) cells in the above said mouse model of retinal degeneration. The stem cells, upon transplantation, exhibited an upregulation of the candidate markers of retinal genes as well as neurotrophic factors. Our study included 3 animals per group which was the limitation of our study. We could also not analyze the neurospheres for prolonged synaptic activity *in vitro,* or validate their neuronal properties physiologically. Further, characterization of these cells *in vitro* may provide insights into neuronal activity. Our study supports the basis for the ciliary epithelial cells to be further examined for their proliferative and differentiation properties and be used for further transplantation. We did not study the translocation and/or synaptic integration of the transplanted cells into the host retina. More studies in this direction need to be done to determine if these cells have only paracrine effects or do they make functional connections with the remaining cells of the host.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Animal Ethics Committee (IAEC) Approval No. 71(69)/IAEC/423, and Institutional committee for Stem cells in Research & Therapy (IC-SCRT) PGIMER, Chandigarh, India with Approval No. PGI-ICSCRT-53-2014/1469).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are basis of this research. All the animals were used in accordance with the US Public Health Service's "Policy on Humane Care and Use of Laboratory Animals," and "Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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CD34 and CD117 Stemness of Lineage-Negative Cells Reverses Memory Loss Induced by Amyloid Beta in Mouse Model

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A majority of the neurodegenerative disorders including Alzheimer's disease are untreatable and occur primarily due to aging and rapidly changing lifestyles. The rodent Alzheimer's disease models are critical for investigating the underlying disease pathology and screening of novel therapeutic targets in preclinical settings. We aimed to characterize the stemness properties of human umbilical cord blood (hUCB) derived lineage-negative (Lin−) stem cells based on CD34 and CD117 expression as well as surface morphology using flow cytometry and scanning electron microscopy, respectively. The efficacy of the stem cells was tested by its capacity to rescue the injury caused by intrahippocampal delivery of varying doses of amyloid beta. The hUCB Lin− stem cells reversed memory loss due to Aβ42-induced injury more effectively at micromolar concentration, and not picomolar concentration. More studies are required to delineate the underlying molecular events associated with hUCB Lin− stem cells.

Keywords: memory loss, alzheimer disease, amyloid beta-peptides, CD117, CD34, stemness

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INTRODUCTION

Neurodegenerative disorders that have affected millions of people worldwide are untreatable and worsen with age. This includes Parkinson disease, ataxia, amyotrophic lateral sclerosis, glaucoma, Lewy body disease, microvascular brain injury, Alzheimer's disease (AD), and other dementias. Dementia has affected almost 47 million individuals at the age of 65 years and older. This number is projected to be more than 131 million in 2050 (Prince et al., 2016).

The drugs and therapies available at present provide only symptomatic relief without alleviating or halting the disease progression. Most of the drugs that have been successful in preclinical studies have failed during clinical trials (Banik et al., 2015a). Eli Lilly developed a drug solanezumab, targeting amyloid with an aim to delay the progression of AD and then subjected it to clinical trials, which eventually were terminated prematurely (Honig et al., 2018). There was repeated failure of clinical trials in AD; this failure calls for reinvigorated effort to discover effective biotherapeutic approaches (Saraf et al., 2011; Innes and Selfe, 2014; Eyre et al., 2016; Minhas et al., 2017). Simultaneously, there is an urgent need to test other alternative therapies.

Stem cell therapy for AD is one of the various alternative therapies for untreatable disorders. In the APP/PS1 transgenic mouse model, the transplantation of mesenchymal stem cells (MSCs) from human umbilical cord blood (hUCB) (Yang et al., 2013) and Wharton's jelly (Xie et al., 2016) has demonstrated the rescue of cognitive impairment by reducing amyloid deposits. However, none of these studies has characterized the cells used in transplantation.

The ultrastructure of embryonic stem cell-derived cardiomyocytes was characterized by visualizing under scanning electron microscopy (SEM) and transmission electron microscopy (TEM; Taha et al., 2012). Similarly, an overall protocol was standardized for TEM to visualize the ultrastructure details of adult MSCs as well (Miko et al., 2015). The analysis of CD34+ cells by TEM depicts the phenotypic immaturity in umbilical cord blood cells in comparison to cells derived from bone marrow (Deliliers et al., 2001). The flow cytometry analysis has shown that lineage-negative (Lin−) cells proliferative slowly but maintained long-term culture and are more primitive than CD133+ and mononucleated (MNC) populations (Forraz et al., 2004). However, further are required for stem cell characterization based on morphological and surface molecular marker assessments that are essential for any advancement in regenerative medicine.

The Lin− population has been identified as a group of cells participating in the reconstitution of formed elements in hematopoiesis. The Lin−Sca1+CD34+Flt+ cells have been shown to differentiate in lineages other than hematopoiesis. This aspect could be interesting in context to our study. The Lin− population comprises cells responsible for the formation of vascular endothelial cells (Asahara et al., 1997). The endothelial progenitor cells in turn can initiate a number of signaling cascades leading to vascularization (Kalka et al., 2000; Gill et al., 2001) in the sites of peripheral vasculature in ischemia (Asahara et al., 1997) or induced ocular injury (Grant et al., 2002). Reports revealed the complete prevention of vascular degeneration in the CNS derivatives (retina) in transgenic mouse models of neuronal degeneration (rd1 & rd10), which correlated to neuronal rescue (Otani et al., 2004). These findings show that Lin− stem cells act like endothelial precursor cells and stimulate neurotrophic responses in healing/to injury. Lin− stem cells, derived from the umbilical cord blood or bone marrow had earlier been used and their efficacy examined in several neurodegenerative mouse models (Banik et al., 2015b; Jindal et al., 2017; Minhas et al., 2017). These studies report the upregulation of the neurotrophic effect by brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) with the downregulation in GFAP (Minhas et al., 2017). It is suggested that Lin− stem cells might modulate neurotrophic factors when transplanted intravitreally near the site of retinal injury artificially induced by N-methyl-D-aspartate (NMDA). (Jindal et al., 2017). Even though our previous report also showed that hUCB-derived Lin− stem cells exert a therapeutic effect in a dose- and time-dependent manner (Banik et al., 2015b), the shape and size of the transplanted cells were not analyzed or characterized adequately.

The hUCB is an underutilized source of stem cells. Cell-based therapies hold an alternative yet untested promise for AD treatment and there are several cord blood banks worldwide promising such therapies to their clients; hence there is a pressing need to investigate the therapeutic potential of hUCB-derived stem cells. The cord blood banks preserve stem cells from hUCB, for its future use in several untreatable neurological disorders. This study was initiated to characterize the stemness of Lin− stem cells based on the surface marker CD34 and CD117 expression and its comparison with MNCs and lineage-positive (Lin+) cells. The 3D visualization, surface morphology, and size of all three cell types were analyzed using SEM. Further, we developed an animal model of memory loss induced by a higher dose (1 μM) of amyloid Beta-42 (Aβ42) and used this model to analyze the therapeutic efficacy of Lin− stem cells derived from hUCB against a higher dose of Aβ insult. This study aimed to increase the toxic effect in the brain, more precisely in the hippocampus, through a higher dose of 1 μM of Aβ42 compared to the dose of 800 pM used in our previous study. It was decided that this would test a better window of therapeutic action of Lin− stem cells in the injured brain. It was hypothesized that the more exacerbated brain pathology could exert an improved therapeutic effect of these naive stem cells to alter disease pathology. Memory loss in mice was induced by injecting an aggregated form of Aβ42 in their hippocampus. Their memory was analyzed by the Morris water maze (MWM) and passive avoidance tests. Significant impairment was observed in the spatial memory. Purified and enriched hUCB Lin− stem cells were also transplanted into the injury-induced hippocampal region of the mouse. The mouse group with the transplanted Lin− stem cells showed a reversal of memory loss caused by Aβ42. When compared to the earlier findings with a dose of 800 pM Aβ42, an improving effect on memory was observed at the same dose of stem cells transplanted but at a 1 μM dose of Aβ42. At present, it is not clear how a higher dose of Aβ42 could play a role in exerting a better effect of Lin− stem cells in improving the memory in the mice. In future studies, the aim is to focus on deciphering the putative molecular mechanism mediating the therapeutic effect of hUCB Lin− stem cells in the reversal of cognitive impairment.

MATERIALS AND METHODS

All experiments were conducted in a good laboratory practice-compliant lab. Standard operating protocols were prepared and used for the study, following the approval by the quality assurance personnel and the study director. All the data entries and protocols were recorded in data recording sheets and the experimental values or results were verified by independent personnel, duly documented in a verifiable and auditable format.

Study Plan

Preparation of Amyloid β Aggregates

An amount of 0.1 mg of Aβ42 with empirical formula $C_{203}H_{311}N_{55}O_{60}S$ and molecular weight 4514.04 (Sigma-Aldrich) was procured. This was dissolved in 100 μl of 1× phosphate buffer saline (PBS) with pH adjusted to 7.4. In accordance with previously published protocols for Aβ42 oligomerization, the peptide was kept in incubation at 37°C for 4 days and 4°C for 6 h (Ahmed et al., 2010).

Isolation of Lin− Stem Cells From hUCB

HUCB was collected after the delivery of newborns from mothers aged 20–35 years (≥28 weeks gestation). The samples were collected in accordance with the ethical guidelines approved by the Institutional Committee on Stem Cell Research and Therapy (ICSCRT; Approval no. IC-SCRT/03/DTM-2979). Samples were collected after obtaining informed consents from the participants. The UCB was taken from placental and umbilical cord blood vessels using 21-gauge sterile needles in an EDTA (anticoagulant) containing vial. The mononucleated cell (MNC) population was isolated by the Ficoll density gradient method. This population of cells was subjected to magnetic assisted cell sorter (MACS) using a cocktail of antibodies for the Lin+ cell surface marker tagged with magnetic beads. The Lin− population was enriched via negative selection when MNCs were incubated with the Lin+ antibody cocktail and allowed to pass through a magnetic column. The Lin+ population was collected after removing the column from the magnetic field.

Characterization of Stem Cells Isolated From HUCB

SEM Analysis

The UCB-derived MNCs, Lin+ cells, and Lin− cells were plated separately on 12 mm circular cover slips in a 24-well culture dish and incubated at 37°C for 1 h. Samples were immersed in 3% glutaraldehyde buffered with 0.1 M Sorensen's buffer at 0–4°C for 48–72 h. This was then rinsed thrice with 1× Sorensen's buffer for 30 min each. The cover slips containing the cells were then subjected to serial dehydration. The coverslips were dipped into 30% → 50% → 70% → 80% → 90% → 95% → 100% → 100% concentrations of ethanol for 10 min each. The cover slips were dried and mounted on the stubs and the samples coated with platinum. Platinum coating was done in an Auto Fine Coater (JEC3000FC, Jeol, Japan) by platinum sputtering. A current of 20 mA was used with an exposure time of 40 s, under vacuum. The samples were then visualized by using a scanning electron microscope (JSM-IT300, Jeol, Japan). They were scanned using a secondary electron detector at a voltage of 9.0 kV and a probe current of 40.0 A, under high vacuum. Images were acquired at 1000×, 2000×, and 5000×.

Flow Cytometric Analysis

A flow cytometric analysis was also carried out to determine the percentage expression of CD45 (nucleated cell marker), CD34, CD117 (stem cell markers), and CD271 (mesenchymal marker) in these cells isolated from hUCB. About one million cells from each population were suspended in 100 μl FACS buffer (PBS-BSA-Azide solution) and incubated with Fc blocker (20 μl for up to 10^7 cells) (Miltenyi Biotech, USA) for 30 min at room temperature (RT). Then fluorochrome-conjugated antibodies (BD Pharmingen, USA) were added in the tubes as per the requirement and incubated for 1 h at RT. CD45 markers were tagged to FITC, CD34 were tagged to PE, CD117 were tagged to APC, and CD271 were tagged to Cy3 or PerCP fluorophore conjugates. Finally, all the tubes were washed twice with FACS buffer and resuspended in 300 μl buffer and analyzed in FACS Calibur (BD Bioscience, USA) within 2–6 h of processing.

Animals

Six- to eight-weeks-old inbred Swiss albino mice were used after approval from the Institutional Animal Ethical Committee (IAEC-473). Animals were maintained in a 12 h light–dark cycle (LD 12:12). These were fed on a standard diet and had free access to drinking water. Mice were sacrificed using an overdose of anesthesia i.e. xylazine and ketamine cocktail. Brains were immediately isolated and stored at −80°C till further use.

Intrahippocampal Delivery of Aβ42 and Lin− Stem Cells Using Stereotaxis

Six- to eight-weeks-old mice were injected with Aβ peptide using stereotaxis. Mice were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg)–ketamine (100 mg/kg) cocktail. The mouse was then positioned on the stereotaxis apparatus in the prone position, with their ears and front teeth were fixed to prevent any head movement. The skull was exposed by giving an incision on the scalp in the median axis. In the exposed skull, Bregma zero was taken as the reference point and a microsyringe needle was moved to specific Bregma coordinates: anteroposterior (AP) +2 mm, mediolateral (ML) ±2 mm, and dorsoventral (DV) −2.5 mm for specific delivery at the dentate gyrus in the hippocampus region, as previously standardized in the lab.

Craniotomies for the bilateral injection, by exposing the skull and injection points, were marked using a 26G needle according to the Bregma scale, following the stereotaxic coordinates (Paxinos and Franklin, 2004). A 1 μM concentration of aggregated Aβ solution, in 5 μl of PBS, was injected at a rate of 1–2 μl/min using a rate-controlled microinjector. After the solution was delivered in the hippocampus, the needle of the microsyringe was kept unmoved for 5 min for proper diffusion of the solution and then slowly removed from the brain by unscrewing the arm of the injection. In the vehicle-treated group, PBS was injected bilaterally and a needle was inserted in the sham surgery group without any Aβ42/vehicle delivery. Similarly, either 50,000 hUCB Lin− stem cells suspended in 1× PBS or 1× PBS alone as vehicle were transplanted at the same site of injury after 21 days of Aβ42 injection using stereotaxic apparatus.

Behavioral Analysis

Evaluation of Spatial Memory by Morris Water Maze (MWM) Experiment

MWM was performed to evaluate the spatial memory of the mice subjected to various experimental conditions, i.e., Aβ42-injected (injury) group, vehicle control, sham control, and stem cell-transplanted group at day 10 post-transplantation. Before subjecting the mice to MWM, the mice were screened for their swimming ability and motor functioning. On day 0, mice were allowed to swim freely for 2 min to examine their swimming ability and subjected to rotarod screening and excluded from the study if found to have irregular muscle coordination. The mice with normal behavioral pattern and vision were included for further experimentation in MWM. The basic MWM protocol for the navigation task included a circular pool where visual cues were placed on the walls and pool side. The tank was divided into four artificial equal

quadrants—T1, T2, T3, T4—and a hidden platform was placed in compartment T1 submerged 1 cm below the water surface. The protocol of seven days was designed and included 6 days acquisition and 7th day retrieval, each day consisting of 4 trials and each trial lasting 120 s. The entire experiment was video-tracked using automated Anymaze software connected with a webcam, which was mounted to obtain an aerial view of the pool. The water temperature was maintained at 21°C (Vorhees and Williams, 2006; Weitzner et al., 2015) and colored black to provide a contrast of white Swiss albino mice so that Anymaze software can identify, distinguish, and track the animal against the background. Using the Anymaze software, the experiment was designed by marking the marginal area of the pool divided into the four quadrants and the hidden platform. In the protocol, events and trials were assigned e.g., entry to platform area, entry to each quadrant etc. The various parameters were analyzed, including escape latency time, mean speed, time spent in each quadrant, distance from each quadrant, and mean distance from the platform (search error). The escape latency time was compared in the different groups. Further, the swimming track plots from all the trials were recorded to analyze their index of learning.

Passive Avoidance

This is a fear-aggravated test in which mice subjected to various experimental conditions are evaluated for learning behavior. The equipment is made up of one lit compartment and one dark compartment. The 3-day experiment was set up and each trial lasted maximum 5 min. On the first day, the mice were kept in a lit chamber and allowed to move freely. After 24 h, in the acquisition/condition phase, mice were kept in the lit chamber. When a mouse moved to the dark chamber, it received a mild foot shock of 20 mA. On the test day, the latency time, i.e. the time taken by the mice for crossing the gate to avoid the stimulus, was calculated and noted; this latency time is associated with memory and learning. The groups were analyzed and compared for the results to analyze the learning-associated memory.

Congo Red Staining

The Aβ42 aggregates were identified using Congo red staining of brain cryosections of the hippocampal region. The cryosections were fixed using histochoice and then hydrated with 90, 70, and 50% ethanol, followed by washing in distilled water. The slides were stained with 1% alcoholic Congo red solution for about 30 min at RT and the nuclei were counterstained with hematoxylin. Excess staining were removed by immersing the slides in 70% alcohol for a few seconds and then cleared in xylene for 30 min before mounting it with a fluorosave mounting medium (Calbiochem, USA).

Immunohistochemistry

To further confirm the Aβ42 aggregates, immunohistochemistry was performed. The Aβ42 primary antibody (Elabscience) was used at 1:100 dilution and incubated overnight at 4°C after serum blocking. The TRITC donkey antirabbit secondary antibody was used at 1:200 dilutions. Washing was done using 1× PBS and counterstained with DAPI. The sections were analyzed using a 532 nm laser line for excitation in confocal microscopy (Olympus FV1000) and the images were merged using its software.

Statistics

All results were analyzed as mean ± SEM in Microsoft Excel. The data were arranged and statistically analyzed using SPSS software version 16.0. In MWM, repeated-measure ANOVA was used for repetitive observation on acquisition days and retrieval trial. Further, a *post hoc* analysis was carried out using least significant difference (LSD). In the passive avoidance test, an independent *t*-test was applied. The values were considered statistically significant if $p \leq 0.05$ in the results.

RESULTS

Standardization of Bregma Coordinates for Hippocampal Injection

Memory loss was induced in 6 to 8-weeks-old Swiss albino mice using intrahippocampal Aβ42 injection by stereotaxic surgery. The schematic represents the skull sutures in the exposed mice brain and the Bregma zero point, from where the axis for hippocampal region was located (**Figure 1a**). For intrahippocampal delivery, bregma coordinates of the skull were standardized by injecting crystal violet dye at anteroposterior axis +2 mm, mediolateral axis −/+ 2 mm, and dorsoventral axis −2.5 mm. The crystal violet dye dispersed throughout the hippocampus with a prominent needle track in the right hemisphere, shown in the coronal section visualized under a dissecting microscope, and only a needle track in the left hemisphere where a needle was inserted without injecting the dye (**Figure 1b**). Further, these coordinates were used for Aβ42 injection and hUCB Lin− stem cell transplantation.

SEM Characterization of Stem Cells Isolated From hUCB

SEM analysis revealed the morphology and size of all the three cell types isolated from hUCB (**Figure 2**). MNCs show heterogeneous populations of immature RBCs and varying lymphocytes. They also show variation in shape, size, and structure. The MNC population was found to be of varying size ranging from 3 to 6 μM in diameter (**Figures 2A,B**). Lin+ cells were found to be in clusters with even-sized microbeads (**Figure 2C**) and they also showed heterogeneous populations with varying size similar to MNCs (**Figure 2D**). Lin− cells showed homogenous population with the same shape, size, and structure. These cells were 5 μM in diameter and uniformly distributed (**Figures 2E,F**). There were no magnetic beads found to be tagged to these cells, confirming their purification by negative selection in a magnetic field.

Flow Cytometric Analysis of Stem Cells Isolated From hUCB

All the three cell types isolated from hUCB were analyzed in a flow cytometer for the presence of nucleated marker (CD45), stem cell markers (CD34, CD117), and mesenchymal markers (CD271; **Figure 3**). When the side scatter population was gated against the CD45-FITC channel, the proportional

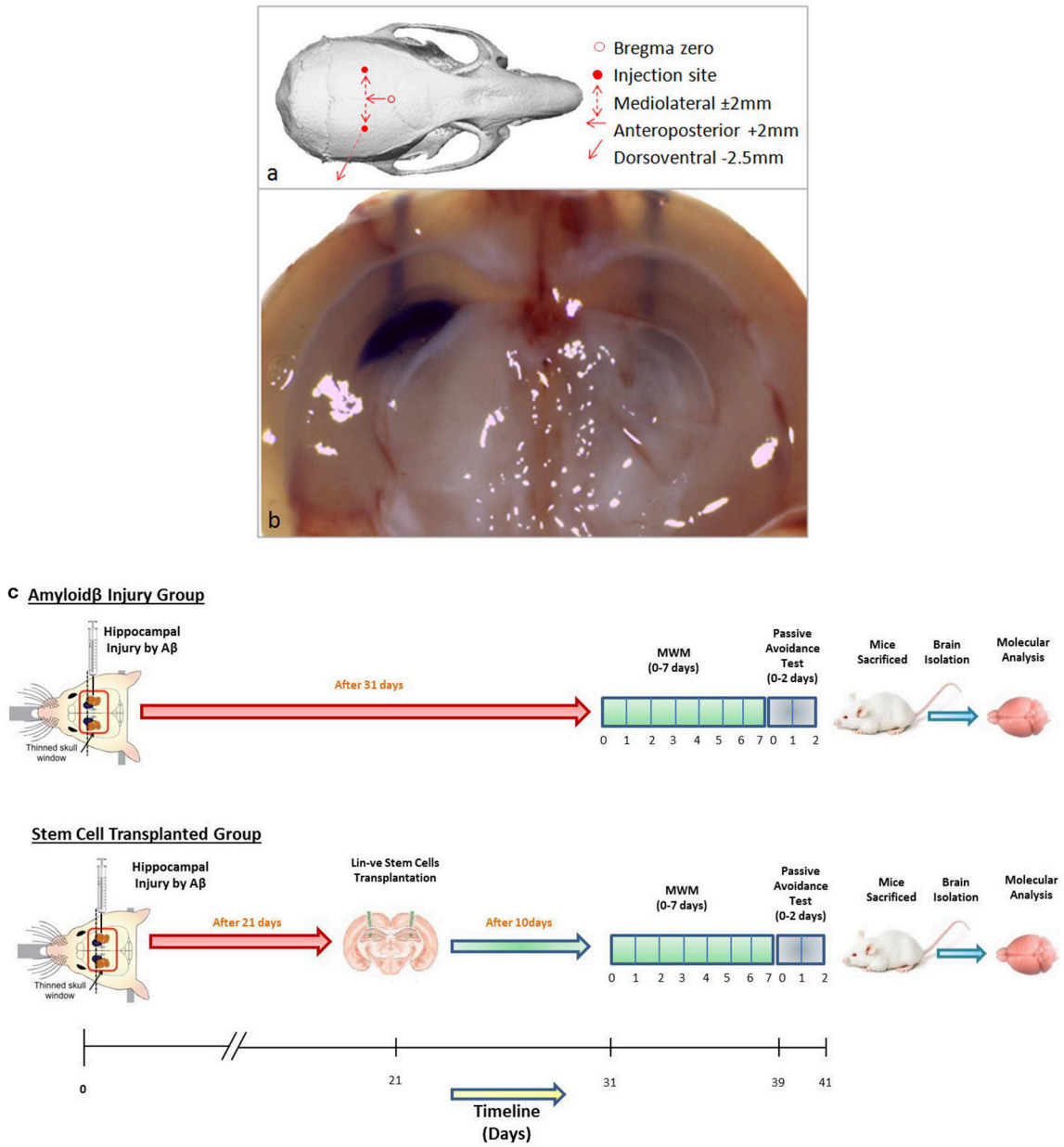

FIGURE 1 | (a) Schematic representation of mouse skull bones showing Bregma zero point and site of injection for hippocampal delivery. **(b)** The gross coronal section of mouse brain shows the injected 2 μl of crystal violet dye diffused throughout the hippocampal area with a needle track on the right hemisphere. In the left hemisphere, a needle was inserted without injecting crystal violet. **(c)** The schematic of the *in vivo* study design of the Aβ injury group and the stem cell-transplanted group.

expression of CD45 was comparable to MNCs (48.68%), Lin+ (41.05%), and Lin− (51.81%; **Figures 3A–D**). Further, CD34 and CD117 percentage expression was gated among the CD45 positive cells from all the cell types. In both the cases, CD34 and CD117 stem cell expression was found to significantly increase in Lin− cells as compared to MNCs and Lin+ cell types. CD34 was highly expressed (24.36%) in Lin− cells, while in MNCs (1.9%) and Lin+ cells (2.8%), reduced expression was noted (**Figures 3E–H**). Similarly, CD117 expression was also found to be significantly high in Lin− cells (19.36%) as compared to MNCs (1.73%) and Lin+ cells (3.15%; **Figures 3I–L**), suggesting that Lin− cells are an enriched population of stem cells. We also examined the presence of CD271, a mesenchymal marker in the stem cell population. The data showed reduced percentage of CD271 (5–12%), which was comparable among all the cell types (**Figures 3M–P**). This shows that Lin− enrichment comprised the nascent hematopoietic stem cell population.

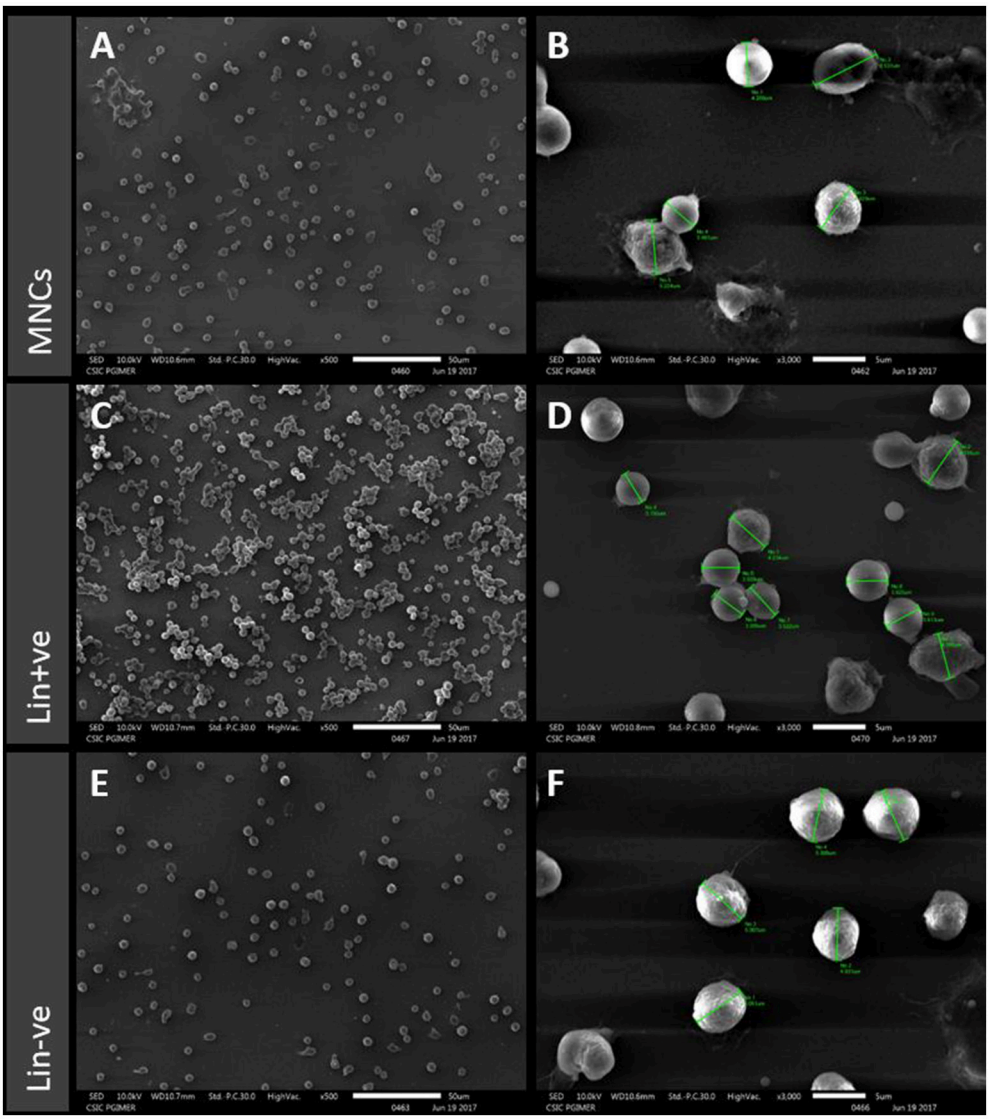

FIGURE 2 | Scanning electron microscopy (SEM) images of MNCs **(A,B)**, Lin+ **(C,D)** and Lin− **(E,F)** from hUCB for morphological characterization. MNCs show heterogeneous populations with variation in shape, size, and structure. The Lin+ cells show similar heterogeneous populations and clusters around even-sized microbeads whereas the Lin− cells show homogenous population and have the same shape, size, and structure.

However, no other lineages such as mesenchymal lineage were observed.

Brain Sections Reveal Aβ42 Deposition and hUCB Lin− Stem Cells Upon Transplantation

Stereotaxic surgery was performed to deliver 1 μM concentration-aggregated oligomers of Aβ42. To identify the amyloid-aggregated brain, sections were stained with alcoholic Congo red (Lorenzo and Yankner, 1994). Congo red binds to the Aβ42 aggregates and imparts dark red/brown color. Stains were identified near the dentate gyrus region of the hippocampus, confirming the deposition of injected Aβ42 in the mouse brain (**Figures 4a–d**). Further, immunohistochemistry confirms the presence of Aβ42 aggregates that were analyzed using confocal microscopy (**Figures 4g–n**). MACS-sorted hUCB Lin− stem cells were pre-labeled with CFDA dye and transplanted at the site of injury. Transplanted cells were identified in brain sections upon 10 days post-transplantation under the confocal microscope (**Figures 4e–f**).

Aβ42-Induced Memory Deficits Were Reversed by hUCB Lin− Stem Cells

After Aβ injection, the mice were tested for memory loss with the Morris water maze (MWM) experiment. Spatial

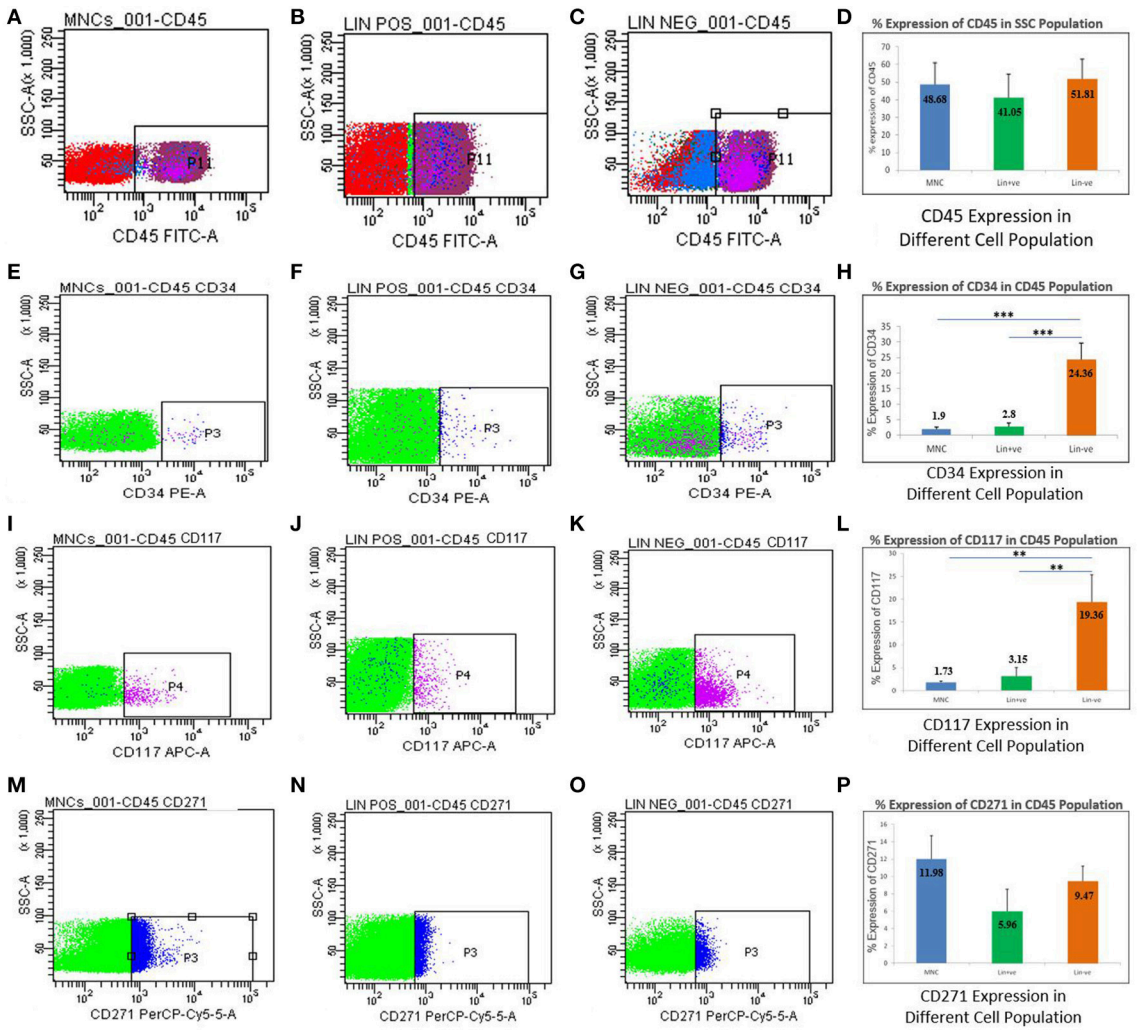

FIGURE 3 | Flowcytometric analysis for MNCs, Lin+ cell, and Lin− cell populations isolated from hUCB. Percentage expression of CD45 showing representative dot plots on three different populations in hUCB **(A–C)** and their average expression shown in a bar graph. **(D)** Representative dot plots showing the expression of CD34 out of CD45 positive cells **(E–G)**. Graph showing the expression of CD34 higher in Lin− cells as compared to MNCs and Lin+ cells **(H)**. Representative dot plots showing the expression of CD117 out of CD45 positive cells **(I–K)**. Graph showing the expression of CD117 higher in Lin− cells as compared to MNCs and Lin+ cells **(L)**. Representative dot plots showing the expression of CD271 out of CD45 positive cells **(M–O)**. Graph showing the expression of CD271 is not significantly different in MNCs,Lin+ and/or Lin− cells **(P)**. $N = 13$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

memory was further tested by subjecting the mice to the MWM on the 10th day post-transplantation of hUCB Lin− stem cells (**Figure 1c**). The increased escape latency time (ELT) indicated the significant spatial memory loss in mice treated with Aβ42. The mice transplanted with hUCB Lin− stem cells showed day-wise decrease in escape latency comparable to the mice in the healthy, vehicle, and sham control groups. The HUCB Lin− stem cell transplantation group showed a significant improvement in special memory as compared to the Aβ42 injury group (**Figure 5A**). The quadrant time spent by mice was calculated in each quadrant of the MWM tank on retrieval day (day 7). In **Figure 5B**, the amyloid injury group mice showed less time spent in target quadrant Q1 as compared to healthy, vehicle, and sham control groups, whereas more time was spent by mice transplanted with hUCB Lin− cells. In **Figure 5C**, the mean distance from the platform traveled on retrieval day (day 7) in four consecutive trials by the mice was calculated and plotted. The results depict that increased mean distance (from the platform) was traveled by amyloid-induced injury mice as compared to mice in control groups, and this distance was again reduced significantly in the stem cell-transplanted group in comparison to the injury group.

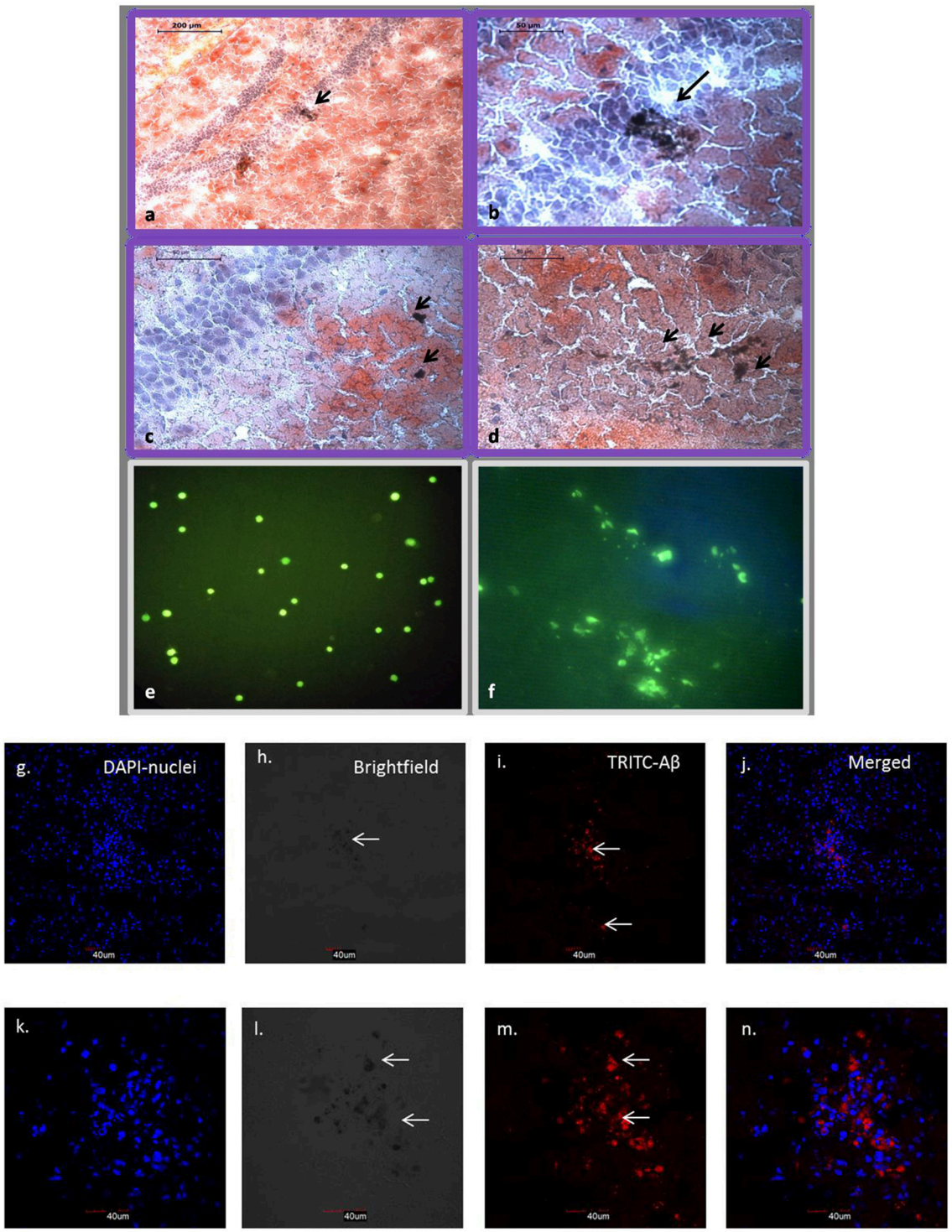

FIGURE 4 | Brain sections with Congo red staining show dark brown Aβ42 aggregates in and around hippocampal region **(a–d)**. **(a)** 10× view of hippocampal region with arrow showing Aβ42 deposition. **(b–d)** 40× view with arrows showing Aβ42 deposition in the dentate gyrus region. CFDA labeled hUCB Lin− stem cells before and after transplantation ($N = 3$; **e,f**). **(e)** Confocal image shows CFDA positive (green) MACS sorted hUCB Lin− stem cells before transplantation. **(f)** Transplanted cells were identified in brain sections upon 10 days post transplantation under the confocal microscope (**Figure 2B**). **(g–n)** Further, Aβ42 aggregates were confirmed by immunohistochemistry at 20× **(g–j)** and 60× **(k–n)** magnification using confocal microscopy ($N = 3$). Aβ42 aggregates were seen in TRITC filter at 532, which gives red fluorescence tagged to secondary antibody **(i,m)**; nuclei were counter stained with DAPI (blue; **g,k**) and aggregates were also seen as dark spots in bright field **(h,i)**.

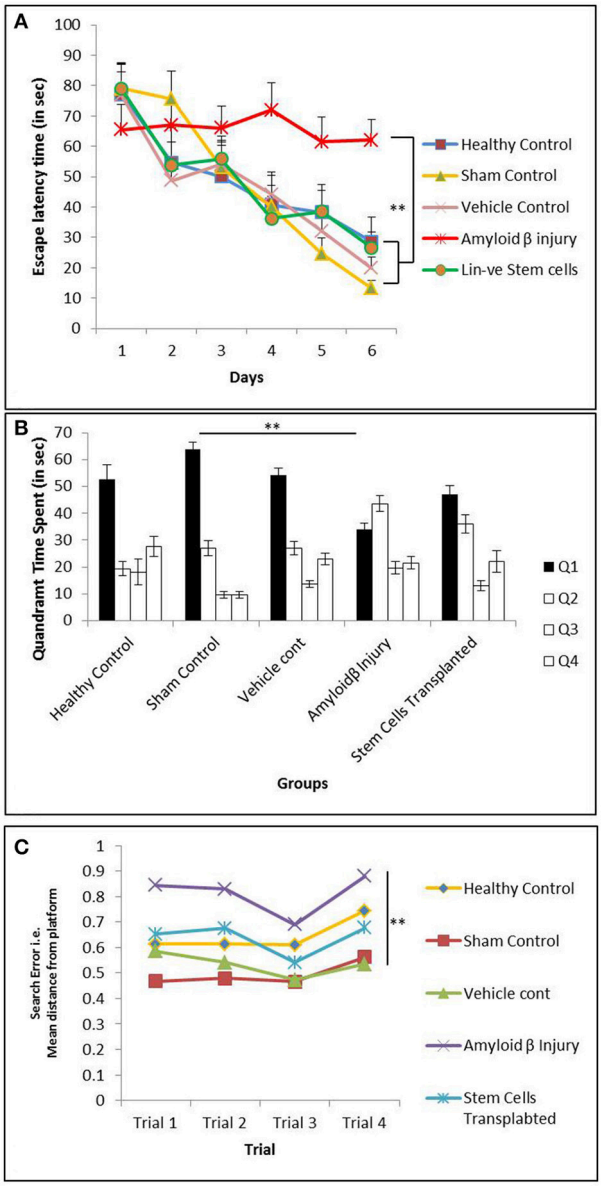

FIGURE 5 | The MWM analysis showing memory impairment in mice injected with Aβ42 and further recovery after hUCB Lin− stem cells transplantation. **(A)** Graph depicts day-wise escape latency time (ELT) (s) during acquisition trials taken by mice in healthy control ($N = 5$), sham control ($N = 7$), vehicle control ($N = 10$), Aβ injury ($N = 7$), and Lin− stem cell-transplanted ($N = 8$) groups. Aβ42-injured mice showed significant memory loss by their never reducing ELT compared to healthy control, whereas hUCB Lin− transplanted mice with Aβ42 injury showed ameliorated memory with reducing ELT along the acquisition days 1–6. **(B)** Retrieval trials on day 7 showed quadrant time (s) spent by mice from different groups in the four Q1, Q2, Q3, and Q4 quadrants. Aβ42-injured mice could not spend maximum time in target quadrant (Q1) as that spent by the control mice, whereas after hUCB Lin− transplantation mice recovered their retrieval memory depicted by their maximum time in Q1 searching for the hidden platform. **(C)** The search error during retrieval trials (day 7) showing the mean distance from the hidden platform was found to be significantly increased in Aβ42-injured mice while it was significantly decreased in Lin− transplanted mice suggesting recovery in memory. Data were analyzed using SPSS repetitive measure ANOVA test followed by LSD *post hoc* analysis (**$p \leq 0.01$).

Swimming Track Plots Confirm Loss of Memory in Aβ42-Injured Mice and Recovery in Memory After hUCB Lin− Stem Cell Transplantation

Anymaze software-assisted swim track plots for all the trials were recorded for analysis using an automated camera. The track plots observed on day two, four, and six of the acquisition trials were evaluated for healthy, sham control, amyloid injury, and stem cell-transplanted groups. The healthy and sham control mice were found to have reduced swimming path with acquisition days. The Aβ42-injured mice were found to be swimming toward the edges of the MWM tank instead of moving near the platform, suggesting their memory impairment. These Aβ42-injured mice after hUCB Lin− stem cell transplantation showed striking reduction in their swimming path to reach the platform along the days of acquisitions as marked in the control groups (**Figure 6**).

Passive Avoidance Analysis Showed Significant Improvement of Fear Conditioning Memory After hUCB Lin− Stem Cell Transplantation

To further confirm the memory and learning in different groups, mice were subjected to another behavioral assay i.e., passive avoidance. The time taken by mice to avoid an aversive stimulus (i.e., electric shock) is proportional to the index of learning and memory. Memory performance is positively associated with the time taken by mice to move from the lit compartment to the dark compartment; more latency denotes a strong recollection of fear conditioning. The results showed a significant difference between control and Aβ42-injured mice ($p < 0.001$), with reduced latency time in Aβ42-injured mice. When these mice were transplanted with hUCB Lin− stem cells, the latency time significantly increased in passive avoidance ($p < 0.001$). This experiment further confirms memory loss in amyloid injury group and recovery by stem cell transplantation (**Figure 7**).

Higher Dose of Aβ-42 (1 μM) Was Found to Be More Effective Than 800 pM in Exerting the Therapeutic Effect of Lin− Stem Cells

A previous study had shown that a lower dose (800 pM) of Aβ42 could exert a cognitive deficit in mice, and a dose- and time-dependent improvement in memory upon hUCB Lin− stem cell transplantation (Banik et al., 2015b). This study aimed to test the efficacy of Lin− stem cells on the impact of a higher dose (1 μM) of Aβ insult. In MWM analysis, although the acquisition and retrieval trials recorded a significantly higher ELT and quadrant time, respectively for groups treated with 800 pM compared to 1 μM Aβ, but interestingly, the stem cell-transplanted groups showed significant cognitive improvement with 1 μM in Aβ-injured mice compared to the mice that received the 800 pM dose, as evident from the recorded ELT, quadrant time, and search error analysis (**Supplementary Figure 1**). In acquisition trials, the difference in ELT between Aβ-1 μM and Aβ-1 μM+Lin−

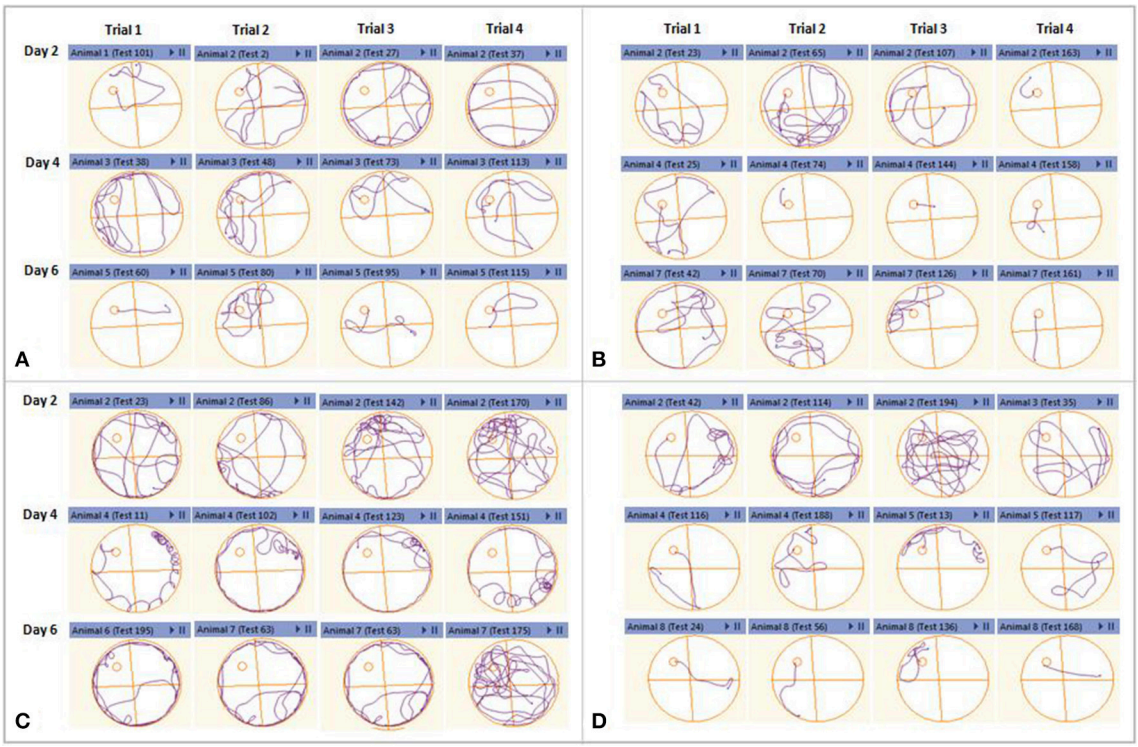

FIGURE 6 | Representative swimming track plots from acquisition day 2, 4, and 6 of different groups as an index of learning. Each day had four consecutive trials. **(A)** Healthy control and sham control. **(B)** Mice showed reduction in swimming path to reach the hidden platform along the acquisition days. **(C)** Aβ42-injured mice could not reduce their swimming path as the acquisition days progress. **(D)** After hUCB Lin− stem cell transplantation mice showed striking reduction in their swimming path to reach the platform along the days of acquisitions as marked in the control groups.

SC was found to be significantly higher ($p < 0.001$) than the difference between Aβ-800 pM and Aβ-800 pM+Lin− SC ($p < 0.05$). In retrieval trials, there was a significant difference between Aβ-1 μM and Aβ-1 μM+Lin− SC groups in quadrant time ($p < 0.05$) and search error analysis ($p < 0.05$) while these differences were found to be non-significant between Aβ-800 pM and Aβ-800 pM+Lin− SC groups. These findings strongly suggest that a higher dose (1 μM) of Aβ could exert an increasing therapeutic effect of Lin− stem cells in this cognitive impairment model.

DISCUSSION

Various animal models are in use for understanding memory loss and dementia and to validate therapies. We created Aβ-induced injury in a mice model of cognitive impairment as it is one of the most common hallmarks of AD. We first validated the effect of Aβ42 on memory loss in the mouse model using the Morris water maze and passive avoidance behavioral tests. The structural analysis of Aβ42 has shown that it exists in monomeric, oligomeric, and fibrillary forms (Hepler et al., 2006). The monomeric form is neuroprotective in nature (Giuffrida et al., 2009), whereas oligomeric forms exert neurotoxicity in the brain by impairing synaptic plasticity (Cleary et al., 2005). The oligomeric preparation was made as per the published protocol (Ahmed et al., 2010). It was then administered stereotactically via intrahippocampal delivery, and the memory loss was evaluated by the behavioral experiments mentioned above. The parameters were analyzed using escape latency time, quadrant time, and the mean distance from the platform, which indicated the memory loss induced by Aβ42. The video track plots further confirmed behavioral alteration in the swimming pattern in the treated mice. The results suggest that the oligomeric form of Aβ42 induces memory loss at 31 days after intrahippocampal injection. As the current therapeutic treatments against Alzheimer's and dementia only provide symptomatic relief without alleviating disease pathology and have failed to show any therapeutic benefits in clinical trials (Doody et al., 2014), Lin− stem cells derived from hUCB were used to simulate the microenvironment in the proximity of artificially induced neurotoxic plaques so that their clearance could be examined (Tong et al., 2015; Wang et al., 2015). The bilateral transplantation of Lin− stem cells from hUCB in the intrahippocampal regions of Aβ-injured mice was carried out. SEM analysis revealed that Lin− cells possess homogenous morphology with similar shape, size, and structure. The absence of magnetic beads around these cells also confirmed that these are the enriched population of stem cells without any markers for Lin+ cells. Further, these cells also showed a significantly higher percentage expression of stem cell markers

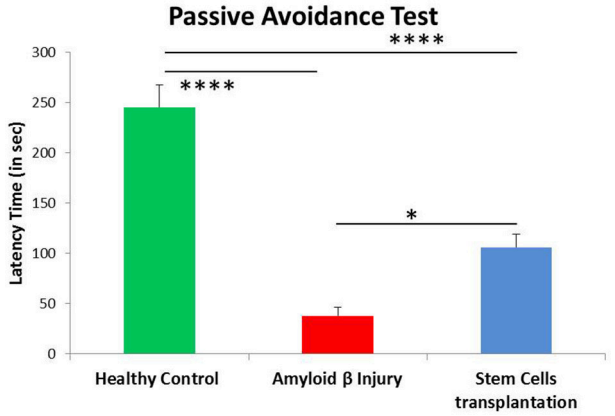

FIGURE 7 | Passive avoidance analysis showed significant improvement of fear conditioning memory after hUCB Lin− stem cell transplantation. Aβ-injured mice ($N = 8$) showed significant reduction in latency time compared to healthy control mice ($N = 7$; ****$p < 0.0001$), whereas latency time was significantly increased after Lin− stem cell transplantation ($N = 8$; *$p < 0.022$). Data were analyzed using SPSS one-way ANOVA.

such as CD34 and CD117 compared to MNCs and Lin+ cell types. Mesenchymal marker, CD271 expression on these cells confirmed the homogeneity of the transplanted cells. These findings suggest that these cells are the naïve cells present in the hUCB population and might be capable of exerting therapeutic effects in the injury model.

Approximately 50,000 hUCB Lin− stem cells were purified and transplanted in Aβ42-injected mice. This showed reversal in spatial memory and working memory after 10 days of transplantation. We used a higher dose (1 μM) of Aβ42 than the previously used 800 pM (Banik et al., 2015b) to test whether Lin− stem cells are able to rescue the behavioral phenotype even at higher doses. We report that 1 μM of Aβ42 is not only a better dose of injury but also provides a better substrate for Lin− cells to significantly improve learning and memory when transplanted. Earlier findings have revealed that aggregated oligomeric forms of Aβ42 are the most neurotoxic in nature (Cleary et al., 2005; Ahmed et al., 2010), whereas a lower dose of Aβ, in nanomolar concentration, can exert a neuroprotective effect through its antioxidative effects evident from the CSF analyzed lipoproteins (Kontush et al., 2001). Aβ in the CNS exerts effects that range from amyloid angiopathy (Attems et al., 2010) to associated neurotoxicity in the AD brain. It was interesting to note that the higher concentration of Aβ (1 μM) displayed a lower decline in cognition as compared to a low concentration of Aβ (800 pM) in the "injury only" mice. Although a high dose of Aβ is expected to exert severe injury in the brain, the duration (21 days) after which the mice were subjected to spatial memory assessment could have provided sufficient time to mount a superior compensatory response as compared to injury at lower concentration at the same time point. It can, therefore, be concluded from the previous findings that severity, location, and time of analysis may impact the neurobehavioral outcomes in patients also. Consequently, this may influence the healing process in an injured brain (Anderson et al., 2011). Although we cannot ascribe the cause of increased cognitive impairment to 800 pM Aβ-injured mice, we speculate that the increased number of mast cells and the microglial cell recruitment at the site of insult after severe injury (1 μM of Aβ) may have more effectively ameliorated the recovery cascade after 3 weeks of injury as compared to the other dose. This could have led to a differential neurobehavioral outcome as compared to 800 pM Aβ-injured mice (Kempuraj et al., 2017).

The extensive literature on stem cell transplantation studies has focused on embryonic and Induced pluripotent stem cells (IPSCs), completely neglecting the potential of autologous transplantation of stem cells banked in commercial cord banks, although there are limited studies. The intravenous transplantation of MSCs shows the modulation of the inflammatory environment in the traumatic brain injury (TBI) animal model, mediated by the enhanced expression of anti-inflammatory cytokines and the reduction in proinflammatory cytokines (Zhang et al., 2013), appearing to indicate the potential of biotherapeutics over synthetic drug development strategies. The bilateral hippocampal transplantation of MSCs isolated from UCB in the APP/PS1 transgenic mice model has also resulted in the reduction of Aβ plaques by an increased expression of neprilysin (Kim et al., 2012), but this has not been followed up with additional studies. Similarly, UCB-MSCs have shown improvement in the cognition and reduction of Aβ in APP/PS1 double transgenic mice at 33 weeks 4 days analysis (Lee et al., 2012). In the amyloid-infused model, MSCs have been shown to increase hippocampal neurogenesis and differentiation of neuronal precursor cells (NPCs) by the Wnt signaling pathway when analyzed at 2 and 4 weeks post-transplantation (Oh et al., 2015). Similarly, the neural stem cell transplantation in the triple transgenic mice model, i.e., APP/PS1/tau, has shown BDNF-induced amelioration of spatial memory and increased synaptic plasticity (Blurton-Jones et al., 2009). The adipose-derived stem cells migrated to the brain by crossing the blood brain barrier when infused intravenously in the Tg2576 mice model of AD and subsequently ameliorated memory loss by upregulated expression of VEGF and IL10. Together, these studies provided the rationale for testing the effects of stem cell transplantation in the Aβ-injury mouse model.

Even though these preclinical studies have shown variable effects using different sources of stem cells, the current study points out that the stem cell effects are either borne out by their paracrine effects or mediated by inducing the proliferation of endogenous stem cells (Ryu et al., 2016). In other models of AD, stem cells have been shown to increase the expression of synaptic proteins and ameliorate the disease pathology, which was not under testing here. Other studies also show the immunomodulatory action of stem cells by changing the expression of anti-inflammatory and proinflammatory cytokines as well as microglia activation. These cells have been shown to translocate at the site of injury and show homing and differentiation in the niche. They also exhibit paracrine effects and trigger endogenous/exogenous healing responses. Our previous study identified transplanted cells even 60 days after

transplantation in the mouse brain, but these cells were not found to be differentiated into any neuronal cell types (Banik et al., 2015b). Although the present study did not elucidate the biochemical or molecular pathway involved in the behavioral outcome, we speculate that this therapeutic effect of the intrahippocampal transplantation of stem cells could be mediated by the paracrine effects of neurotrophic factors, such as GDNF, CNTF, and BDNF rather than direct replacement of damaged neurons. The secretion of neurotrophic factors, especially BDNF, has been shown to result in increased CREB phosphorylation via the TrkB pathway in other systems (Guo et al., 2017). This might activate genes that regulate cognitive function, neurogenesis, cell proliferation, differentiation, cell migration, or synaptogenesis. Future mechanistic studies could be carried out by administering the inhibitors of BDNF and CREB to investigate how Lin− stem cells promote learning through the BDNF–CREB pathway, if at all.

ETHICS STATEMENT

The stem cells were used after approval by Institutional Committee on the Stem Cell Research and Therapy (ICSCRT; Approval no. IC-SCRT/03/DTM-2979). Swiss albino mice were used after approval from Institutional Animal Ethical Committee approval (IAEC-473).

AUTHOR CONTRIBUTIONS

PB conducted all the experiments, data analysis, and writing. SB conducted work of stem cell characterization and data analysis. AB was involved in manuscript writing and data analysis. BN contributed to co-conceptualization of manuscript. AA was involved in the complete conceptualization and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00222/full#supplementary-material

Supplementary Figure 1 | MWM analysis shows the difference between 800 pM and 1 μMAβ treated groups ($N = 7$) with their respective Lin− stem cell transplantation groups. Stem cell-transplanted groups showed significant improvement in cognition in 1 μM of Aβ-injured mice ($N = 8$) compared to 800 pM dose. **(A)** In acquisition trials, difference in ELT between Aβ-1 μM and Aβ-1 μM+Lin− SC was significantly higher (**$p < 0.001$) compared to difference between Aβ-800 pM ($N = 5$) and Aβ-800 pM+Lin− SC ($N = 5$; *$p < 0.05$). **(B)** In retrieval trials, the quadrant time was significantly improved in Aβ-1 μM+Lin− SC groups compared to Aβ-1 μM (*$p < 0.05$), whereas it was non-significant between Aβ-800 pM and Aβ-800 pM+Lin− SC groups. **(C)** In search error, the distance from the hidden platform was significantly reduced in Aβ-1 μM+Lin− SC groups compared to Aβ-1 μM (*$p < 0.05$), whereas it was non-significant between Aβ-800 pM and Aβ-800 pM+Lin− SC groups. Data were analyzed using SPSS repetitive measure ANOVA test followed by LSD *post hoc* analysis.

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Article

Alteration of neurotrophic factors after transplantation of bone marrow derived Lin-ve stem cell in NMDA induced mouse model of retinal degeneration.[†]

Running head: Lin-ve stem cells in retinal degeneration

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Abstract

Retinal ganglion cell layer (RGCs) is one of the important layers of retina, depleted in Glaucoma. Loss of RGC neurons is a major cellular mechanism involved in its pathogenesis resulting in severe vision loss. Stem cell therapy has emerged as a potential strategy to arrest the apoptotic loss of RGCs and also replace the degenerative cells in damaged retina. Here we have investigated the incorporation and survival of mouse bone marrow derived Lin-ve stem cells in N-methyl-D-aspartate (NMDA) induced mouse model of retinal degeneration. Two days after intravitreal injection of NMDA (100mM) showed significant decrease in ganglion cell number and increase in TUNEL positive apoptotic cells in retinal layers. The injury was further characterized by immunohistochemical expression of Brn3b, GFAP, Bcl2, pCREB, CNTF, GDNF and BDNF in retinal layers. Lin-ve cells (100,000 dose) were intravitreally transplanted after 2 days of injury and evaluated after 7, 14 and 21 days of transplantation. Transplanted cells were found to have migrated from intravitreal space and incorporated into injured retina at 7, 14 and 21 days post transplantation. At 21 days Brn3b, CNTF and BDNF expression was found to be upregulated whereas GDNF was downregulated when compared to respective injury time points. Molecular data showed decrease in the expression of Brn3b, BDNF, CNTF and GDNF post transplantation when compared with injury groups. This study reveals that Lin-ve stem cells may exert neuroprotective effect in damaged retina mediated by participation of neurotrophic factors induced by stem cell transplantation at the site of injury.

Keywords: Bone marrow, NMDA, Lin-ve, Stem cell, BDNF, CNTF

Introduction: Neuronal degeneration is the major cause of visual impairment associated with various retinal disorders such as glaucoma, age related macular degeneration (AMD), and retinitis pigmentosa**.** In Glaucoma, there is selective loss of retinal ganglion cells (RGCs). Interestingly RGC neurons are protected in AMD. Damage to RGC layer in Glaucoma occurs either due to damage to ganglion cell axons (Osborne et al, 1999) or insufficient supply of neurotrophic factors in RGC (Quigley et al, 2000; Pease et al, 2000) or due to overexcitement of ganglion cells (Dreyer et al, 1996; Siliprandi et al, 1992). Excessive excitation of NMDA receptor in retina results in the increased calcium influx that activates various enzymes which destroy the cell structure and DNA. The structural and functional integrity of RGCs are critical for visual perception because these neurons transmit visual signals to the brain. Therefore, a major therapeutic aim in glaucoma is to facilitate the survival of RGCs. NMDA activity resembles glutamate neurotransmitter, high concentration of which results in excitotoxicity of neurons through NMDA receptor. Therefore, NMDA induced retinal degeneration model is optimum good source to investigate disease pathophysiology and novel therapeutic interventions in preclinical settings.

Currently, there is no effective treatment to retrieve vision loss or alter underlying disease pathology in diseases of retinal degeneration. Cell based therapeutic approach may help in restoration of vision to some extent. It was shown that release of neurotrophic factors at the site of injury by stem cells enhances the regenerative capacity of host tissue (Tobias et al, 2003; Zhang and Wang, 2010; Garcia et al, 2004).

Neurotrophic factors are chemicals that stimulate and control neurogenesis. They provide a therapeutic strategy for the treatment of various retinal and CNS degenerative disorders. These are actually proteins that promote the survival of specific cell population during development and maintain the health of mature cells. They may act upstream in the programmed cell death molecular cascade to prevent or delay cell death.

BDNF shows expression in Muller cells and RGCs (Seki et al, 2003) and is important for the survival of retinal ganglion cell (Martin et al, 2003). BDNF is essential for the development of neurons as well as for cell survival and synaptic activity (Binder et al, 2004). BDNF exerts its pro-survival effects by binds to its receptor TrkB and activate the phosphatidylinositol 3-kinase (PI3K)/Akt, which leads to deactivation of proapoptotic targets, and the extracellular signal-regulated kinase (ERK), which results in phosphorylation of the cAMP response element binding protein (CREB) that induces transcription of various genes associated with neuronal survival (Kimura et al, 2016).

CNTF was found to be supportive for the survival of ganglionic neurons. In the retina, CNTFR-ά has been localized to retinal pigmented epithelial cells, rods and cones, inner nuclear layer, and retinal ganglion cells and their axons (Beltran et al, 2003). BDNF and CNTF have supportive effects on retinal recovery from ischemia in a rodent model of anterior ischemic optic neuropathy (rAION) (Goldenberg-Cohen et al. 2014).

GDNF is expressed only in the retina in the eye and further localized specifically to ganglion cells, photoreceptor outer segments and RPE while Muller cells show the predominant expression of GFRa-1, hence establishing themselves as the major target for GDNF (Jing et al. 1996) Several investigators have shown its potential therapeutic value by providing neuroprotection in retinal degeneration diseases (Harada et al 2003, Hauck et al. 2006).

There are number of neurotrophic factors that are being tested in human clinical trials for neurodegenerative disease e.g. CNTF, BDNF, FGF, GDNF etc. Moreover stem cell secretes several neurotrophic factors that promotes the cell survival.

Bone marrow stem cells are autologous in nature, can be easily isolated and therefore are better choice for cell-based therapies as compared to other stem cell types. Transplantation of bone marrow derived stem cells may help in restoring the vision by forming new retinal cells and replacing the degenerating retinal neurons and/or by rescuing retinal neurons from further

degeneration by releasing various growth factors. Zhang *et al* showed the inhibition of photoreceptor damage and retinal damage by sub-retinal delivery of stem cells derived from bone marrow (2010). Bone marrow cells contain primitive cell population that are not committed to any lineage, called as Lineage negative cells (Otani et al, 2004). These cells are depleted of CD5, TER-119, CD2, CD3, CD14, CD15, CD16, CD19, CD56, CD123, CD11b and GR-1 antigens in case of human and CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter 119 in case of mouse. In 2004, Otani *et al* showed the preservation of nuclear layers of retina in rd1 and rd10 mouse models after intravitreal injection of bone marrow derived Lin-ve stem cells (2004). Our earlier study on characterization of these cells from mouse bone marrow showed higher expression of primitive stem cell markers such as CD34 and CD117 (Jindal et al, 2014). Hence we wanted to investigate the efficacy of these bone marrow derived Lin-ve stem cell population in delaying the progression of retinal cell death induced by NMDA.

Materials and Methods:

Animal groups: All experiments were done in GLP compliant laboratory using calibrated instruments as per standard operating procedures. Animals were broadly divided into following groups: **Group1**: Injury group. In left eye, 2µl of 100mM of NMDA was injected intravitreally to the mouse eye. To the right eye, 1X PBS was injected in same volume, which was acting as a control. **Group 2**: In Lin-ve transplantation group, both eyes of mouse were NMDA injured. 100,000 Lin-ve cells in a volume of 2µl were injected intraviteally to left eye. In the contralateral eye PBS was injected, which served as an injury control (Figure A).

Animals: Animals were procured from institute's central animal facility vide Registration no. 47/1999/CPCSEA. All experiments were conducted with the approval of Institute Animal Ethical Committee (Approval no. 48/IAEC/228). Experiments were performed using

C57BL/6J mice (6-8 week old), weighing 20-25gm mice of either sex. All efforts were made to minimize the number of animals used and their suffering. 12-h light/dark cycle (LD 12:12) was maintained for animals throughout the experiments. Chow diet was given to the animals and they had free access to drinking water. We have used 5 animals per group for H/E analysis, 3 animals per group for the IHC quantification analysis, 4 animals per group for the qCR.

Anaesthesia and euthanasia: Cocktail of xylazine (10 mg/Kg) (Sigma, USA) and ketamine (100 mg/Kg) (Ketomac, India) was administered intraperitoneally as anaesthetic agent. Animals were sacrificed by overdose of same anaesthesia.

Establishment of NMDA injury Model and Morphometric Analysis: To the anesthetized mice, using microsyringe with 23G needle (Exmire, Japan), 2µl of 50mM or 100mM NMDA (Sigma, USA) in 0.1M PBS (pH 7.4) (Sigma, USA) was injected intravitreally to left eye of each mouse and to the contralateral eye (right eye), equal volume of 1X PBS was injected, which served as control. To characterize the injury, 2 days after NMDA injection, animals were sacrificed. Eyes were enucleated and processed for cryo-sectioning. Cryo- sections (6µm thick) were stained with H&E. To study the toxicity induced by NMDA, morphometric and TUNEL analysis was done.

Enrichment and characterization of Lineage negative cells from mouse bone marrow: Lineage cell depletion and FACS characterization was conducted following our previously published article (Jindal et al. 2014). Briefly, bone marrow cells were isolated from tibia and femur of C57BL/6J mice (6-8 week old) and mononuclear cells were collected through 70µm cell strainer (BD Biosciences, USA). Lineage negative cells were obtained using magnetic beads labelled lineage-specific antibodies against CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter 119 (Miltenyi Biotec, Germany). Cells were characterised for CD34, CD117 and Sca1 in lineage negative population using Flow cytometry (BD FACS Canto II).

CFDA Labelling: To track the cells after transplantation, Lineage negative cells were labelled with carboxyfluorescein diacetate- succinimidyl ester. 2 ml cell suspension was added to 2 ml of CFDA-SE (Invitrogen) solution (final conc. of CFDA in cell suspension was between 5-10 µM). Cells were incubated for 15 min at 37°C with mild shaking. Excess dye was excluded from the cell suspension by incubating cells in fresh medium for another 30 minutes. Again, cells were washed with 1XPBS and then counted with Cell counter.

Transplantation of Lin-ve stem cells in NMDA injured mouse model: About 2µL of Lin-ve CFDA-SE labelled stem cells (100,000 cells), suspended in PBS were injected intravitreally in left eye of the animal with NMDA injury. As a control, normal saline was injected into the fellow injured eye (right eye).

Immunofluorescence: Frozen retinal sections of 6µm thickness were taken in Cryostat (Leica, Germany). Sections were blocked with serum for 20 minutes and then incubated with primary antibody (Table 1) in a humidified chamber, overnight at 4°C. Sections were washed in 1X PBS three times (10 minutes each) followed by incubation with fluorophore-conjugated secondary antibodies (Jackson, USA) for one hour. All sections were counter stained with DAPI and were mounted with antifade mounting medium, fluorsave (Calbiochem, USA). Images were recorded with Fluorescence microscope (Olympus, Japan). Quantitation of immuofluorescence was done by imagej software.

Retinal mRNA estimation by qPCR: Total RNA was isolated from the retinal tissue using RNeasy Mini kit protocol (Qiagen, USA). 100 ng of RNA was used to synthesize first-strand cDNA by using cDNA synthesis kit (Thermo Scientific, USA) and PCR amplification was performed using suitable primers (Table 2). The thermal cycler conditions were 10 min at 95°C and then 35 cycles of 1 minute at 95°C, 1 minute at 61°C, and 1 minute at 72°C, followed by 10 minute at 72°C.

Statistical Analysis: Data was presented as the mean±SE of number of mouse per group. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Real time PCR data was represented as fold change or relative expression of mRNA transcript expression of particular marker. Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant.

Results:

Validation of NMDA injured retinal degeneration mouse model: Drug or PBS was injected in the intravitreal space using a micro-syringe. The position of the needle was shown in Figure 1A. FITC-Dextran (Sigma, USA) was injected in the eye to localize the dye (Fig.1B). Two doses (50mM and 100mM) of NMDA were used for model standardization. Two days after intravitreal injection of NMDA to the mouse eye revealed that there was significant decrease in number of retinal ganglion cells in 100mM injected NMDA retina (51.81±26.46) compared to 50mM injected (96.19±34.17) and PBS injected retina (129.57±42.83) (Fig.1C-F). Positive control of TUNEL assay showing brown colored apoptotic cells (Fig.1G). TUNEL assay showed more number of apoptotic cells in 50mM and 100mM NMDA injected retina as compared to PBS injected control (Fig.1H-J).

Expression of anti-apoptotic and injury markers in NMDA injured retina: The Brn3b expression, a marker for retinal ganglion cell, was found to be significantly downregulated in 50mM and 100mM NMDA injected retinal sections compared to PBS control sections (Fig.2A-I). There was increased pCREB expression in NMDA injured retina compared to control (Fig.2J-R). The expression of anti-apoptotic marker, such as Bcl2, was found to be downregulated after 2 days of 50mM and 100mM NMDA injection (Fig.2S-AA). GFAP (Glial fibrillary acidic protein), the muller glia cell marker or injury marker was also found to

be upregulated after NMDA injury compared to control retina (Fig.2BB-HH). Further, with the increased dose of NMDA, i.e. 100mM of NMDA, GFAP staining was found to be more intense at muller glia end feet extending towards inner layers of retina (Fig.2II).

Quantitation of expression of anti-apoptotic and injury markers in NMDA injured retina by imagej software: There was decrease in expression of Brn3b, BCl2 and increase in expression of pCREB, GFAP when the NMDA concentration was increased from 50mM to 100mM (Fig 3A-D).

Expression of neurotrophic factors in control versus injured retina: Expression of various neurotrophic factors were also analyzed after NMDA injection. Immunohistochemical expression of BDNF and CNTF was found to be decreased after 50mM and 100mM of NMDA injection retina as compared to the PBS- injected retina (Fig.4B-H, Fig 4T-Z). However, the expression of GDNF was increased at 50mM NMDA and then decreased at 100mM NMDA concentration. (Fig.4 K-Q). Quantitation showing decreased BDNF expression in NMDA treated retinal sections as compared to control (Fig. 4BB). As compared to control, there was increased expression of GDNF at 50mM NMDA and then decreased GDNF expression at 100mM NMDA concentration(Fig. 4CC). Decreased CNTF expression in NMDA treated retinal sections as compared to control (Fig. 4DD).

Characterization of mouse bone marrow derived Lin-ve stem cells: Marrow was collected by flushing tibia and femur of mouse in sterile condition, after which Lin-ve cells were purified from total bone marrow cell population using magnetic cell sorter. This type of enrichment resulted in depletion of committed hematopoietic precursors from total bone marrow. Lin-ve cells were further characterised with the help of flow cytometry to analyse the expression of certain haematopoietic stem cell markers e.g. CD34, Sca1 and CD117 (Fig.5A-L). It was seen that the expression of CD34 (Fig.5D-F) and CD117 (Fig.5J-L) was

significantly increased in lineage negative cell population as compared to total bone marrow and lineage positive cells. Before transplantation, Lin-ve cells were labelled with fluorescent CFDA dye. 100% cells were CFDA stained as confirmed by FACS (Fig.5O-P). CFDA labelled lineage negative cells appeared in bright green colour under fluorescent microscope (Fig.5N).

Effect of Lin-ve stem cell transplantation in NMDA injured mouse retina: Further, 100mM injured retina was selected to study the effect of Lin-ve stem cell transplantation. A dose of 100,000 cells was transplanted through intravitreal route in NMDA injured mouse eye and evaluated at day7, day14 and day21 post transplantation. CFDA labelled transplanted cells were tracked throughout the retina. Transplanted cells were found near the sclera at 7day post-transplantation (Fig.6B) and after 14 and 21 days, they were found to have migrated to the inner layers (Fig.5E,H).

Expression of neurotrophic factors in injured eye after stem cell transplantation: We have checked the expression of a series of neurotrophic factors such as GDNF, CNTF, BDNF and Brn3b in retinal sections by immunohistochemistry as well as their mRNA levels by qPCR.

Brn3b expression was reduced with increasing days of NMDA injury (Fig.7B-N) which was reversed after Lin-ve cell transplantation at all the time points (Fig.7E-Q). Brn3b expression was maximum at 7 day of transplantation (Figure 7S).

CNTF and GDNF expression was found to be up-regulated at 14 and 21 day of injury (Fig.8 and 9H,N) as compared to 7 day (Fig.8 and 9B) of injury. After Lin-ve cell transplantation, there was further increase in the expression of CNTF at all time points as compared to the respective injury groups (Figure 8S) whereas in GDNF there was increase in expression at 7 and 14 day but decrease in expression at 21 day in comparison to respective injury groups

(Fig.9S). CNTF and GDNF showed maximum expression at 14day of Lin-ve cell post-transplantation (Fig 8 and 9S).

However, BDNF immunoreactivity was found to be decreased at 14 and 21 day (Fig.10B, H, N) of injury. Lin-ve stem cells transplantation led to increase in the BDNF immunorecactivity at all the time points (Fig.9E-Q) as compared to injury group. BDNF showed maximum expression at 7 day of Lin-ve cell post- transplantation (Fig 10S).

Molecular expression of BDNF, GDNF, CNTF and Brn3b was also analysed by real time PCR. We found significant decrease in the expression of Brn3b after NMDA injury as compared to control. Also, after Lin-ve cell transplantation, there was further decrease in expression of Brn3b compared to injury alone (Fig.7T). The expression of CNTF, GDNF and BDNF was also found to be decreased after Lin-ve cell transplantation, but this was not statistically significant (Fig.8T,9T,10T).

Discussion:

Stem cell based regenerative medicine is an emerging field of treatment based on the concept of generating new cells or replacing the non-functional damaged cells. Many neurodegenerative diseases occur due to loss or non-functional nature of a particular cell type in diseases like Age related macular degeneration (AMD), Parkinson's disease, Huntington's disease and Alzheimer's diseases. Adult bone marrow stem cells have been shown to differentiate into various cell types of central nervous system in various animal models and in *in-vitro* conditions raising hopes for treatment for various neurodegenerative diseases. It has been shown that Lin-ve enriched population possess significantly higher number true stem cells compared to bone marrow derived mononuclear cell population (Jindal et al, 2014). The present work was planned to study the mobilisation of Lin-ve stem cells in N- methyl-D aspartate (NMDA) injured mouse retina.

NMDA injury model of mouse retina was established to study the homing of bone marrow derived Lin-ve stem cells. Recently the effect of bone marrow derived Lin-ve cells was evaluated in a NaIO3-induced retinal injury model to check their neuroprotective and antiapoptotic activity (Machalińska et al, 2015). In various CNS as well as retinal disorders like glaucoma and retinal ischemia**,** increased glutamate levels in vitreous cavity results in oxidative stress (Dong et al, 2009). Effect of NMDA is similar to that of glutamate and acts only on NMDA receptor. NMDA induced injury model has been previously used by many research groups (Lam et al, 1999). Also, the mechanism or the pathophysiology of NMDA induced neurotoxicity has been studied well. It has already been reported that when NMDA acts on its receptor, apoptotic cell death of retinal ganglion cells is ensured by excitotoxicity due to increased intracellular calcium levels and increased oxidative stress (Dong et al, 2009; Zhou et al, 2013).

Previous studies have shown that, during cell death, the expression of anti-apoptotic markers like Bcl2 was reduced (Zhou et al, 2013). Similar expression pattern was observed in our study. Phosphorylated cAMP (Adenosine 3'5' Cyclic Monophosphate)-Response Element Binding protein (pCREB) regulates cell death, functions as an apoptotic protein. In this study, the expression of p-CREB was increased after 50mM and 100mM NMDA injection. Glial fibrillary acidic protein (GFAP), a marker for muller glia cells, is expressed at end feet of muller cells under normal conditions. Any traumatic injury to the retina, like retinal detachments or ischemic insult, results in upregulation of GFAP throughout the retina (Chang et al, 2007; Lewis and Fisher, 2003). In our PBS injected control retina, weak expression of GFAP was observed in INL and GCL. However, in NMDA injured retina, strong GFAP signal was seen in both INL and GCL. These findings are consistent with the previous studies (Honjo et al. 2000). Level of BDNF and CNTF was found to be decreased 2 day after injury with increased dose of NMDA due to reduced number of survived cells. GDNF expression

was found to b increased 2 days after 50mM NMDA because more amount of neurotrophic factors (GDNF) were released by retinal cells to protect themselves from injury. Decreased expression of GDNF after 100mM NMDA insult was due to reduced number of survived cells. Hence less amount of neurotrophic factors were released.

The expression of various stem cell markers like Sca1, CD117 and CD34 in Lin-ve stem cell population was evaluated using FACS. The expression of CD117 and CD34 was higher in Lin-ve population as compared to total bone marrow and lin+ve cells population (jindal et al, 2014). These findings indicate that Lin-ve population has increased expression of stem cell progenitor markers and could be useful for tissue repair and regeneration. In our previous study, different doses of Lin-ve stem cells (50000, 1000000 and 200000 cells) and different routes such as intravitreal and intravenous route was used for transplantation in laser injured retinal mouse model. 100,000 cells dose showed homing and survival for up to 21 days at the site of injury after transplantation (Singh et al, 2012). We believe that when ganglion cells need to be repaired, the intravitreal route is preferred while in the case of photoreceptor or RPE degeneration, intravenous or subretinal route has been used more frequently for transplantation of stem cells. In various studies, stem cell incorporation has been shown in transgenic as well as injury models (Singh et al, 2012; Tucker et al, 2011; Castanheira et al, 2008). In present study, Lin-ve BMCs were found to have migrated and incorporated at 7 and 21 day of transplantation period. Injury plays a major role in stem cell migration and their incorporation (Chacko et al, 2003). It may result in release of several cytokines or inflammatory molecules that attract the stem cells to damaged site (Kaur et al, 2008).

Retinal injury is necessary for the stem cell homing and differentitation. Previous studies showed no incorporation of the cells in control eyes when stem cells were injected. (Machalinska et al, 2015, chacko et al, 2003).

Injury is known to result in alteration in neurotrophic factors. The expression of CNTF and GDNF was increased at 7,14 and 21 day of injury. Honjo and his colleagues also showed increased expression of CNTF after NMDA injury in rat model (2000). Valter et al also showed the upregulation of neurotrophic factors in time dependent manner against light induced damage after optic nerve transaction (2005).

Stem cell incorporation has been shown to elevate the level of growth factors in injured retina. Various neurotrophic factors are secreted by stem cells that promote the survival of neurons (Wilkins et al, 2009; Lu et al, 2003). Otani et al showed rescue of retinal damage when bone marrow derived lineage negative stem cells were injected intravitreally. This group also showed vasculopathic and neurotrophic effects of Lin-ve stem cells in rd10 mouse (2004). In MCAO models, mesenchymal stem cells were shown to secrete neurotrophic factors (Kurozumi et al, 2005). Our results also revealed the presence of transplanted Lin-ve cells in retinal layer followed by upregulation of neurotrophic factors after transplantation such as BDNF, GDNF and CNTF. There are several studies that are showing functional outcomes of stem cell transplantation in various retinal degeneration diseases (Jindal et al, 2012). The protein expression of GDNF, CNTF, BDNF and CNTF were found to be increased after transplantation of Lin-ve stem cells as compared to injury when quantification of immunofluoresence images was done with imagej software. But real time data showed decreased expression of GDNF, CNTF, BDNF and Brn3b after transplantation as compared to injury. Protein levels are not necessarily proportional to mRNA levels. Less mRNA expression and high corresponding protein might happen because after translation that particular mRNA gets degraded while its protein has half life and remains in the cellular pool. Protein stability may be increased by acetylation , phosphorylation, glycosylation etc.

The expression of Brn3b is more at 7 day of transplantation of Lin-ve stem cell as compared to 14 and 21 day of transplantation. There may be two reasons for this decrease in expression of Brn3b during time course of transplantation.

1. Endogenous neurotrophic factors may not be sufficient for the stem cell survival. For the survival of stem cell in retina, exogenous delivery of survival or neurotrophic factors may be required (Nickerson et al, 2008).
2. 100,000 cells may sufficient for injury occurred at 7day. More number of cells (i.e. more than 100,000 cells) will be required to overcome from the injury occurred at 14 and 21day (Singh et al, 2012).

Mouse ES were delivered in the subretinal space of Royal College of Surgeons (RCS) rats rescue photoreceptor cells from degeneration (Haruta et al. 2004). Pigment epithelial cells derived from Embryonic stem cells (ESPEs) have been reported to have characteristics of RPE in both *in-vitro* and *in-vivo* conditions. ES cells from Cynomolgus monkeys were induced to form ESPEs and these were then transplanted into 4 day old RCS rats subretinally. 8 weeks after transplantation, it was seen that the transplanted ECPE cells enhance the survival of host photoreceptors by histology and IHC (Wang et al.2010). Functional recovery of vision was also seen by Wang et al. This group initially generated RPE-like structures on transgenic feeder layer and then transplanted them subretinally in mouse model of retinitis pigmentosa. Transplanted eyes showed visual improvement in a time frame of 7 months (Aoki et al. 2008). The in-vitro models of ES cell derived eyes, have structures complimentary to retinal ganglion like cells. These structures upon transplantation into NMDA injury model, get incorporated and regenerate the RGCs of the host retina (Meyer et al.2004).

Early born neurons like RGCs and cone cells and late born neurons have been shown to be generated from iPSCs on exposure to particular environmental conditions, displaying therapeutic implications in retinal degeneration disorders (Tucker et al. 2011).

Functional restoration of retinal cells was also seen through ERG by Tucker et al in a rhodopsin null mice when they subretinally transplanted differentiated iPSCs generated from mouse fibroblasts via induction of the Sox2, c-Myc, Oct4 and Klf4 and depletion of SSEA1 (stage-specific embryonic antigen 1-enriched) (Kokkinaki et al. 2011).

Conclusion: Results from our study demonstrate that Lin-ve isolated from mouse bone marrow can reverse NMDA induced retinal degeneration with the help of various neurotrophic factors. Furthermore, our study also strengthens the existing reports of time dependent response in transplantation studies. It has potential to translate into clinical benefits offering future insights for exploring other sub sets of bone marrow as well as examining the preclinical effects. Additional approaches may include investigating the effect of other doses, routes and head to head comparisons with other cell types against varying severity of injuries in animal models. Future studies may also focus not only on associated functional molecular links but also functional outcomes, using electrophysiological approaches.

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Conflict of Interest: None

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Figure legends:

Figure A: Study Design.

Figure 1: Establishment of NMDA injury model of mouse retina. (A) Site of intravitreal injection. (B) FITC-Dextran dye injected in the eye to locate it in the intravitreal space. (C) H&E staining of control retinal sections (D-E) H&E staining of control retinal sections revealing loss of ganglion cells (arrow head) after 50mM and 100mM concentration of NMDA injection. (F) Morphometric analysis showing significant loss of ganglion cells in 50mM and 100 mM injected retinal sections. Data represent as Mean±SE. (G) Positive control of TUNEL assay showing brown colored apoptotic cells. (H-J) More number of apoptotic cells in 50mM and 100mM NMDA injected retina as compared to PBS injected control (H) in TUNEL assay.

Figure 2: Expression of anti-apoptotic and injury markers in NMDA injured retina. (A-I) Decreased Brn3b expression with increased concentration of NMDA in retinal section. (J-R) Increased expression of pCREB with increased concentration of NMDA in retina section. (S-AA) Decreased Bcl2 expression with increased concentration of NMDA in retinal section. (BB-GG) GFAP expression was increased with the increased NMDA concentration in retinal sections. DAPI was shown to understand the morphology of the retina section. Merged images was shown to see the expression of each marker in retinal layer.

Figure 3: Quantitation of expression of anti-apoptotic and injury markers in NMDA injured retina by imagej software: (A-D) Decreased expression of Brn3b, BCl2 and increased expression of pCREB, GFAP with increased concentration of NMDA. Comparisons between the mean variables of different groups was done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further,

Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Figure 4: Expression of neurotrophic factors in control versus injured retina and Quantitiation of retinal sections based on fluorescence intensity by imagej software. (B, E, H) BDNF expression was decreased after 50mM and 100mM of NMDA injection compared to the PBS- injected retina. (K, N, Q) The expression of GDNF was increased at 50mM NMDA and then decreased at 100mM NMDA concentration. (T, W, Z) CNTF expression was found to be decreased after 50mM and 100mM NMDA injection. (BB) Quantitation showing decreased BDNF expression in NMDA treated retinal sections as compared to control. (CC) As compared to control, there was increased expression of GDNF at 50mM NMDA and then decreased GDNF expression at 100mM NMDA concentration. (DD) Decreased CNTF expression in NMDA treated retinal sections as compared to control. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Figure 5: Characterization of bone-marrow derived lineage negative stem cells. Flow cytometric expression of CD45 (A-C) and haematopoietic stem cell markers such as CD34 (D-F), Sca1 (G-I) and CD117 (J-L) in total bone marrow, lineage negative and lineage positive cells. (M) Quantitative analysis of different cell markers. Data represent as Mean±SE. (N-P) Lin-ve cells labelled with fluorescent CFDA dye and further confirmed by flow-cytometry.

Figure 6: CFDA labeled Lin-ve stem cells in retina after transplantation. Lin-ve cells were found in retinal layers after 7 days (B), 14 days (E) and 21 days (H) of transplantation. (A), (D) and (G) are bright field images of (B), (E) and (D).

Figure 7: Brn3b expression in NMDA injured and lineage negative transplanted retina at different time points. No expression of Brn3b was found in retina sections after 7, 14 and 21 day of 100mM NMDA injury (B,H,N). Weak expression of Brn3b was found at 7 and 21 day of Lin-ve transplantation (E,Q). Brn3b immunoreactivity was visualized using a Cy3-conjugated secondary antibody. Sections were also counter stained with DAPI. (S) Quantitiation of retinal sections for Brn3b marker based on fluorescence intensity by imagej software showing increased Brn3b expression after Lin-ve cells transplantion at7,14 and 21 day as compared to injury at same respective time points. (T) Molecular expression of brn3b in Lin-ve transplanted retina by RT PCR showed decrease in Brn3b expression in NMDA injured retina. But after Lin-ve transplantation, real time PCR showed further decrease in the expression of Brn3b. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Figure 8: CNTF expression in NMDA injured and lineage negative transplanted retina at different time points. At 21 day of 100mM NMDA injury, the expression of CNTF was increased as compared to 7 and 14 day of injury (B,H, N). At 14 day of Lin-ve transplantation, the expression of CNTF was increased (K) but at 21 day of transplantation, its expression was found to be decreased (Q). CNTF immunoreactivity was visualized using a Cy3-conjugated secondary antibody. Sections were also counter stained with DAPI. Quantitiation of retinal sections for CNTF marker based on fluorescence intensity by imagej software showing increased CNTF expression after Lin-ve cells transplantion at 7,14 and 21

day as compared to injury at same respective time points. 14 day lin-ve transpantation showed more expression of CNTF as compared to 7 day and 21day of Lin-ve transplantion. (T) Molecular expression of CNTF in Lin-ve transplanted retina by RT PCR showed increase in CNTF expression in NMDA injured retina. But after Lin-ve transplantation, real time PCR showed further decrease in the expression of CNTF. This decrease in CNTF expression was not statistically significant. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Figure 9: GDNF expression in NMDA injured and lineage negative transplanted retina at different time points . GDNF expression in retina sections was increased at 14 day and it was further increased at 21 day of 100mM NMDA injury (H,N). This expression is significantly decreased after Lin-ve cell transplantation (E, K, Q). GDNF immunoreactivity was visualized using a Cy3-conjugated secondary antibody. Sections were also counter stained with DAPI. Quantitiation of retinal sections for GDNF marker based on fluorescence intensity by imagej software showing increased GDNF expression after Lin-ve cells transplantion at 7,14 and decreased GDNF expression at 21 day as compared to injury at same respective time points. (T) Molecular expression of GDNF in Lin-ve transplanted retina by RT PCR showed increase in GDNF expression in NMDA injured retina. But after Lin-ve transplantation, real time PCR showed decrease in the expression of GDNF as compared to respective injury points.This decrease in GDNF expression was not statistically significant. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Figure 10: BDNF expression in NMDA injured and lineage negative transplanted retina at different time points by Immunofluoresence. BDNF expression was found to be was decreased at 14 and 21 day of injury (H,N). After Lin-ve cells transplantation, the expression of BDNF was increased as compared to respective injury points. (E,Q). BDNF immunoreactivity was visualized using a Cy3-conjugated secondary antibody. Sections were also counter stained with DAPI. Quantitiation of retinal sections for BDNF marker based on fluorescence intensity by imagej software showing increased BDNF expression after Lin-ve cells transplantion at 7,14 and 21day as compared to injury at same respective time points. (T) Molecular expression of BDNF in Lin-ve transplanted retina by RT PCR showed significant decrease in the expression of BDNF at 7, 14 and 21 day as compared to injury group. Comparisons between the mean variables of different groups were done by one way analysis of variance (ANOVA). Data was statistically analysed using 16.0 versions of SPSS. Further, Sidak test was used for post-hoc analysis. P-value < 0.05 was considered as statistically significant. Data represent as Mean±SE.

Table1: List of antibodies used for Immunofluoresence.

Target Protein	Make	Catalogue	Reactivity
BDNF (N-20)	Santa Cruz Biotechnology, USA	SC-546	Rabbit Polyclonal IgG
GDNF (B-8)	Santa Cruz Biotechnology, USA	SC-13147	Mouse Monoclonal IgG
CNTF (R-20)	Santa Cruz Biotechnology, USA	SC-1912	Goat Polyclonal IgG
Brn3b (H-18)	Santa Cruz Biotechnology, USA	SC-31989	Goat Polyclonal IgG
Bcl2 (N-19)	Santa Cruz Biotechnology, USA	SC-492	Rabbit Polyclonal IgG
GFAP (N-18)	Santa Cruz Biotechnology, USA	SC-6171	Goat Polyclonal IgG
pCREB (Ser 133)	Santa Cruz Biotechnology, USA	SC- 7978	Rabbit Polyclonal IgG

Table 2: List of primers used in qPCR

Target Gene	Target length (bp)	Primer Sequence (5'→3')	Annealing Temp (^{O}C)
BDNF	131	F: TGGCTGACACTTTTGAGCAC R: CAAAGGCACTTGACTGCTGA	61
CNTF	62	F: TTGATTCCACAGGCACAAAA R: CCCTGCCTGACTCAGAGGT	61
GDNF	142	F: TGGGCTATGAAACCAAGGAG R: CAACATGCCTGGCCTACTTT	61
Brn3b	149	F: GCAGTCTCCACTTGGTGCTTACTC R: TTCCCCCTACAAACAAACCTCC	61
B-actin	228	F: AGCCATGTACGTAGCCATCC R: CTCTCAGCTGTGGTGGTGAA	61

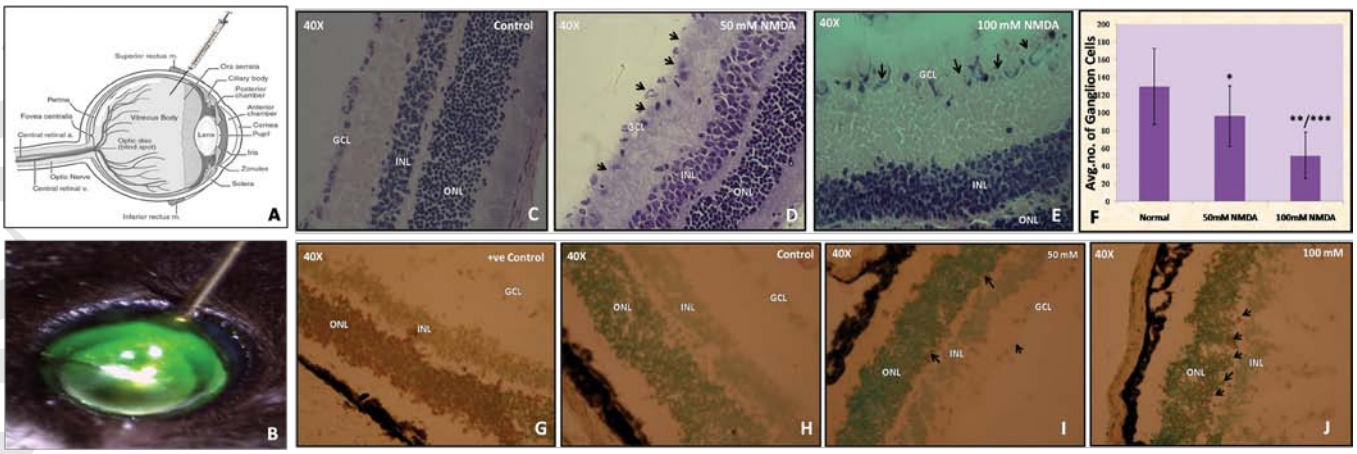


Figure 1

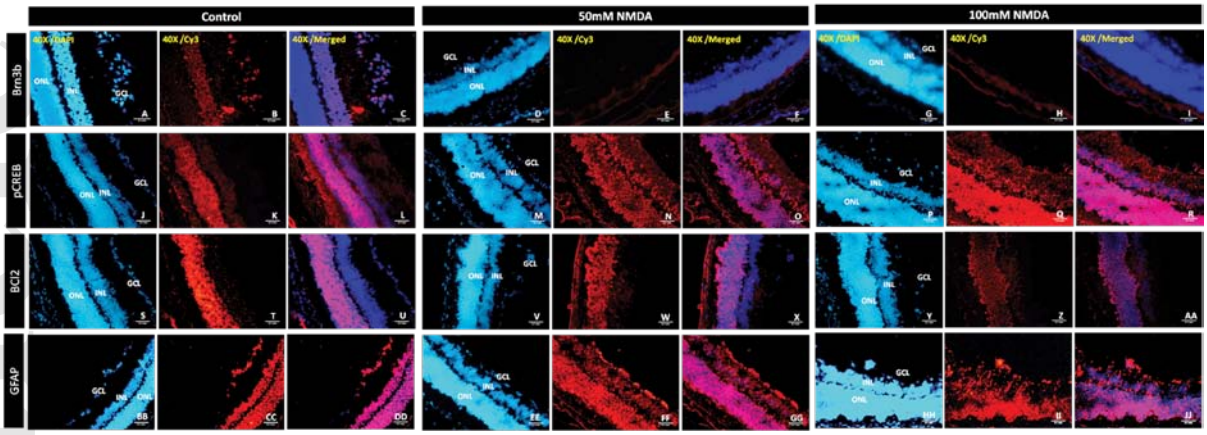
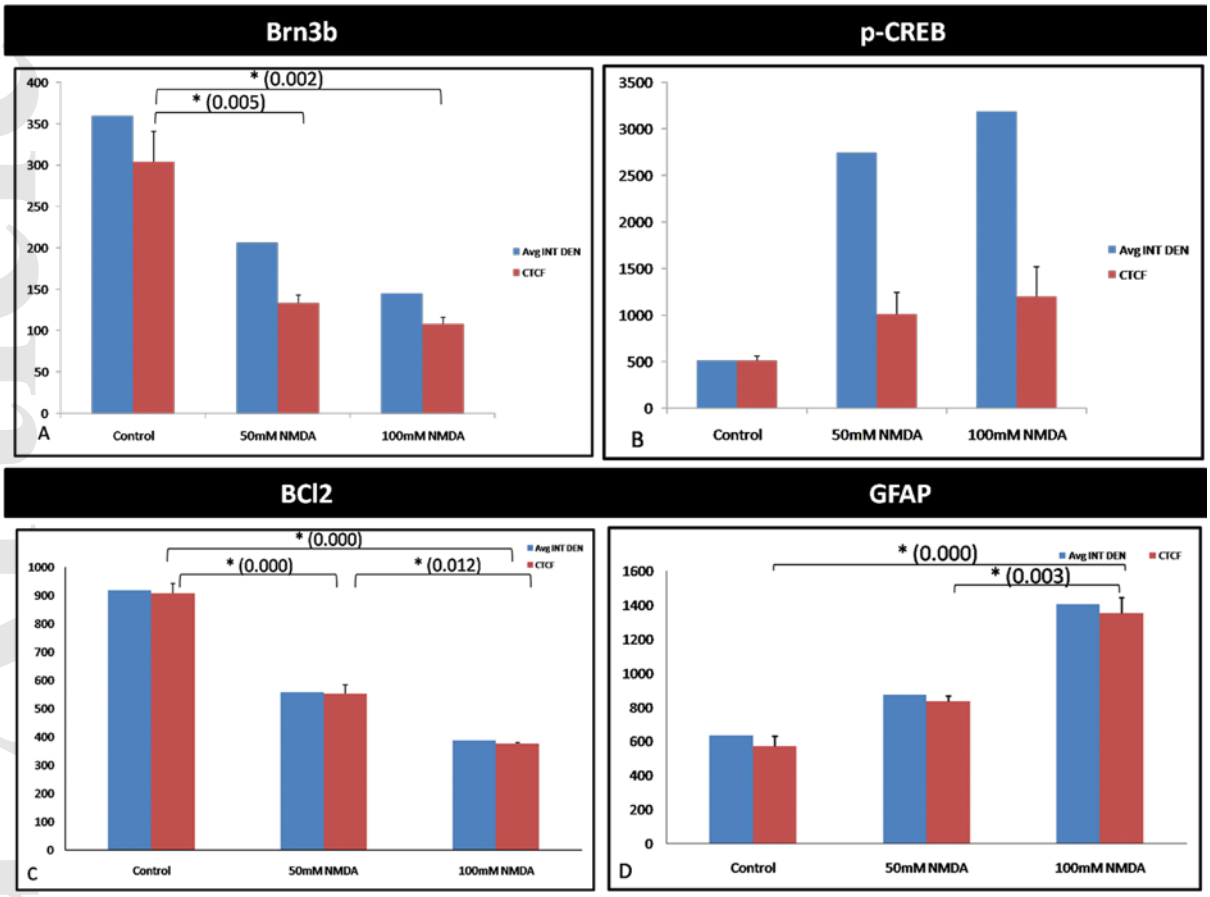


Figure 2



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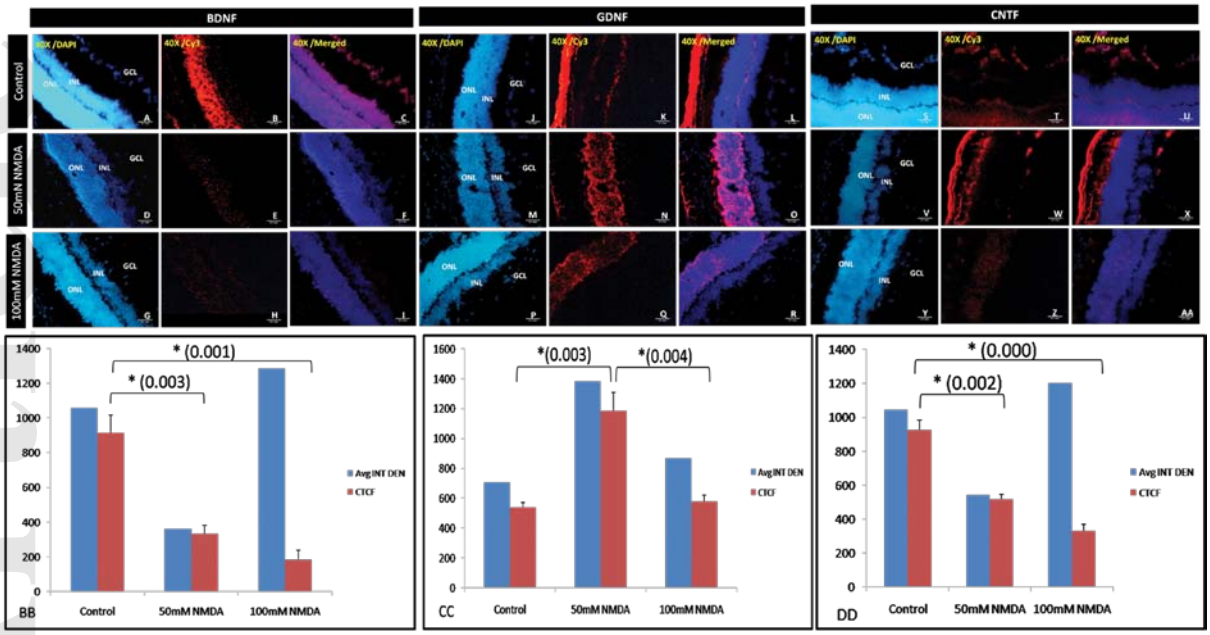

Figure 4

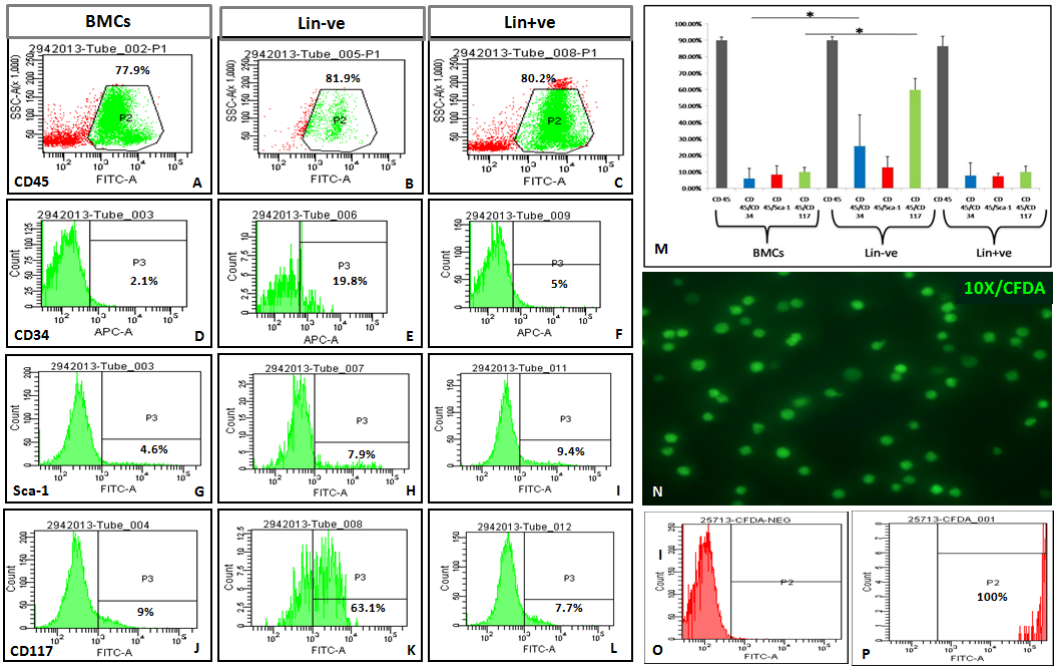

Figure 5

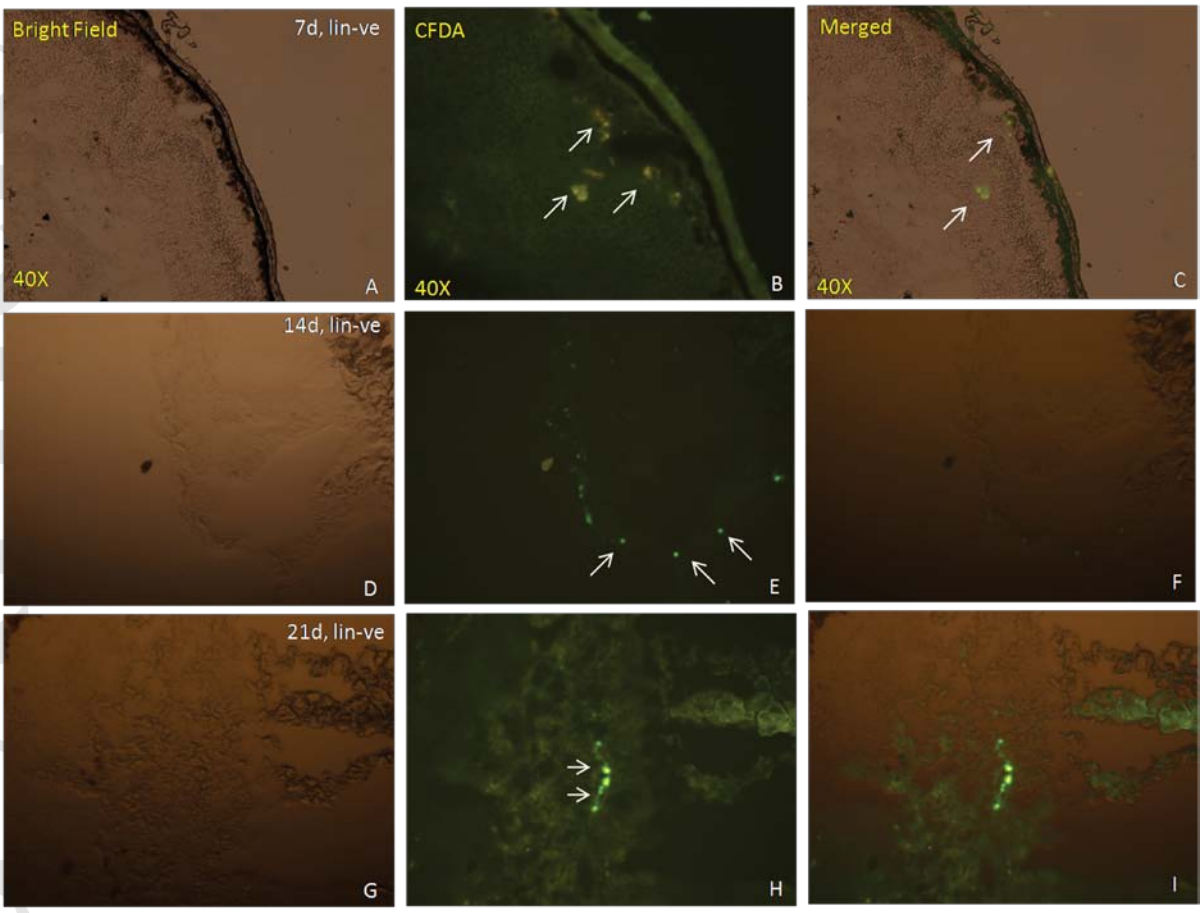

Figure 6

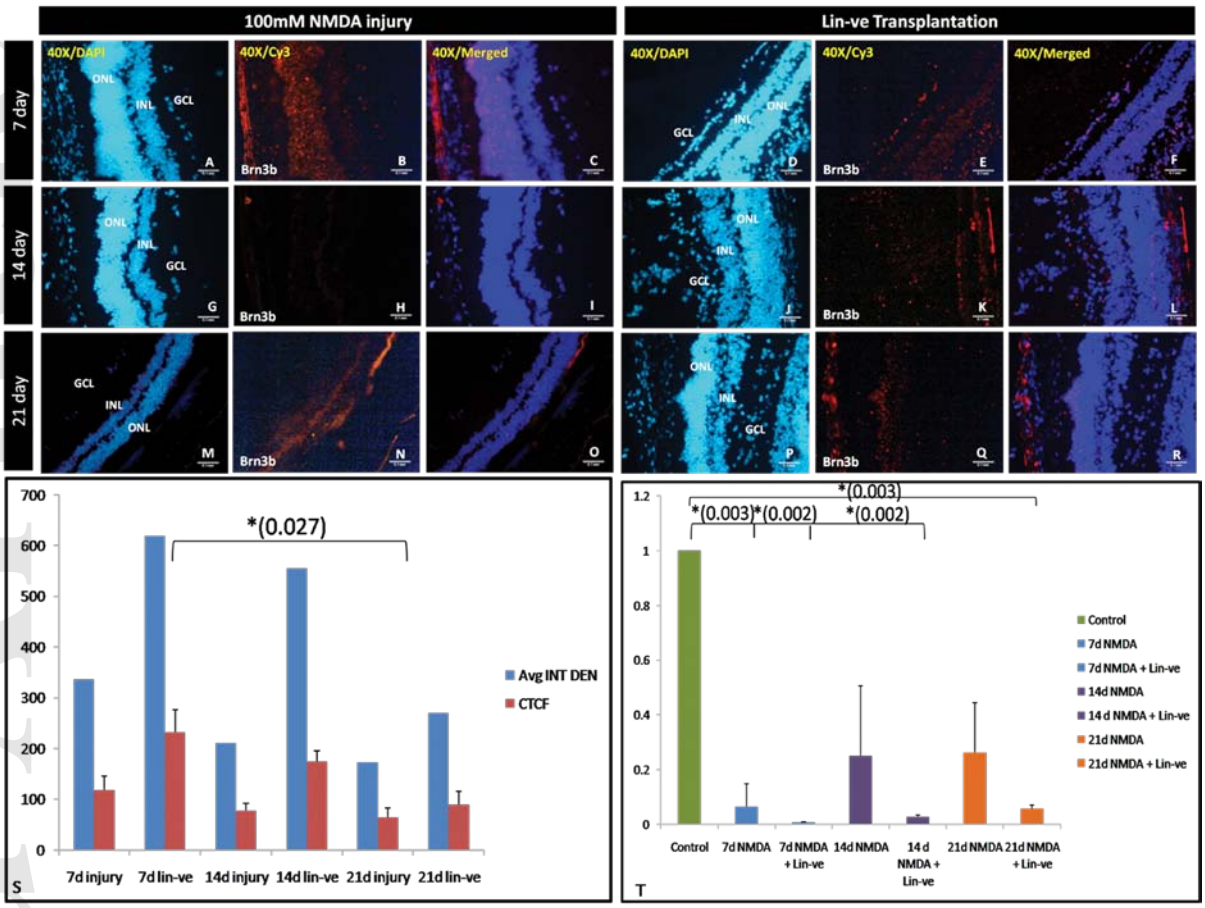

Figure 7

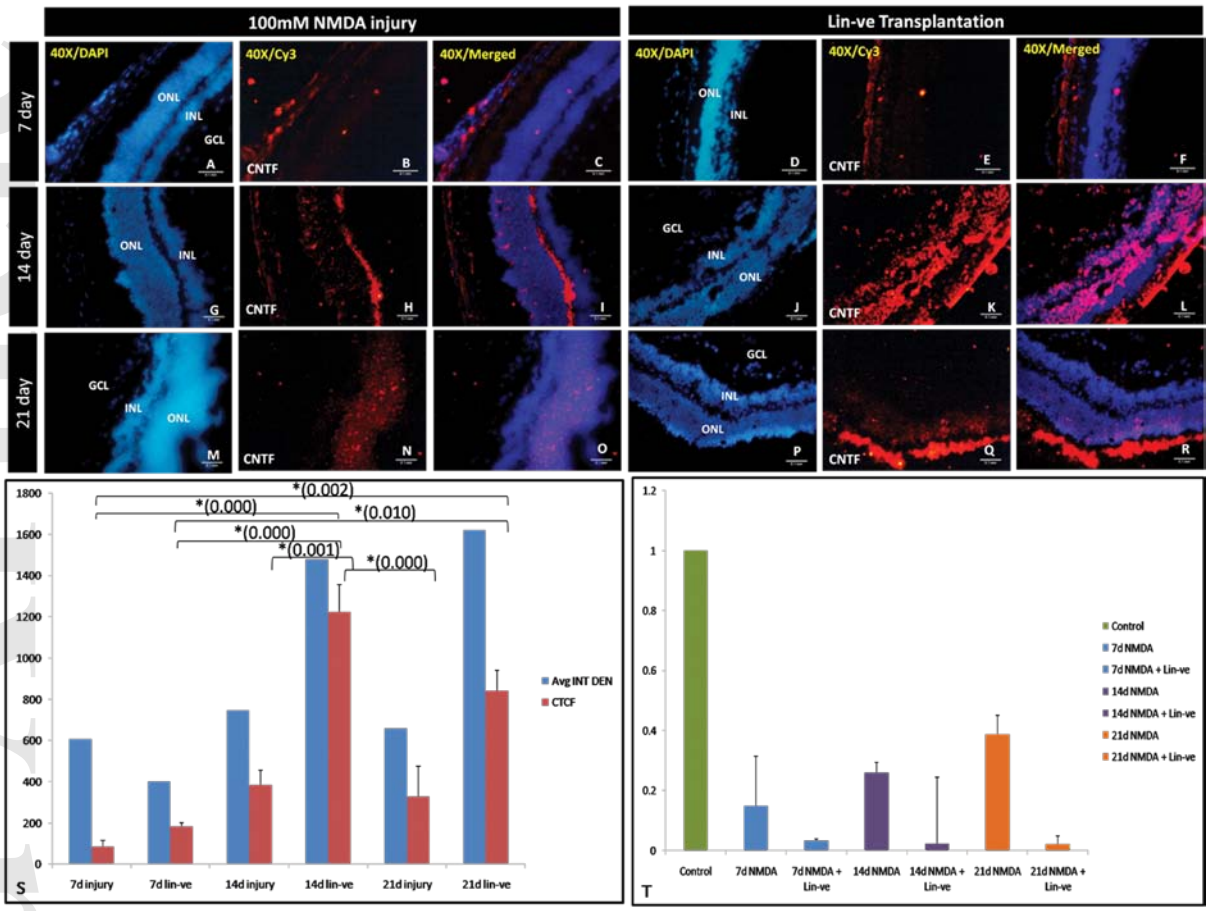

Figure 8

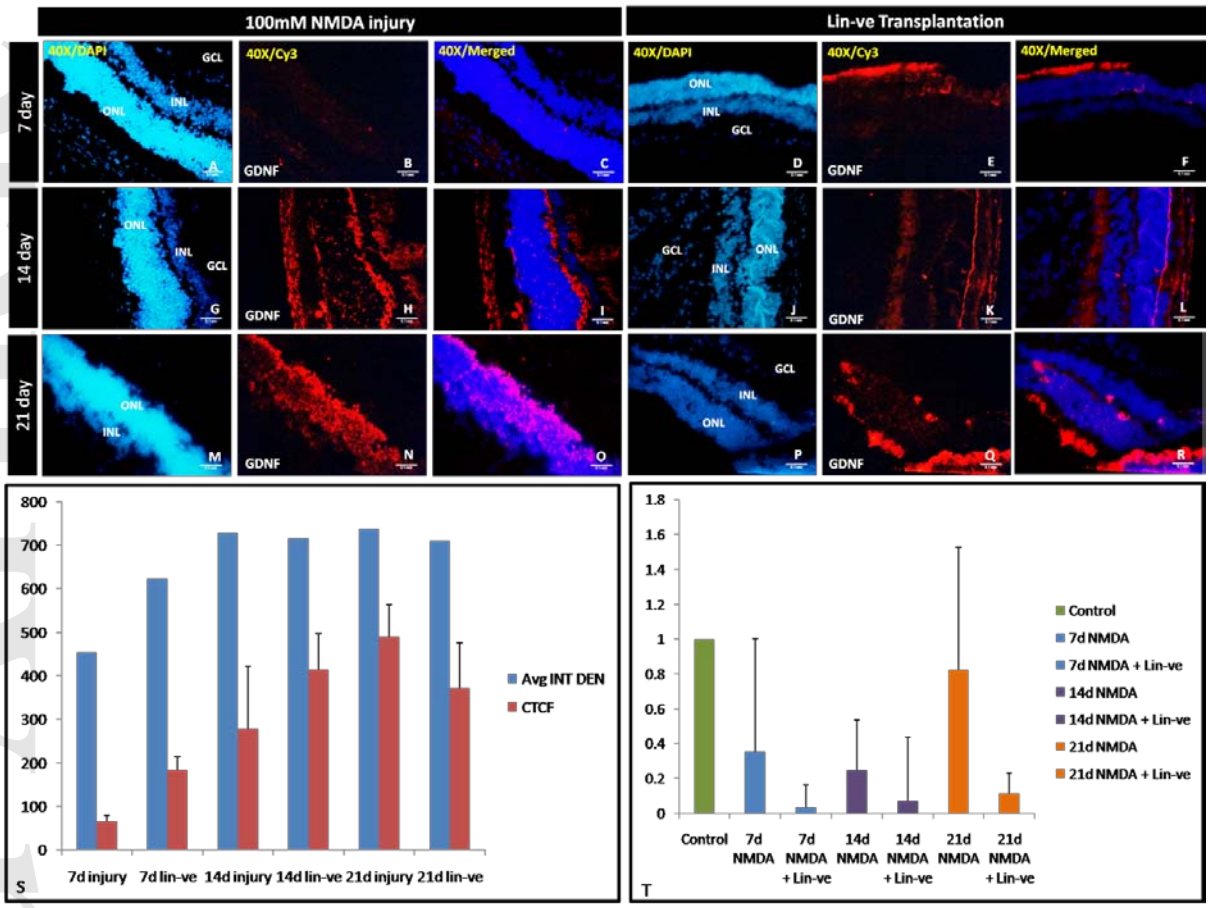

Figure 9

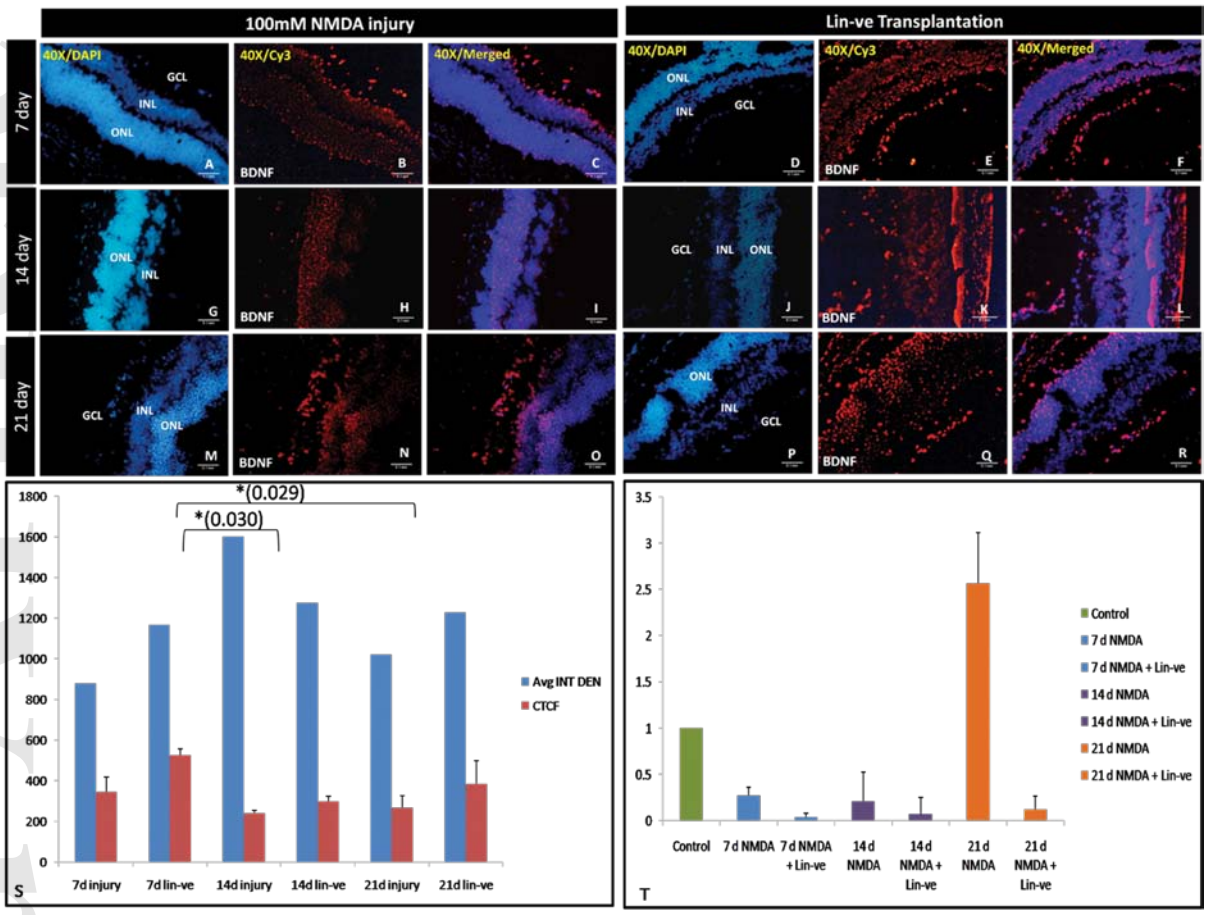

Figure 10

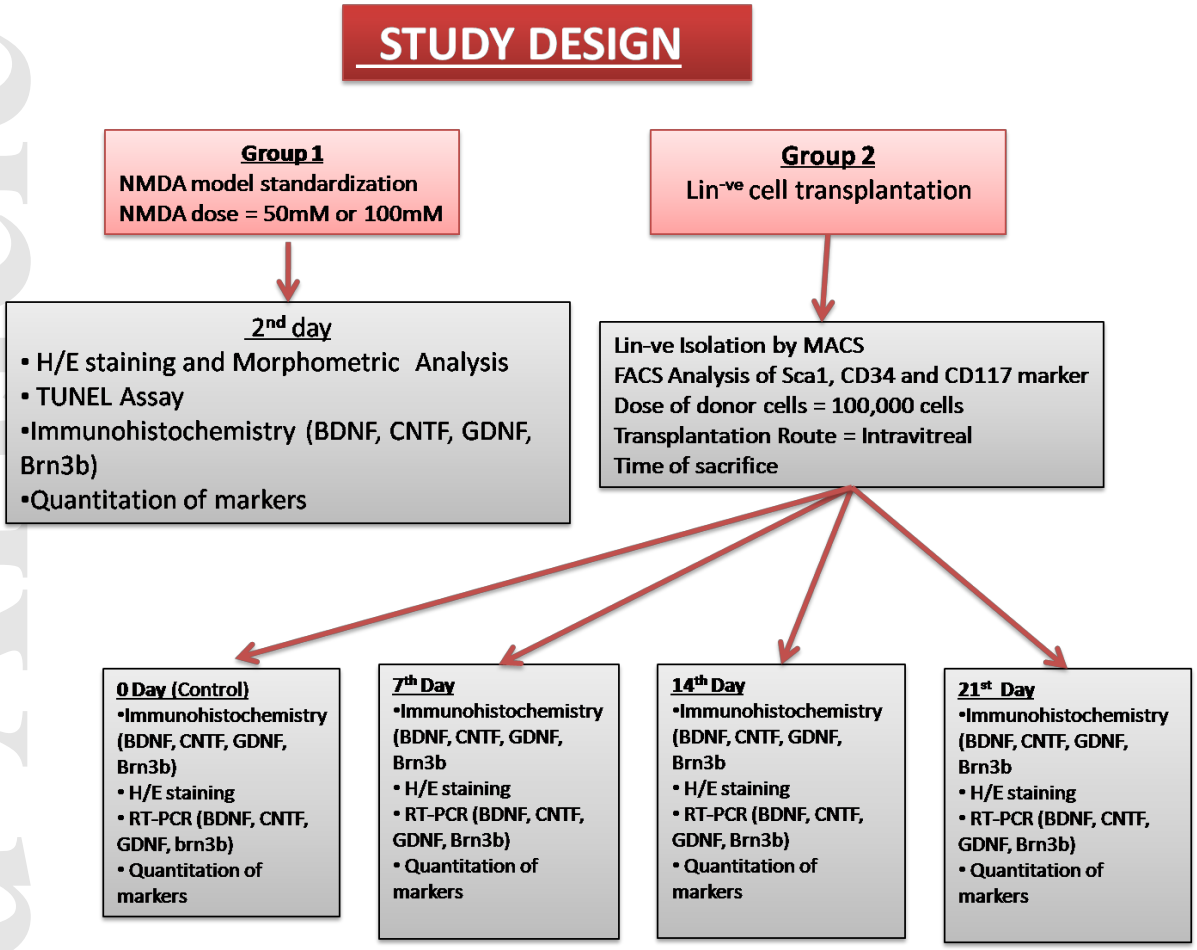

Figure A

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Transplantation of lineage-negative stem cells in pterygopalatine artery ligation induced retinal ischemia–reperfusion injury in mice

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Abstract Retinal ischemia is a condition associated with retinal degenerative diseases such as glaucoma, diabetic retinopathy, and other optic neuropathies, leading to visual impairment and blindness worldwide. Currently, there is no therapy available for ischemic retinopathies. Therefore, the aim of this study was to test a murine model of pterygopalatine artery ligation-induced retinal injury for transplantation of mouse bone marrow-derived lineage-negative (lin-ve) stem cells. The mouse external carotid artery and pterygopalatine artery were ligated for 3.5 h followed by reperfusion. The model was validated through fundus fluorescein angiography, laser Doppler and FITC dextran perfusion in whole-mounts. Lin-ve stem cells isolated from mouse bone marrow were transplanted through tail-vein, which showed migration to retina leading to decrease in GFAP expression. The neurotrophic factors such as BDNF and FGF2 showed enhanced expression in the retina. The functional analysis with electroretinogram did not demonstrate any significant changes before or after injury or stem cell transplantation. This study shows a neuroprotective potential in lin-ve stem cells in the retinal ischemia induced by pterygopalatine artery ligation and presents a practical model for validating therapies for ischemic disorders of the retina in future.

Keywords Retinal ischemia · Pterygopalatine artery · External carotid artery · Stem cells · Neurotrophic factors · BDNF · GFAP

Abbreviations

ANOVA	Analysis of variance
BCCAO	Bilateral common carotid artery occlusion
BDNF	Brain-derived neurotrophic factor
CCA	Common carotid artery
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CNTF	Ciliary neurotrophic factor
CRAO	Central retinal artery occlusion
CREB	cAMP response element binding protein
ECA	External carotid artery
ERG	Electroretinogram
FACS	Fluorescence-activated cell sorting
FGF2	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
IAEC	Institutional animal ethics committee
ICA	Internal carotid artery

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IC-SCRT	Institutional committee for stem cell research and therapy
ILM	Inner limiting membrane
INL	Inner nuclear layer
IOP	Intraocular pressure
IPL	Inner plexiform layer
iPSC	Induced pluripotent stem cells
Lin-ve	Lineage-negative
MACS	Magnetic activated cell sorting
MAP2	Microtubule-associated protein 2
MCAO	Middle cerebral artery occlusion
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PPA	Pterygopalatine artery
RPE	Retinal pigment epithelium
Sca1	Stem cell antigen 1

Introduction

Retinal ischemia is a common clinical condition linked with a vast range of retinal diseases leading to blindness worldwide. It is caused by the disruption of blood supply to the retina which results in low oxygen and glucose. As retina has a high metabolic demand, any disruption in blood supply can initiate a cascade of excitotoxicity, free radical generation, inflammation, which ultimately causes cell death [1].

Many different animal models of retinal ischemia have been established which include high intraocular pressure (IOP) [2], central retinal artery occlusion [3], optic nerve injury, laser photocoagulation of retinal vessels [4], vascular models such as bilateral common carotid artery occlusion (BCCAO) [5, 6] and middle cerebral artery occlusion (MCAO) [7, 8]. These models have been used to study pathophysiology and to test new therapeutics. However, a reproducible model that can result in damage due to retinal ischemia alone still eludes us. Pressure-induced ischemia has been shown to cause non-uniform damage to the retina [9]. Another study demonstrated that high IOP might also result in retinal damage by increased pressure apart from the effects of ischemia [2]. Similarly, MCAO induced retinal ischemia can cause more damage and mortality through cerebral ischemia than retinal injury. A recent development is the vascular models based on ligation of the external carotid artery and pterygopalatine artery. The ligation of these two arteries obstructs the blood supply to the ophthalmic artery and hence, the retina [10, 11].

Currently, there is no treatment available for retinal ischemia and associated vision loss. Cell therapy is therefore considered an attractive strategy as a replacement to the degenerating neurons. Bone marrow-derived stem cell therapy hence is an attractive approach for retinal regeneration especially when the mobilization of resident stem cells has failed to provide promising results. Stem cells are believed to act through different mechanisms including modulation of anti-inflammatory effects and apoptosis, increasing angiogenesis in ischemic areas, releasing cytokines and neurotrophins and stimulating the resident stem cells in the injured tissue [12]. Various clinical trials have been carried out using bone marrow-derived mononuclear cells as well as CD34-positive cells in different conditions such as ischemia, macular degeneration, and retinitis pigmentosa [13]. With growing need for better treatment efficacy, it is compelling to investigate the functional and physiological parameters along with the molecular and histological readouts in pre-clinical models such that functional efficacy of the stem cell therapy is analyzed in tandem with molecular studies.

Lineage-negative (lin-ve) stem cells are the cell population which is derived from bone marrow and is devoid of any mature cell markers such as Mac-1 (myeloid), CD4/CD8 (T-cells), CD19 (B-cells), Ter119 (erythrocytes) making them amenable for transplantation [14]. We, therefore, in this study sought to determine the efficacy of lineage-negative population of stem cells in the rescue of a murine model of external carotid artery (ECA) and pterygopalatine artery (PPA) ligation induced retinal ischemia injury.

Materials and methods

Animals

Sex- and age-matched, male C57BL6/J mice were used for the experiments. All animals were caged in 12 h light/dark cycle. All experiments were conducted under the Institutional ethical guidelines and approved by both Institutional Animal Ethics Committee (IAEC) and Institutional Committee for Stem Cell Research and Therapy (IC-SCRT).

External carotid artery and pterygopalatine artery ligation mouse model

The mouse model was adapted to a previously established procedure [7, 10, 11]. Briefly, mice were anesthetized by administering xylazine-ketamine (10 and 100 mg/kg, respectively) and a small midline incision was made in the neck. The right common carotid artery (CCA) was exposed by carefully removing and retracting the muscles. The external carotid artery (ECA), a branch of CCA was exposed and ligated near the bifurcation using a thread. After that, the other branch of CCA, internal carotid artery (ICA) was located and cleared. A further branch of ICA, PPA was located and ligated using a

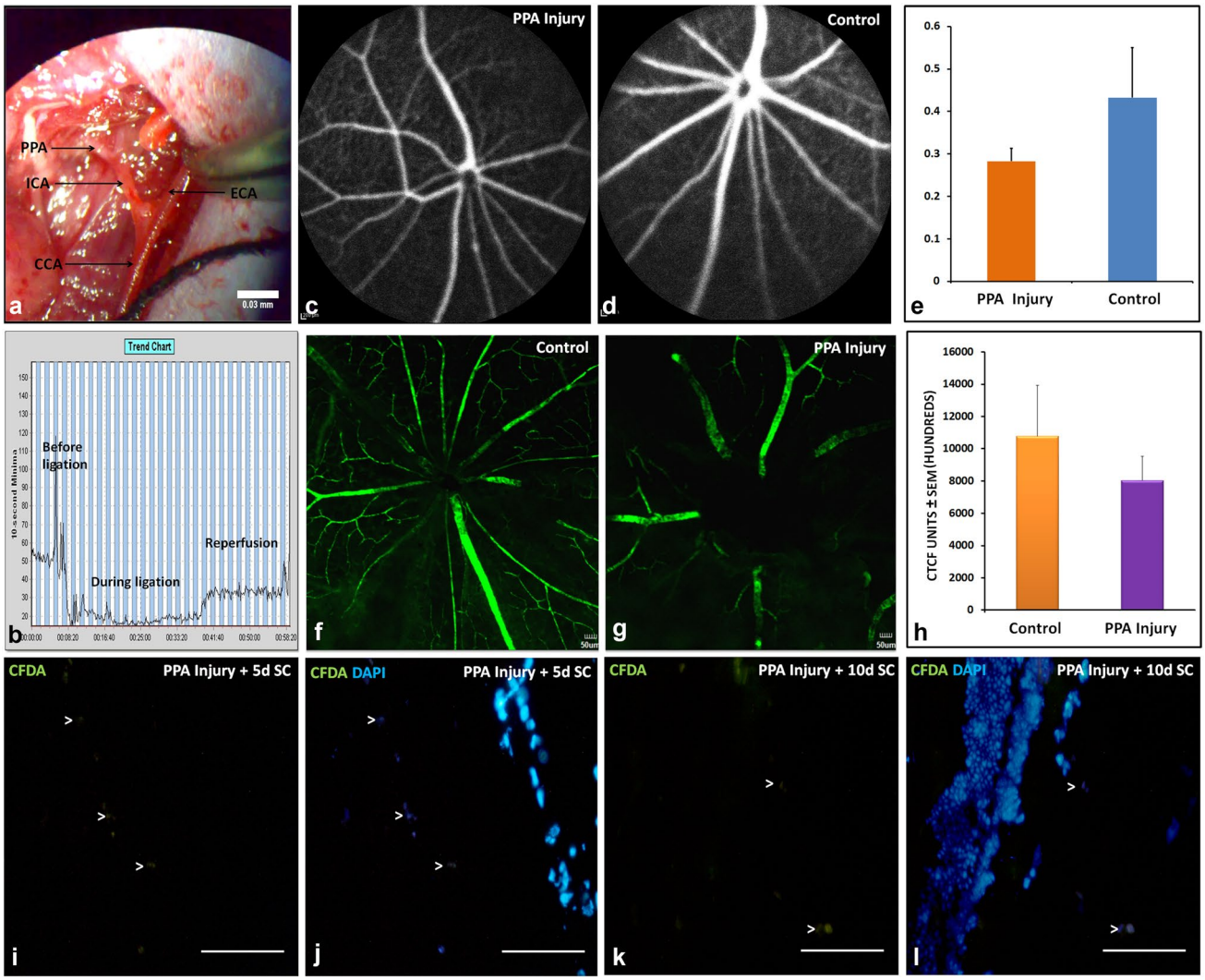

Fig. 1 Transplantation of bone marrow-derived lineage negative stem cells in ECA and PPA ligation-induced retinal ischemia (**a**). The retinal ischemia was created by ligation of the ECA and PPA for 3.5 h and reperfusion of 5 days. (*CCA* common carotid artery, *ECA* external carotid artery, *ICA* internal carotid artery, *PPA* pterygopalatine artery). **b** The trend chart from Laser Doppler blood-flow meter illustrates the changes in the cerebral blood flow. **c–h**. The retinal vasculature was examined using fundus angiography and FITC-dextran perfusion. **c** The right eye (ipsilateral) fundus after the ligations. **d** The left eye (contralateral) fundus of the same animal where there is no ligation. **e** Quantitative comparison of vessel diameter in contralateral and ipsilateral eyes (Student's *t* test, n = 4; p = 0.264). **f**, **g** Retinal vasculature in control and injured retina respectively after 3.5 h of ligation through FITC-dextran perfusion (*Scale bar 50 microns*). **h** Quantitative comparison of fluorescence in contralateral and ipsilateral eyes (Student's *t* test, n = 2, p = 0.442). **i**, **j** CFDA labeled cells in the retinal section after 5 days of transplantation and corresponding merged image with DAPI counterstain. **k**, **l** The retinal section after 10 days of transplantation and the corresponding merged image with DAPI counterstain (>mark the CFDA labeled cells; *Scale bar 250 microns*)

suture (size 7–0; Ethicon, USA) for 3.5 h (Fig. 1a). After ligation of 3.5 h, both PPA and ECA were re-opened and allowed to reperfuse. The incision was sutured, and local anesthesia was applied to avoid any discomfort. Mice were then placed in padded cages and kept warm until recovery. Laser Doppler blood flow meter (Moor Instruments, UK) was used to record any changes in cerebral blood flow.

Fluorescein fundus angiography

To validate the model, fundus fluorescein angiography of right and left eye of the mouse was done after an intravenous injection of sodium fluorescein. Fundus photographs were captured within an hour of ECA and PPA ligation, using Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany). The thickness of the vessels was

measured using Image J software and compared between contralateral and ipsilateral eye.

FITC-dextran imaging

The retinal vasculature was visualized through perfusion of fluorescein isothiocyanate (FITC)-dextran. Mice were kept under anesthesia and were perfused through left ventricle with FITC-dextran (Sigma-Aldrich, USA; 20 mg/ml) dissolved in 1× PBS. Both the eyes were enucleated after sacrifice and fixed overnight. Retinal whole mounts were prepared under the dissection microscope, and the mounts were visualized with a confocal microscope (Olympus, Japan). Fluorescence intensity was quantified with Image J and compared between contralateral and ipsilateral eye.

Isolation of lineage-negative bone marrow-derived stem cells

For bone marrow isolation, the syngenic strain of mice was used. Briefly, bone marrow was flushed from femur, tibia, humerus and radio-ulna. Red blood cells (RBCs) were lysed using lysis buffer (BD Biosciences, USA). Lineage-negative cells were isolated using lineage cell depletion kit (Miltenyi Biotec, Germany) that allows depletion of cells that express lineage antigens (negative selection). Bone marrow cells were incubated with a cocktail of biotin-conjugated monoclonal antibodies and anti-biotin conjugated monoclonal antibodies conjugated to microbeads. Lineage-positive cells were magnetically labeled and separated using magnetic-associated cell sorter, MACS (Miltenyi Biotec, Germany), where these cells were retained on the MACS column, and the lineage-negative population was eluted. These isolated cells were counted and then further used for flow cytometric characterization or transplantation.

Transplantation of stem cells

The lineage-negative cell population was labeled with fluorescent dye–carboxyfluorescein succinimidyl ester (CFDA-SE) before transplantation to track the cells post-transplantation. Labeled cells were counted and suspended in PBS for transplantation. CFDA-labeled cells were transplanted in mouse intravenously through tail vein after 24 h of injury. About 100,000 cells in 0.1 ml of PBS were injected. The analysis was done at day 5 and day 10 post-transplantation.

Electroretinography

The electroretinogram (ERG) was used to record a and b waves at different time-points. The mice were dark-adapted overnight. Before the recordings, the mice were anesthetized and placed on a heating pad. The pupils were dilated using tropicamide and sodium carboxymethylcellulose was applied on the cornea to keep it moistened. The ground and the reference electrodes were placed subcutaneously in the tail and between the ears respectively. The ERG was recorded using a gold cornea electrode with continuous white light flashes. The four parameters were recorded: a-wave amplitude, b-wave amplitude, implicit time to a-wave and implicit time to b-wave. The signals generated were digitized and analyzed using the ERG software (LabScribe, iWorx Systems, Dover, NH, USA).

Histological analysis

After 5 days of ischemia–reperfusion injury, mice were sacrificed with a high dose of anesthesia. Both eyes were enucleated and stored at −80 °C. The whole eye was embedded in tissue-freezing medium (Leica, Germany) and cryosectioned with a thickness of 8–10 microns and obtained on poly-L-lysine coated slides. These cryosections were subjected to histological analysis and immunofluorescence staining. Sections were fixed with tissue fixative (Histochoice tissue fixative; Sigma, USA) and washed with 1× phosphate buffered saline (PBS). The sections were then stained with Haematoxylin (Sigma, USA) and Eosin (Sigma, USA) to record histological and cytoarchitecture related changes in the retina. The sections were subsequently observed under a light microscope. The total retinal thickness and inner plexiform layer thickness were measured using Image J in different groups. Thickness was compared between control, injured, day 5 and day 10 stem cell transplanted groups.

Immunohistochemical analysis

To validate the injury and to study the change in expression levels of various markers and neurotrophic factors, immunofluorescence analysis on retinal cryosections was carried out using specific antibodies after injury and transplantation. Sections were incubated with primary antibody (1:100) overnight at 4 °C, washed and then incubated with fluorochrome-labeled secondary antibody (1:200) for 1 h at room temperature. Sections were counter-stained with DAPI (1:1000), which stains the nuclei and mounted with FluorSave reagent (Calbiochem, USA). The slides were subsequently observed under the fluorescent microscope. Images have been edited using Adobe Photoshop to enhance visibility in compliance with publishing ethics.

The primary antibodies used in the study were: glial fibrillary acidic protein, GFAP (BD Biosciences, USA), brain-derived growth factor, BDNF (BD Biosciences, USA), basic fibroblast growth factor, bFGF (BD Biosciences, USA), Nestin (BD Biosciences, USA), MAP2

(Santa Cruz, USA). The secondary antibody used was Cy3-labeled (Jackson ImmunoResearch, USA).

The immunofluorescence intensity of various markers was quantitated as corrected total cell fluorescence (CTCF) at different time-points using the Image J software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Real-time PCR

mRNA expression of various injury and cell markers, as well as neurotrophic factors, was analyzed by real-time PCR. The whole retina was isolated from enucleated mouse eye. Retina was homogenized and processed for total RNA isolation using RNA isolation kit (Qiagen, Netherlands). This RNA was then converted into cDNA using cDNA synthesis kit (Thermo Scientific, USA). cDNA was subjected to PCR reaction using primers specific for relevant markers (Table 1). The reaction was set up in triplicates. B-actin acted as an internal control and was used to normalize the expression levels. Expression levels were quantified and analyzed using real-time PCR software (StepOne, Applied Biosystems, USA).

Statistical analysis

All the study data was presented as mean ± SEM and statistically analyzed using SPSS 16.0 and p-value < 0.05 was considered to be statistically significant. Student's *t* test and ANOVA (followed by LSD posthoc analysis) were used to compare between different groups and time-points. The ERG data was analyzed using Wilcoxon Signed Ranks test.

Results

External carotid artery and pterygopalatine artery ligation results in retinal ischemia:

Laser-Doppler blood flow meter was used to map the cerebral blood flow changes during the ECA and PPA ligation surgery. There was a reduction in blood flow after the ligation of ECA and PPA (Fig. 1b). After 3.5 h, when the ligation was removed, the blood flow increased, depicting a successful reperfusion.

After ligation of ECA and PPA, narrowing of blood vessels in the retina was observed by fluorescein angiography. Right eye fundus which was subjected to ligation and imaged after 30–60 min of ligation showed narrowing of vessels as compared to the fellow contralateral eye, without any ligation (Fig. 1c–d). The thickness of vessels in contralateral versus ipsilateral eye was measured using Image J software. The comparison was made using Student's *t* test between the contralateral and ipsilateral eyes (Fig. 1e).

The retinal vasculature was visualized through cardiac perfusion of FITC-dextran in the anesthetized mouse. Obstructed vasculature was noted in FITC dextran perfused retinal flat mounts in the injured retina after 3.5 h of ligation as compared to the contralateral retina (Fig. 1f–g). Fluorescence intensity for whole-mounts was compared between the contralateral and ipsilateral eye by Student's *t* test (Fig. 1h).

Tracking transplanted cells in retina

CFDA-labelled donor cells were noted near ILM against DAPI counterstain after 5 days of intravenous transplantation were noted (Fig. 1i–j). CFDA-labelled cells were also observed 10 days post-transplantation (Fig. 1k–l).

Histological changes in retina after injury and stem cell transplantation:

After 3.5 h of ECA and PPA ligation and 5 days of reperfusion, a significant decrease was seen in total retinal thickness as well as the thickness of the inner plexiform layer (IPL). The hematoxylin and eosin-stained retinal cryosections for control, injured, and day 5 and day 10 transplanted groups were recorded (Fig. 2a–d). The total retinal thickness after stem cell transplantation increased at day 5 and day 10 and was almost similar to that of control (Fig. 2e). In the case of IPL, there was an increase in the thickness after transplantation at both the time-points, but the increase was not significant (Fig. 2f).

Table 1 Primer sequences for various markers

1	GFAP	ACAGACTTTCTCCAACCTCCAG	CCTTCTGACACGGATTTGGT
2	BDNF	TGGCTGACACTTTTGAGCAC	CAAAGGCACTTGACTGCTGA
3	FGF2	AGTGCCTTACACAATGGTTC	ACCACGCTTCTGACATCG
4	Nestin	AACTGGCACACCTCAAGATGT	TCAAGGGTATTAGGCAAGGGG
5	NeuN	GTTGCCTACCGGGGTGCACAC	TGCTCCAGTGCCGCTCCATAAG
6	B-actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA

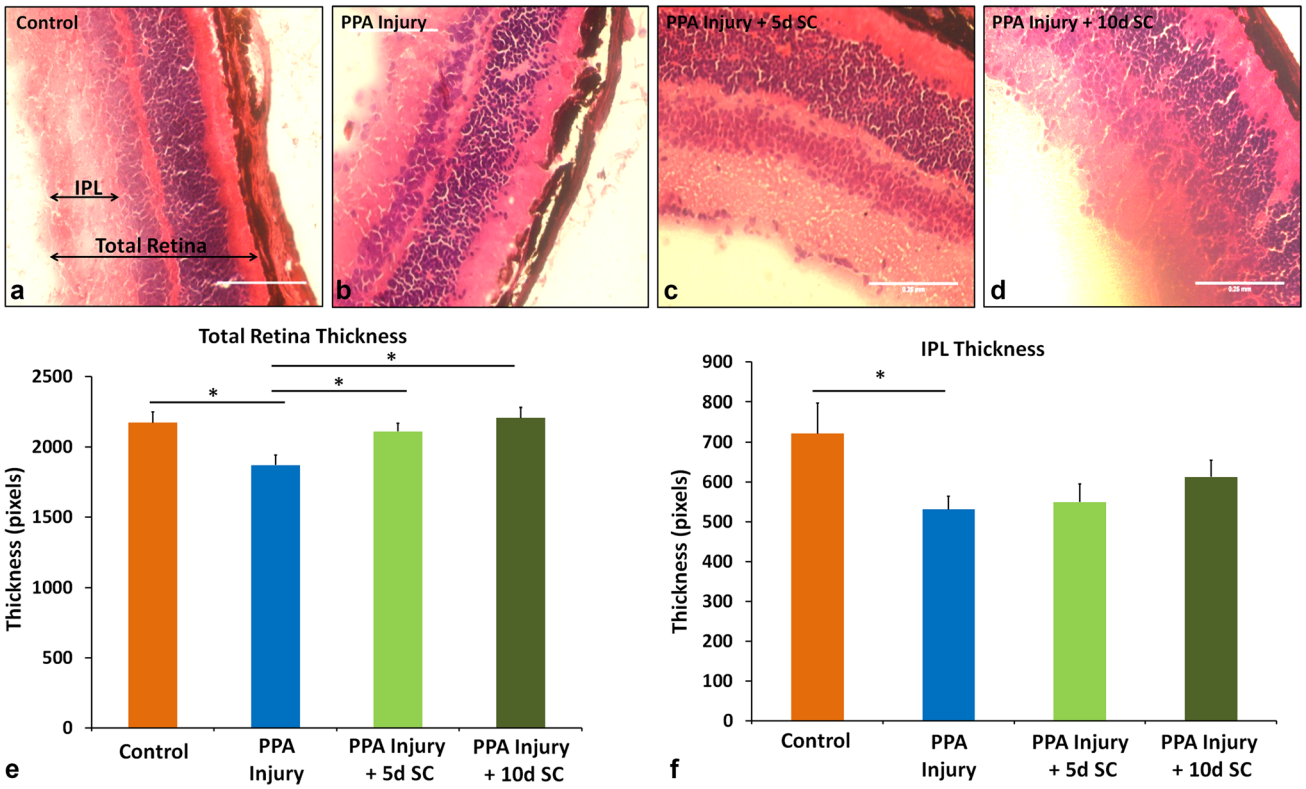

Fig. 2 Histological analysis of retina. Figure showing change in histology of retinal layers after injury and stem cells transplantation through hemotoxylin and eosin staining of retinal cryo-sections. **a** Control retina. **b** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **c**, **d** Injured retina with stem cells transplantation after 24 h of injury analysed at day 5 and day 10 post transplantation respectively *(Scale bar 250 microns)*. **e**, **f** The quantitative analysis of inner plexiform layer and total retinal thickness respectively between different groups (ANOVA, followed by post-hoc analysis by LSD, $p=0.020$, $F=4.216$ for total retinal thickness; $p=0.084$, $F=2.599$ for inner plexiform layer thickness)

Change in expression of neurotrophic factors and other cell markers after stem cells transplantation

Retinal ischemia injury was also validated by molecular analysis of glial injury marker, GFAP. An increase in expression GFAP after retinal ischemia was observed in retinal cryosections. After 3.5 h of ligation and 5 days of reperfusion, GFAP expression showed a considerable increase with glial processes spanning the inner nuclear layer (INL) and outer nuclear layer (ONL) in the ipsilateral injured eye as compared to the control eye. In the latter, the expression was only seen in the ganglion cell layer (GCL) towards the inner limiting membrane, ILM (Fig. 3a–d). GFAP expression was found to be reduced after stem cells transplantation. (Fig. 3e–h). GFAP mRNA expression analyzed by qPCR was found to be reduced, although not significant, after transplantation (Fig. 3i). Nestin, a marker for neural stem cells, showed increased immunofluorescence after injury and 10 days of stem cell transplantation as compared to the control (Fig. 3j–q). Similarly, the qPCR data showed an increase in Nestin expression after transplantation (Fig. 3r). We also observed an increase in expression of neurotrophic factors, such as BDNF and FGF2. FGF2 showed an increased immunofluorescence after PPA ligation induced injury as well as after transplantation of stem cells (Fig. 4a–h). The real-time PCR data also showed an increase in expression levels for FGF2 after injury as well as after day 5 and day 10 post-transplantation in the ipsilateral retina as compared to the contralateral retina (Fig. 4i). The brain-derived neurotrophic factor (BDNF) also increased after ligation and reperfusion injury (Fig. 4j–q). The qPCR data showed an increase in BDNF expression in the three groups (Fig. 4r).

No change in expression of microtubule-associated protein (MAP2) (Fig. 5a–h) and rhodopsin, a marker of rod cells (Fig. 5i–l) was seen after the PPA injury and stem cells transplantation.

The expression of HIF1α was found to be decreased after stem cell transplantation. Immunofluorescence analysis showed reduced HIF1α levels after 5 and 10 days post transplantation as compared to the injured retina (Fig. 6a–h). The mRNA levels of NeuN, which marks the neuronal nuclei was found to be decreased (although not significant) after the ischemia–reperfusion injury with

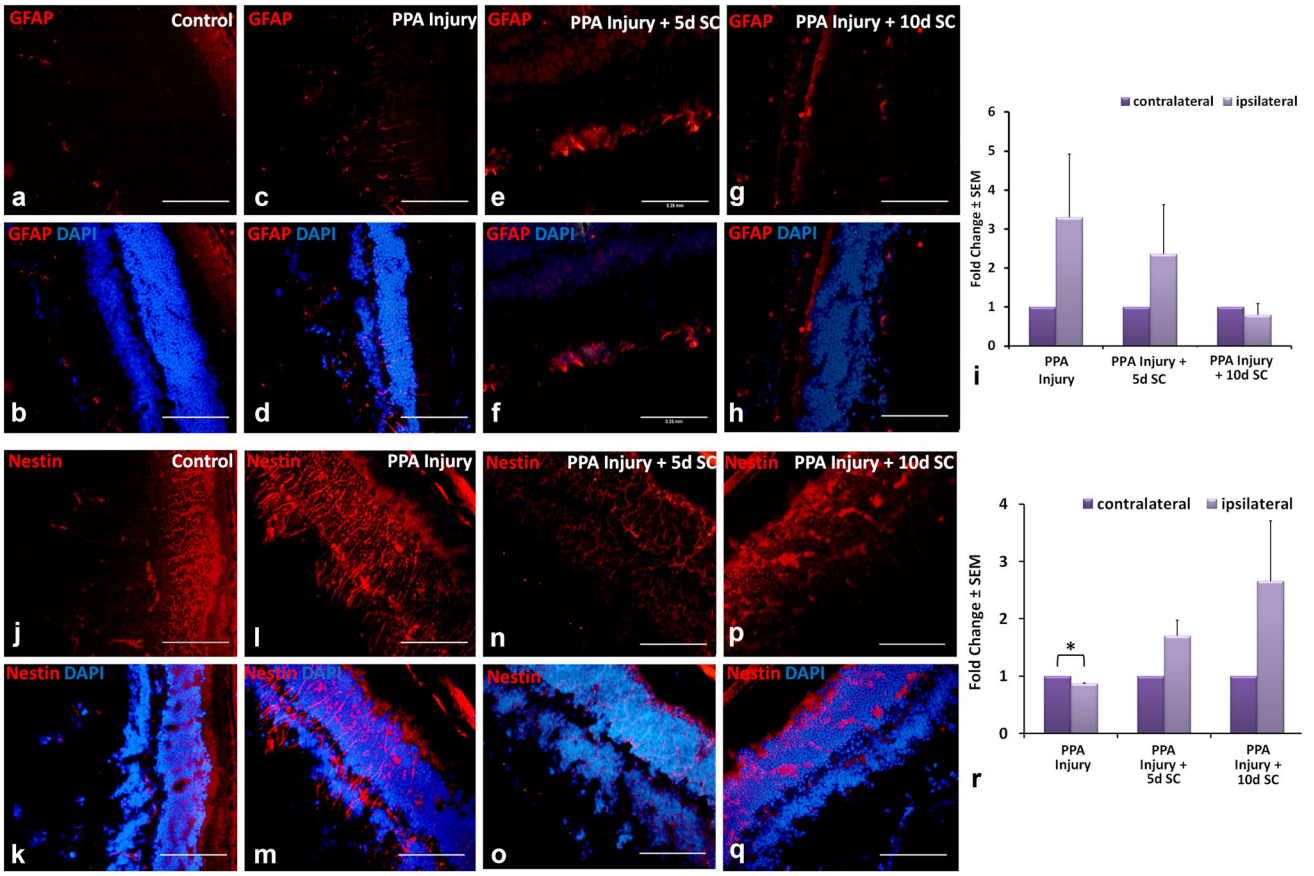

Fig. 3 GFAP and Nestin expression in retina. **a–i** Change in GFAP expression in retina after stem cells transplantation through immunofluorescnece and qPCR. **a**, **b** Control retina sections stained for GFAP and merged image with DAPI counterstain respectively. **c**, **d** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **e**, **f** Immunofluorescence images of retina after 5 days of transplantation. **g**, **h** Immunofluorescence images of retina after 10 days of transplantation. **i** Real-time PCR analysis of GFAP expression in retina (Student's *t* test, n = 3, p = 0.293 for PPA injury; n = 4, p = 0.322 for PPA injury + 5d SC; n = 4, p = 0.535 for PPA injury + 10d SC). The comparison between three groups was also done by ANOVA, followed by LSD test (p = 0.335, F = 1.259). **j–r** Change in Nestin expression in retina after stem cells transplantation through immunofluorescnece and qPCR. **j**, **k** Control retina sections stained for Nestin and merged image with DAPI counterstain respectively. **l**, **m** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **n**, **o** Immunofluorescence images of retina after 5 days of transplantation. **p**, **q** Immunofluorescence images of retina after 10 days of transplantation. **r** Real-time PCR analysis of Nestin expression in retina (Student's t-test, n = 3, p = 0.008 for PPA injury; n = 5, p = 0.063 for PPA injury + 5d SC; n = 4, p = 0.214 for PPA injury + 10d SC). The comparison between three groups was also done by ANOVA, followed by LSD test (p = 0.235, F = 1.707). *Scale bar 250 microns*

contralateral eye serving as the control. The NeuN expression showed an increasing trend at 5 and 10 days after transplantation of stem cells (Fig. 6i).

The fluorescence intensity was also compared in immunofluorescence images for different molecular markers and neurotrophic factors between control and injured retina after 5 days of ischemia–reperfusion injury as well at 5 and 10 days post transplantation (Fig. 6j–m). The expression of glial injury marker, GFAP, increased at 5 days of injury. After 10 days of stem cell transplantation, reduced expression of GFAP (Fig. 6j) could be seen. HIF1α showed a significant increase in the fluorescence intensity in the injured retina as compared to the control, which was found to be reduced after the transplantation (Fig. 6k). The neurotrophic factors, namely FGF2 and BNDF, were found to be increased after injury and transplantation (Fig. 6l–m).

Changes in Electroretinogram trends after ischemic injury

The electroretinographic responses were recorded after the overnight dark adaptation. The four parameters were investigated, i.e. a-wave amplitude, b-wave amplitude, implicit time to a-wave, and implicit time to b-wave which did not show any significant change either after injury or transplantation. The left eye was used as a contralateral control (Fig. 7a–r).

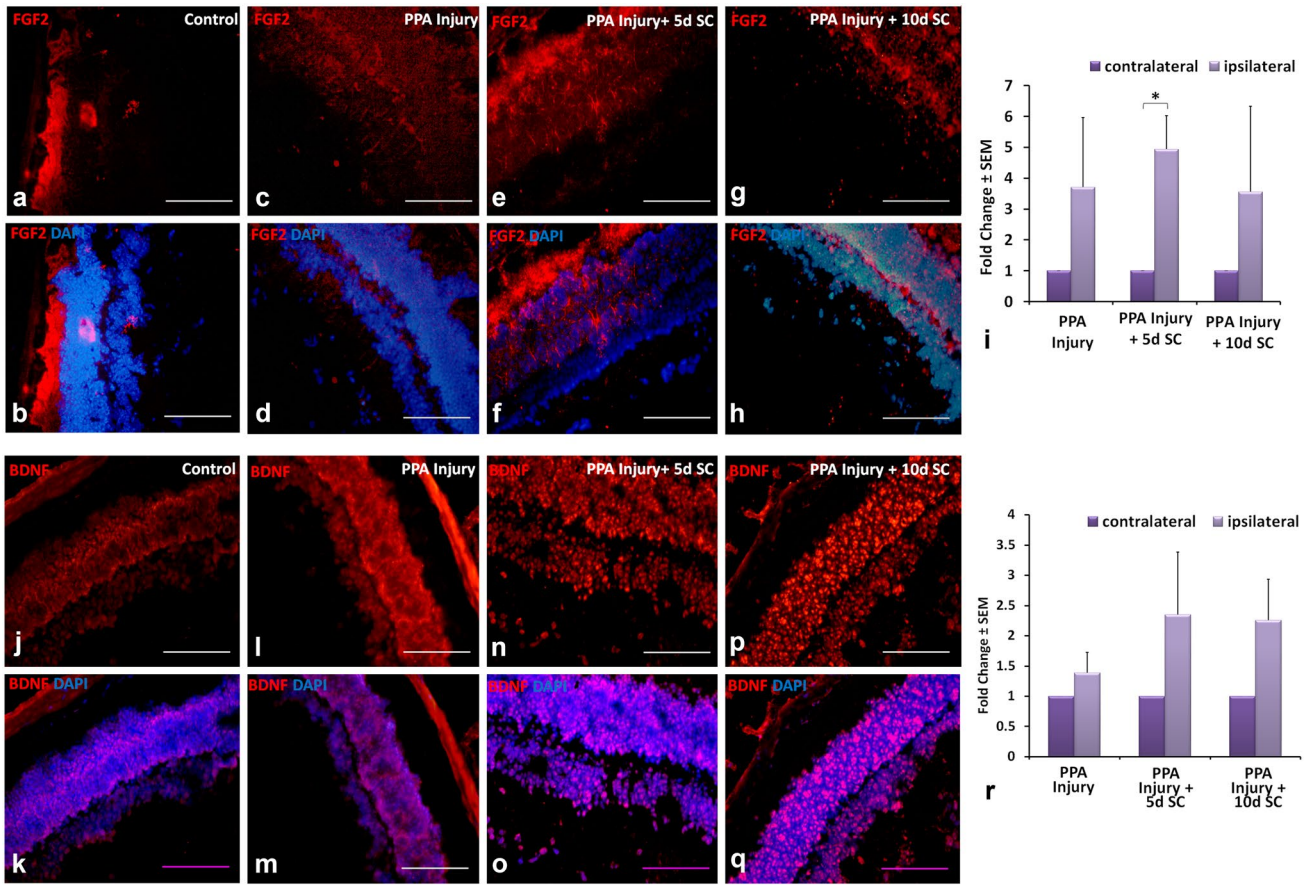

Fig. 4 Neurotrophic factors—FGF2 and BDNF expression in retina. **a–i** Change in FGF2 expression in retina after stem cells transplantation through immunofluorescence and qPCR. **a**, **b** Control retina sections stained for FGF2 and merged image with DAPI counterstain respectively. **c**, **d** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **e**, **f** Immunofluorescence images of retina after 5 days of transplantation. **g**, **h** Immunofluorescence images of retina after 10 days of transplantation. **i** Real-time PCR analysis of FGF2 expression in retina. (Student's *t* test, n = 3, p = 0.352 for PPA injury; n = 5, p = 0.022 for PPA injury + 5d SC; n = 4, p = 0.355 for PPA injury + 10d SC). The comparison between three groups was also done by ANOVA, followed by LSD test (p = 0.857, F = 0.157). **j-r**. Change in BDNF expression in retina after stem cells transplantation through immunofluorescnece and qPCR. **j**, **k** Control retina sections stained for BDNF and merged image with DAPI counterstain respectively. **l**, **m** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **n**, **o** Immunofluorescence images of retina after 5 days of transplantation. **p**, **q** Immunofluorescence images of retina after 10 days of transplantation. **r** Real-time PCR analysis of BDNF expression in retina. (Student's *t* test, n = 3, p = 0.359 for PPA injury; n = 5, p = 0.262 for PPA injury + 5d SC; n = 5, p = 0.135 for PPA injury + 10d SC). The comparison between three groups was also done by ANOVA, followed by LSD test (p = 0.738, F = 0.313). *Scale bar 250 microns*

Discussion

Retinal ischemia is a condition associated with many retinal disorders such as diabetic retinopathy, central retinal artery occlusion (CRAO), glaucoma, and various optic neuropathies [1]. Our data was found to be consistent with earlier studies on retinal ischemia showing a decrease in thickness of IPL after injury. The inner retina has been earlier shown to be more sensitive than the outer retina [15]. In a rat model of increased IOP, a study showed a decrease in inner retinal thickness after ischemia [16]. Flat-mounts have been previously analyzed to map the vascular architecture of retina. Oghishima et al. showed an interruption in blood supply through FITC dextran perfusion imaging of retinal flat-mounts after the ligation of different arteries supplying blood to the retina [11]. In another study by Lelong et al. FITC-dextran perfused retinal whole mounts showed an interrupted blood supply after retinal ischemia [7]. Consistent with previous studies, we illustrate a decrease in the FITC-dextran perfusion in the retina after the ligation of PPA and ECA when compared to the contralateral retina.

Glial fibrillary acidic protein (GFAP), is an intermediate filament protein expressed in astrocytes in CNS. In CNS, astrocytes are activated after injury, causing increased GFAP expression [17]. But, in the case of the retina, the expression is seen in Muller cells and ganglion cells. Any insult to the retina increases the expression of GFAP and is observed as glial processes present throughout the retina

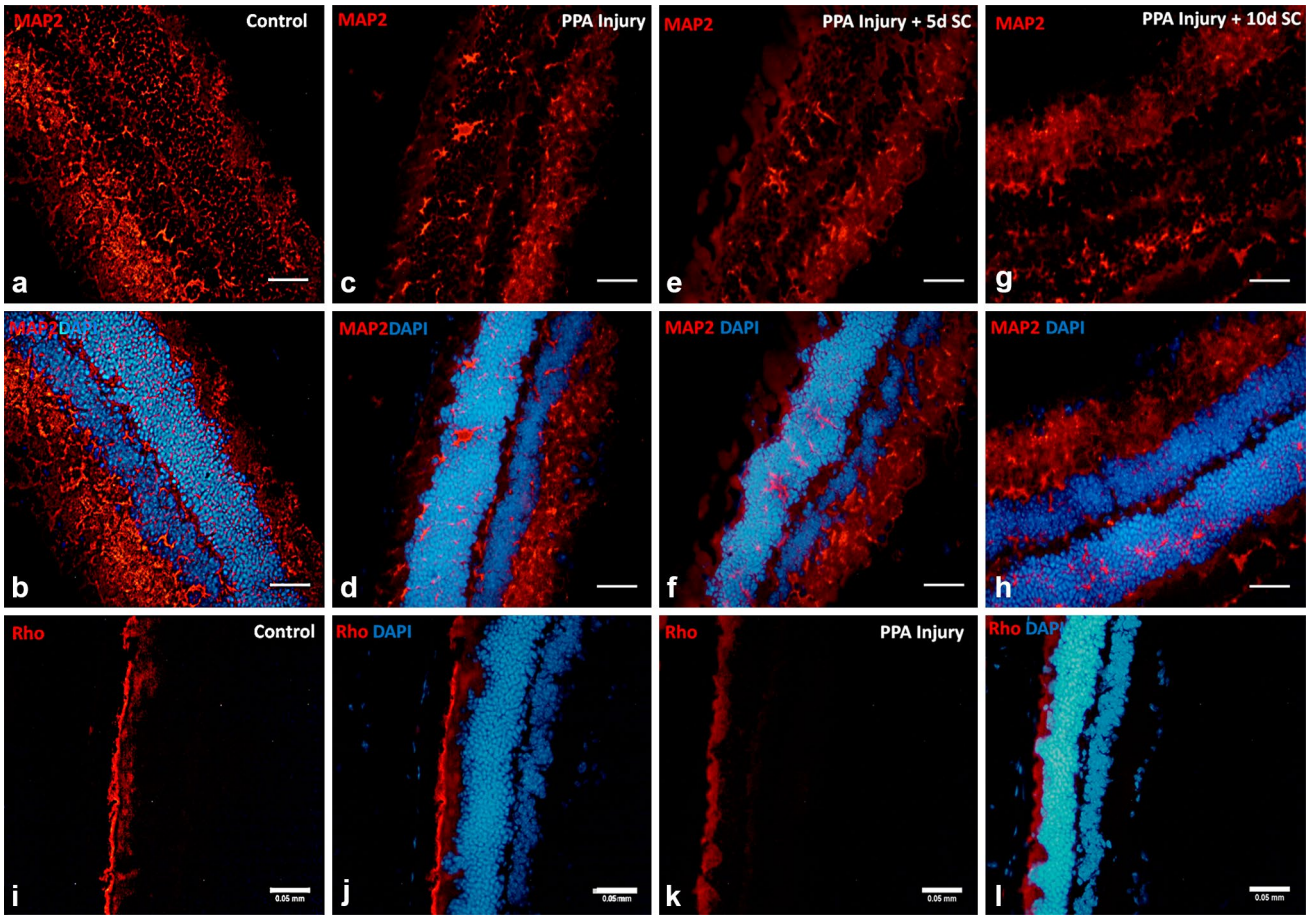

Fig. 5 No change in expression of MAP2 and rhodopsin. **a**, **b** Control retina sections stained for MAP2 and merged image with DAPI counterstain respectively. **c**, **d** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **e**, **f** Immunofluorescence images of retina after 5 days of transplantation. **g**, **h** Retina after 10 days of transplantation. **i**, **j** Control sections. **k**, **l** Injured retina after 3.5 h of ligation and 5 days of reperfusion stained for rhodopsin and merged image with DAPI counterstain respectively. *Scale bar 50 microns*

[18, 19]. In this mouse model of ligation and reperfusion, a similar increase in GFAP immunoreactivity was observed in the retina. This elevated expression further showed a decrease upon stem cells transplantation. Hypoxia-inducible factors or HIF (another marker for ischemia), which are known transcription factors, have also been reported to be activated in response to change in oxygen levels [20]. These are involved in the transcription of many genes activated in response to hypoxia such as vascular endothelial growth factor (VEGF) and other genes that are responsible for angiogenesis [21–24]. Similarly, our study also shows that the decrease in blood supply to retina results in upregulation of HIF1α expression after injury which is found reduced after the transplantation of stem cells.

No existing therapies have succeeded in addressing this problem. Stem cell transplantation has previously shown good results to some extent in various animal models of retinal diseases with successful migration, incorporation, and differentiation of transplanted cells isolated from various sources in different studies. These have been used in various models of neuronal injury. Prabhakar et al. [25] have demonstrated the migration of bone-marrow cells to ischemic brain in a mouse stroke model. Similarly, Harris et al. also demonstrated homing of bone-marrow derived hematopoietic stem cells in a mouse model of retinal pigment epithelium (RPE) damage induced by physical and chemical means. The transplanted cells also showed RPE cell-like morphology [26]. Bone-marrow stem cells were shown to migrate and rescue the rat model of retinal ischemia–reperfusion injury, induced by elevated intraocular pressure. The intravitreally transplanted cells showed increased expression of neurotrophic factors such as BDNF, bFGF, CNTF for 4 weeks after injury [27]. Earlier Tomita et al. showed successful differentiation of bone marrow-derived stem cells into retinal cells in another rat model of mechanical injury. The transplanted cells grafted in the retinal layers and expressed GFAP, vimentin, calbindin and rhodopsin 2 weeks after transplantation [28]. In another

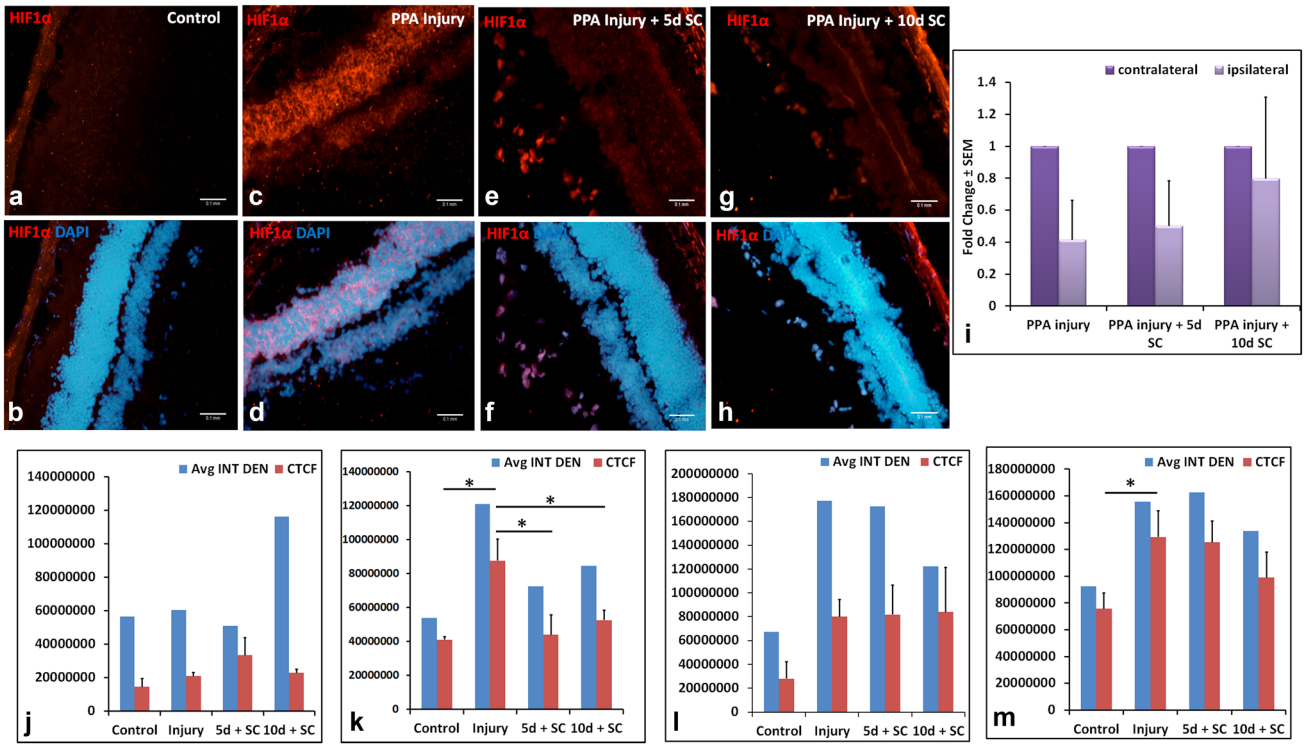

Fig. 6 HIF1α and NeuN expression in retina and fluorescence intensity of different molecular markers **a–h** Change in *HIF1*α expression in retina after stem cells transplantation through immunofluorescence. **a**, **b** Control retina sections. **c**, **d** Injured retina after 3.5 h of ligation and 5 days of reperfusion. **e**, **f** Immunofluorescence images of retina after 5 days of transplantation. **g**, **h** Immunofluorescence images of retina after 10 days of transplantation stained for *HIF1*α and merged image with DAPI counterstain respectively. *Scale bar 100 microns*. **i** Real-time PCR analysis of NeuN expression in retina (Student's *t* test, n = 3, p = 0.079 for PPA injury; p = 0.220 for PPA injury + 5d SC; p = 0.731 for PPA injury + 10d SC). **j–m** Change in expression of different markers in retina through quantitation of immunofluorescence intensity at different time-points. **j** GFAP (p = 0.536, F = 0.802) **k**. HIF1α (p = 0.015, F = 6.522). **l** FGF2 (p = 0.645, F = 0.576). **m** BDNF (p = 0.153, F = 2.073). The data is presented as corrected cell total fluorescence (CTCF) units and was analysed with ANOVA followed by post-hoc analysis with LSD

interesting study Chung et al. [29] in a laser-induced retinal injury model, showed the migration of bone marrow-derived cells and repair of damaged retina. These studies have also led to development of several patents. The use of peripheral blood hematopoietic stem cells or granulocyte—colony stimulating factor (G-CSF) to mobilise resident stem cells in retinal disorders has been patented [30]. In another patent published in 2013, stem cell-based therapy has been shown to rescue retinal degeneration [31]. Friedlander et al. patented the use of lineage-negative stem cell population from bone marrow for the treatment of vascular disorders of the eye [32] depicting the growing interest in stem cell applications. Since the lineage-negative population is known to be more enriched for stem cells as compared to total bone marrow population, as also shown in our previous study, the lineage-negative cells isolated from mouse bone marrow expressed elevated levels of CD34, CD117 and Sca-1, as markers for stem cells [33]. In a study by Atmaca-Sonmez et al. [34] lineage-negative, Sca-1 positive stem cells derived from bone marrow, when injected in a mouse model of sodium iodate induced degeneration, migrated to sub-retinal space and displayed expression of retinal pigment epithelium-specific markers. The lineage-negative population of stem cells has shown successful migration in a model of laser-induced retinal injury in time and dose-dependent manner when transplanted through intravitreal as well as intravenous routes. Better migration was observed in intravenously transplanted cells in this model [35]. In a separate study, we also showed that lineage-negative population, isolated from human umbilical cord blood, when transplanted in amyloid-beta-induced memory loss model reversed the memory loss [36].

The functional efficacy of stem cell transplantation in retinal degeneration has, however, been intensely debated, especially in view of the rising hopes and diminishing pace of clinical success. Some studies have shown that the transplanted stem cells not only replace the damaged neurons but also form functional synapses. On the other hand, investigations have also reported molecular changes which are not reflected at physiological levels. Electroretinogram

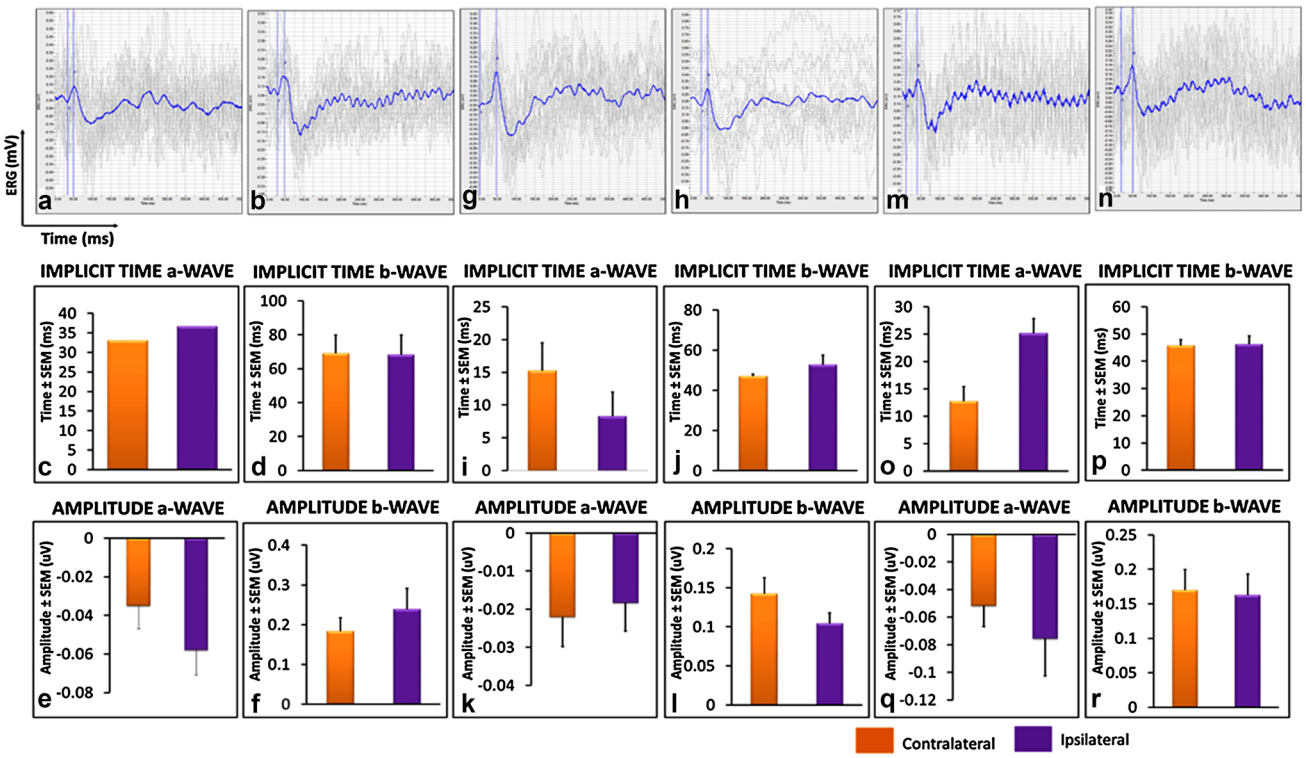

Fig. 7 Electroretinogram analysis **a–f**. Representative electroretinogram wave of contralateral eye (**a**), ipsilateral eye (**b**), a-wave amplitude (**c**), b-wave amplitude (**d**), a-wave implicit time (**e**), b-wave implicit time (**f**) after injury (n = 4). **g–l** Representative electroretinogram wave of contralateral eye (**g**), ipsilateral eye (**h**), a-wave amplitude (**i**), b-wave amplitude (**j**), a-wave implicit time (**k**), b-wave implicit time (**l**) after 5 days of stem cells transplantation (n = 7). **m–r** Representative electroretinogram wave of contralateral eye (**m**), representative electroretinogram wave of ipsilateral eye (**n**), a-wave amplitude (**o**), b-wave amplitude (**p**), a-wave implicit time (**q**), b-wave implicit time (**r**) after 10 days of stem cell transplantation (n = 5). The data was analysed through Wilcoxon signed ranks test (*orange bar* contralateral and *purple bar* ipsilateral eye). (Color figure online)

analysis is a useful tool to map the functional responses of different cells types in the retina [37] but it's role in RGC function remains controversial [38]. In this study, as we did not observe any change in any of the electroretinogram parameters we believe that this could be due to several reasons. ERG variations in retinal degeneration are well described. For example, Kim et al. [39] investigated the effect of injury and failed to demonstrate any significant change in a-wave amplitude at earlier time-points even when histological changes were observed in the retinal layers. In another model of retinal ischemia, the molecular changes were visible as early as 1 h after ischemia, but no changes were demonstrated in a-wave and b-wave implicit times [7]. Our ERG responses were recorded at 5 and 10 days after transplantation. These studies do not necessarily suggest the futility of functional analysis. These can also be interpreted as possible dissociation of functional responses from molecular changes.

In this study, we also observed an increase in expression of neurotrophic factors, i.e., BDNF and bFGF through immunofluorescence and qPCR, after both injury and after stem cell transplantation. The neurotrophic factors are involved in development, growth, injury and regeneration. Interestingly, Wilkins et al. [40] have shown that the bone marrow-derived stem cells secrete BDNF and protect the neuronal cells in-vitro on exposure to nitric oxide. In another study by Gao et al. [41] the authors have demonstrated increased BDNF levels in a rat model of optic nerve injury. The therapeutic effect of neurotrophic factors was also shown in a transgenic model of retinal degeneration, where transgenic expression of BDNF in retina slowed down the damage caused to the photoreceptors [42]. Basic fibroblast growth factor (bFGF) has also been shown to be neuroprotective in a rabbit model of retinal damage induced by laser. The bFGF treatment resulted in recovery to some extent as well as less damage to the cells in the retina [43]. The mechanism through which the lineage-negative stem cells exert neuroprotection to the damaged retina after the ischemic injury is postulated to be mediated by the release of these trophic factors. The stem cell transplantation in this study resulted in the increase in expression of BDNF, FGF and Nestin. The neurotrophic factors involved in the retinal regeneration act through different

signaling pathways including MAPK, PI3K, pSTAT pathways and it was postulated that cross-talk between various growth factors is critical for neuroprotection [44–46]. Figure 8 summarizes the putative mechanism showing the damage caused by artery ligation, along with the proposed mechanism of how stem cell transplantation could be exerting protection through the release of neurotrophic factors. The neurotrophic factors, whether they are released by transplanted cells or by endogenous cells, remains debatable was not analysed in this study. Studies have also shown that the neuroprotective modulation by bone-marrow cells in hypoxia may not be dependent

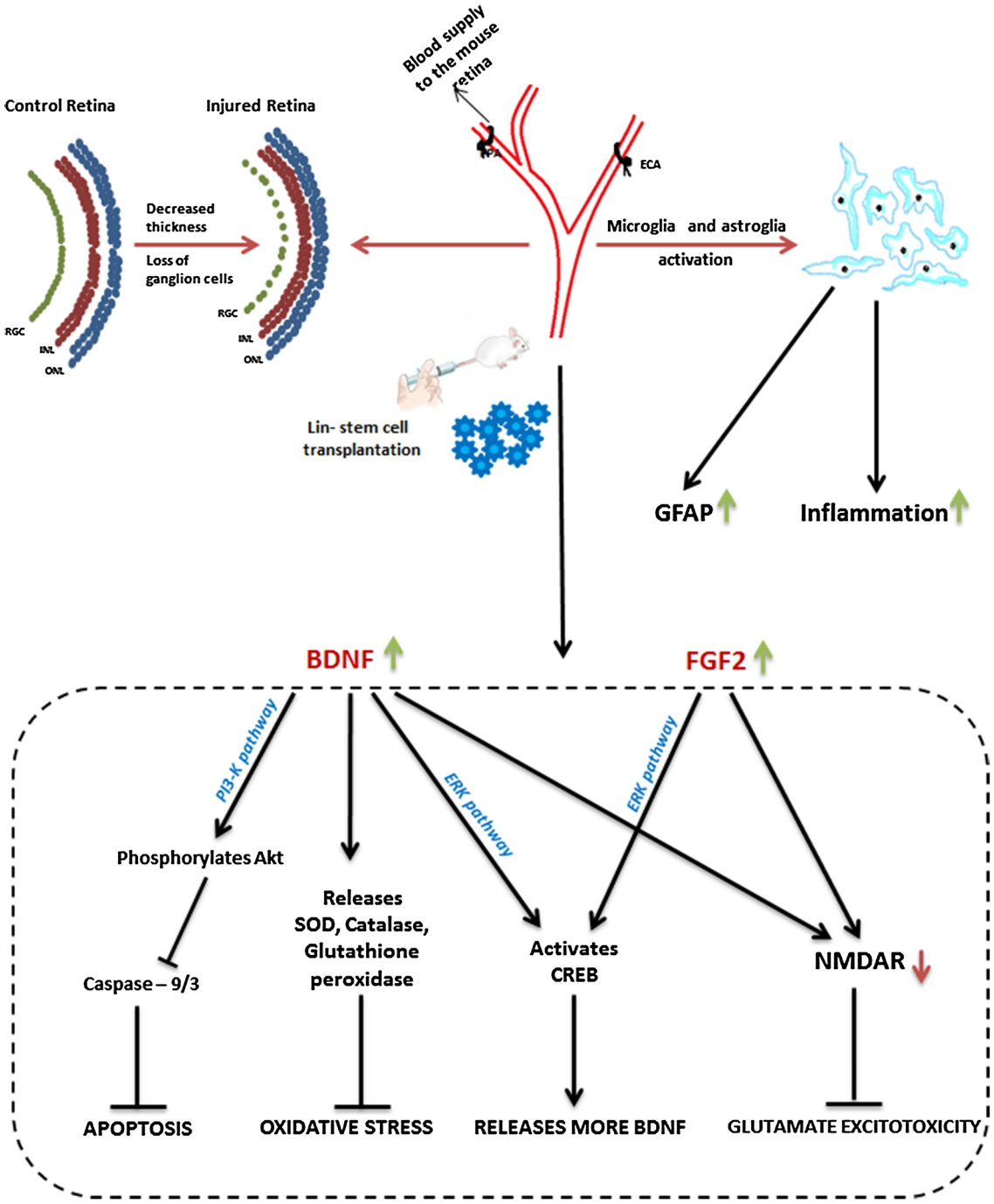

Fig. 8 Mechanism of action Lin-ve stem cell transplantation after the ECA and PPA ligation induced injury is proposed to provide neuroprotection through BDNF and FGF2. The *dotted area* depicts the postulated pathways through which BDNF and FGF2 may lead to less apoptosis, excitotoxic damage, and oxidative stress

on direct cell contact and involves a cross-talk between the transplanted cells and the host cells through secreted factors [47]. Further studies could help delineate the role of stem cells transplantation and to identify their role in neuroprotection through production of neurotrophic factors, differentiation or stimulation of resident cells to secrete neuroprotective cytokines. The study was limited to shorter duration transplantation endpoints. Additional studies, with longer duration time-points, could be conducted to analyse the efficacy of stem cells.

Our study, therefore, highlights the fact that the molecular changes, manifest as immunohistochemical changes, do not necessarily correspond to functional amelioration after stem cell transplantation until better time points are chosen or higher doses are used. It is also important to revisit the utility of ERG in the context of RGC function evaluation. Future studies with intensity based ERG measurements will be helpful once the sensitivity of ERG for RGC is validated. Experimental set-ups at such time points as to evoke responses that can be matched to molecular changes can be planned. Additional functional assays including evaluation of damage to blood vessels, quantification of donor cell engraftment, cell proliferation, and quantitative demonstration of increases in vascular density can be used for future studies to assess the cellular repair. Further studies are warranted to demonstrate the mechanisms behind the neuroprotective effect of neurotrophic factors by using pharmacological inhibition or ectopic expression of BDNF and FGF2 in retinal ischemia. Also there stands a need to study the long-term efficacy of stem cells. In conclusion, this study demonstrates that the ECA and PPA ligation model successfully mimics the characteristics of retinal ischemia with many advantages over other established models of retinal ischemia.

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Author contributions GM performed the surgery; SP is a co-investigator in grant application; RM and MS provided training, RB supervised the FFA and AA designed, supervised and provided resources for the study.

Compliance with Ethical Standards

Conflict of interest No conflicting relationship exists for any author.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Potential for Stem Cells Therapy in Alzheimer's Disease: Do Neurotrophic Factors Play Critical Role?

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Abstract

Alzheimer's disease (AD) is one of the most common causes of dementia. Despite several decades of serious research in AD there is no standard disease modifying therapy available. Stem cells hold immense potential to regenerate tissue systems and are studied in a number of brain-related disorders. For various untreatable neurodegenerative disorders, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) (current-approved drugs provide only symptomatic relief), stem cell therapy holds a great promise and provides a great research opportunity. Here we review several stem cell transplantation studies with reference to both preclinical and clinical approaches. We focus on different sources of stem cells in a number of animal models and on molecular mechanisms involved in possible treatment of neurodegenerative disorders. The clinical studies reviewed suggest safety efficacy and translational potential of stem cell therapy. The therapeutic outcome of stem cell transplantation has been promising in many studies but no unifying hypothesis exists for an underlying mechanism. Some studies reported paracrine effects exerted by these cells via release of neurotrophic factors, while other studies reported immunomodulatory effects by transplanted cells. There are also reports supporting stem cell transplantation causing endogenous cell proliferation or replacement of diseased cells at the site of degeneration. In animal models of AD, stem cell transplantation is also believed to increase expression of synaptic proteins. A number of stem cell transplantation studies point out great potential for this novel approach in preventing or halting several neurodegenerative diseases. The current challenge is to clearly define the molecular mechanism by which stem cells operate and the extent of actual contribution by the exogenous and/or endogenous cells in the rescue of disease.

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AUTHOR'S CONTRIBUTION

PB and AB contributed in writing of manuscript. AA participated in the concept of review, it's designing and editing. DKL has done concept designing, editing, rephrasing of manuscript and manuscript writing.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Keywords

Stem cells; Alzheimer's disease; neurodegeneration; synaptogenesis; differentiation; proliferation; therapeutics; transplantation

INTRODUCTION: THE POTENTIAL OF STEM CELL TRANSPLANTATION IN ALZHEIMER'S DISEASE

A number of neurodegenerative disorders, such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) are untreatable, and they progressively worsen with age, resulting in death. The world Alzheimer Repot 2015 reported over 46 million individuals in the world's population suffer from dementia, and this number is estimated to increase upto 131.5 million by 2050 [1]. Dementia is associated with multiple causes that include alcoholism, AD, stroke, PD and drug/medication intoxication. It is the fifth leading cause of death in the US with age of 65 years or above. In 2015 AD prevalence in USA was estimated to be close to 5.3 million, and this is expected to rise up to 11 to 16 million in 2050 [2]. In India, the number of individuals which are suffering from AD and other dementia is estimated to be approximate 3.7 million and this number is expected to double by the year 2030 [3].

The most common form of dementia, AD, is characterized by different stages of cognitive and functional impairment. Patients suffering from AD lose autonomy in their daily normal activities, and this progressively deteriorates with age. In 1901, Alois Alzheimer, a German psychiatrist, diagnosed a 51 year-old woman with a condition he called "amnestic writing disorder" [4]. Her psychosocial abnormalities included aphasia and memory impairment. Later, in 1910 when Alzheimer's supervisor published his book *Psychiatrie,* he reported this case and mentioned this condition as Alzheimer's disease [4]. Since then, extensive research has progressed worldwide to understand several aspects of the disease, ranging from its pathology, disease onset, prevalence, diagnosis and treatment in various cellular, pre-clinical and clinical studies. Currently, AD pathophysiology is based on several important hypotheses i.e., including the cholinergic hypothesis, protein misfolding, and amyloid cascade hypotheses [5–7].

The hippocampus plays a significant role in memory encoding and retrieval. Hippocampus is the first region of the brain to be affected in AD. Injury to brain tissue has not been seriously considered for treatment by cell replacement strategies as compared to the other organs e.g. skin and liver tissues. Earlier, neuroanatomists considered that the nervous system is incapable of regeneration. In 1962 Joseph Altman provided the first evidence of neurogenesis in the cerebral cortex and later, in 1963 he showed the occurrence of neurogenesis in the dentate gyrus of rat and cat hippocampus [8]. In some animals, neuronal precursors originate from the subventricular zone (SVZ) to the main olfactory bulb via specialized migratory route known as the rostral migratory stream (RMS). More recently, various strategies are being employed to activate these lesser population of stem cells by various methods [9]. Currently available FDA-approved drugs for AD provide symptomatic relief to the patients without alleviating elusive disease pathology. Alternative strategies such

as herbal remedies [10–12] and cell based therapies [13, 14] are being tested in preclinical settings with the hope of halting disease progression. The underlying mechanism is either replacement of degenerating neurons or exerting neuroprotection by the paracrine effect of transplanted cells by the secretion of neurotrophic factors (Fig. 1) [15]. The efficacy of stem cells has been studied in various pre-clinical studies by transplanting these cells into the disease- specific animal models. However, there is a gap of knowledge describing the underlying molecular mechanisms involved in the rescue of disease by transplanted cells.

PATHOPHYSIOLOGICAL FEATURES IN ALZHEIMER'S DISEASE

Several animal studies and human brain biopsies have revealed the pathological hallmarks of AD, including extracellular amyloid-β (Aβ) plaque deposits and formation of intracellular neurofibrillary tangles (NFT). NFT are misfolded structures produced by aberrant phosphorylation of microtubule-stabilizing tau proteins. The process of Aβ formation is known to play a significant role in AD etiology [16]. Amyloid plaques may trigger a pathological cascade resulting in neurofibrillary tangles and neuroinflammation causing neuritic dysfunction, which ultimately leads to neuronal death. In AD patients, excessive accumulation of amyloid plaques is likely to be due to dysregulation of activity of β-site Amyloid Precursor Protein-Cleaving Enzyme 1 (BACE1). BACE1 gives rise to Aβ from the membrane-spanning Aβ precursor protein (APP). This is the rate limiting step of Aβ production. This cleavage occurs at the N-terminus of Aβ to form soluble APPβ, and the C-terminus is further cleaved by γ-secretase complex, which yields Aβ40/42 [17]. Aβ fragments thus generated aggregate to form amyloid fibrils. Aβ40 (with 40 amino acid residue) is the predominant form but Aβ42 (with 42 residues) is more fibrillogenic than the shorter species and is involved in disease pathology.

Several environmental factors cause epigenetic changes in individuals. It plays a significant role in regulating the gene expression *via* modification of DNA and histone protein modification leading to genetic dysregulation thereby causing various disease pathologies. In AD, amyloid fibril-induced neuroinflammation is believed to increase expression of epigenetic factors such as. methyl-CpG-binding protein 2 and histone deacetylase 2 and their interaction further suppresses the expression of synaptic protein leading to amyloid induced memory deficiency [18].

Tau is an intracellular microtubule associated protein that plays an essential role in microtubule stabilization. Abnormal phosphorylation of tau leads to microtubule disruption. The formation of neurofibrillary tangles may be triggered by amyloid plaque. In addition, the cholinergic hypothesis postulates a reduction in neurotransmitter acetylcholine in the AD patients [19] as the primary cause of AD. Besides amyloid plaque deposition and neurofibrillary tangle formation, vascular dysfunction also appears in AD pathophysiology.

Both genetic and environmental factors contribute to etiology of AD. Genetic factors linked to autosomal dominant inherited mutations include presenilin 1 (PS1), presenilin 2 (PS2), APP and enzymes involved in amyloid processing, such as BACE1. This genetic form, also called familial AD (FAD) contributes marginally towards prevalence (no more than 5% of AD cases [20]), whereas most AD cases are sporadic, with an unknown cause. The E4

variant of APOE is largely known as a major genetic risk factor for the late onset of AD [21]. Studies suggest that there are some interactions with amyloid to cause this dramatic effect [22]. Some studies also propose that sporadic cases are the result of various environmental and epigenetic factors which lead to an etiology based upon “Latent Early-life Associated Regulation” (LEARn). LEARn describes effects resulting from exposure of stressors in early life e.g. nutritional imbalance, toxic metals (such as lead) and other stressors, which induce epigenetic alterations on disease associated gene chromatin or histones [23]. These changes remain latent as (de)methylation of promoter or chromatin modifications by (de)acetylation, (de)methylation and (de)phosphorylation. Upon one or more additional hits in the later life, expression of modified gene(s) alters sufficiently to induce pathology.

CELL TYPE CONSIDERATIONS FOR DISEASE MODIFYING THERAPIES

The requirement of a suitable cell type with particular characteristics for specific disease types is needed for proper and effective cell transplantation. Stem cells from several tissues such as bone marrow and umbilical cord blood are well characterized for their proliferation and differentiation properties and can be an optimum source for transplantation [24, 25]. Current strategies emphasize culturing of isolated cells in an optimum medium with suitable nutrient environment to obtain the desired disease phenotype. The microenvironment also provides suitable niche for selective expression of desirable markers to trigger these cells for a specialized cell type [26, 27]. Long-term culture and characterization of primary neurons isolated from rodent and human fetal tissue is essential for undertaking comparative studies. Abundant tau and amyloid-β production in human brain cultures provides a powerful cellular model for AD. In a recent study Ray *et al* provide a well-characterized methodology for fetal human primary brain cell culture, which is useful to test the therapeutic efficacy of drugs targeting AD [28]. Cultures of induced pluripotent stem cell (iPSC) generated from fibroblasts of FAD patients with presenilin 1 and presenilin 2 mutations were characterized after acquiring neuronal lineage [29]. Apart from increased Aβ42 expression, the iPSC model also showed variable drug response and alleviation of stress induced response by docosahexaenoic acid (DHA) treatment [30]. Likewise, RNA silencing has also been used in this cellular model. Therapeutic strategies primarily focus on targeting production of Aβ by identifying key molecular regulators of BACE1 expression. The researchers have also elucidated the role of human micro-RNA (miR)-339-5p which negatively modulates BACE1 in primary human brain cultures, and expression of miR-339-5p is reduced in AD patients [31].

PRE-CLINICAL STUDIES TO PROBE REGENERATIVE POTENTIAL OF STEM CELLS

At present, there are no consensus measures to accurately diagnose and monitor progression of AD [32]. This significantly hinders effective treatments against AD. To study AD pathologies and its targets, different animal models of AD have been established and tested in preclinical settings. These model systems range from laboratory animals like zebrafish, murid rodents and nonhuman primates to model invertebrates such as *Drosophila* and *C*

elegans. Among these, rats and mice are widely used, and their transgenic counterparts are the most-established system to evaluate disease pathophysiology as well as effective treatment strategies. Several strategies have been adopted to establish AD like pathologies and induced memory impairment in these models [33]. These include predetermined brain injury, neurotoxin induced cell loss in brain and intra-cerebroventricular injection of Aβ peptides [34].

Current treatments for AD includes blocking neurotransmitter degradation, which provide temporary symptomatic relief without alleviating the pathophysiological burden of the disease [35, 36]. Therefore, alternative cell based studies for transplantation have been carried out in the belief that either these cells replace degenerating neurons or secrete trophic factors that provide a protective environment to the endogenous cells. Various neurotrophic factors are secreted by the cells to modulate the synaptic functioning in brain. In particular, BDNF is synthesized by neurons and highly expressed in cortex and hippocampus; these regions are crucial for learning and memory in brain [37].

The animal models associated with Aβ-induced memory loss have been widely studied in understanding pathophysiology of AD and testing therapeutic efficacy of various drug targets. Prakash *et al.* use intracerebroventricular (ICV) injection of Aβ to study the role of pioglitazone, a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, on neurotrophic factor BDNF in a rat model of AD with neuroinflammation. Aβ-injured animals showed significant impairment in memory as well as reduced levels of BDNF, which were reversed by administration of pioglitazone [38]. Tang *et al.* demonstrated fibrillar Aβ40 induced neurotoxicity in rat hippocampus, characterized by congo red plaques and degenerating neurons at the site of injection. This pathological outcome was supported by impaired cognitive performance in the rats, tested in Morris water maze. Further, they have used this model to validate cell replacement efficacy of neural precursor cells derived from human embryonic stem cells. The neural precursor cells are partially differentiated, as these cells are more precisely committed to their lineage [39]. The transplanted cells were found to ameliorate Aβ-induced cognitive impairment in these rats and further survived, integrated and differentiated into GFAP and NF-200 positive neuronal cells after 16 weeks of transplantation [40].

Blurton-Jones *et al.* explored the role of neural stem cell transplantation in reversal of memory impairment. To study the effect of neural stem cells (NSCs) in AD pathology and cognitive functions, these cells were transplanted into aged triple transgenic mice that express mutant presenilin, tau and APP with aggressive Aβ load. Remarkably, transplanted NSCs were found to ameliorate loss in spatial learning and memory *without* altering Aβ and tau pathologies. Further, these cells increased synaptic density in diseased brain, which was assisted by BDNF. Loss of function studies have revealed that NSCs exert regenerative effects mediated by BDNF. It was further found that restoration of memory loss occurred when recombinant BDNF was additionally supplemented [15]. The same group recently reported that when these NSCs were genetically engineered to stably release the Aβ degrading enzyme neprilysin (NEP), they could augment synaptic plasticity as well as ameliorate underlying Aβ pathology in triple transgenic mice [41]. Neuralstem, Inc. had announced the first data on neural stem cells transplantation studies in an animal model of

AD. This group reported that HK532: IGF1 (NSI-532.IGF) cells ameliorate spatial learning deficits and improved memory in AD mice. To generate human insulin-like growth factor 1 (IGF-1), a cortical neural stem cell line was engineered. IGF-1 cells also impart a wide-range of neuroprotective properties [42]. Notably, the cells, which were administered in the peri-hippocampal region showed survival up to ten weeks. Also, mice with stem cell transplantation performed better than did control mice at fourteen weeks after the surgery. It would be reasonable to conclude that such preliminary studies point toward a potentially feasible therapeutic approach to treat AD in the future, and that the therapeutic effect of stem cells upon transplantation into the brain is supported by a combination of approaches and largely mediated or at least significantly influenced by paracrine effects.

IMMUNOMODULATORY EFFECTS OF STEM CELLS TARGETING AD PATHOLOGY

Reports also suggest that transplanted stem cells exerts some immunomodulatory response at the site of injury, leading to release of cytokines that further target the underlying AD pathology. Jin *et al.* highlighted the phenomenon of crosstalk between transplanted cells and endogenous neuroproliferative cells by the transplantation of neural precursor cells (NPCs) in focal cerebral ischemia of rat brain. In their earlier study they found reduced infarct volume and improved behavioral outcomes upon transplantation of NPCs in middle cerebral artery occlusion model of rat. In a more recent study neurogenesis was shown by an increase in BrdU labeling and expression of neuronal migration protein doublecortin in the ipsilateral SVZ whereas not in contralateral SVZ or subgranular zone (SGZ) in young and aged rats [43]. In another study, authors have administered umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) in double transgenic mice of PS1 and APP, which substantially ameliorated loss of spatial learning and memory by microglia activation. Further, levels of Aβ peptide, hyperphosphorylation of tau and BACE1 activity were reduced significantly. This neuroprotective effect by UCB-MSCs involved modulation of neuroinflammation due to reduction in pro-inflammatory and increase in anti-inflammatory cytokines, induced by microglia activation [44]. These findings suggest that UCB-MSC may act as a therapeutic agent to ameliorate decline in cognitive functions in AD model mice.

Besides amyloid plaque deposition and neurofibrillary tangle formation, vascular dysfunction also contributes to the AD pathophysiology. Vascular endothelial growth factor (VEGF) is also implicated in AD related neurodegeneration. Therefore Garcia *et al* used the strategy of providing VEGF by transplantation of overexpressing bone marrow derived mesenchymal cells into the lateral ventricles of brain using stereotaxic surgery in double transgenic mouse model with $APP_{SWE}/PS1_{dE9}$ mutations [45]. Behavioral and molecular parameters were assessed for vascularization and amyloid plaque deposition. Outcomes included reducing behavioral deficit and amyloid deposition besides inducing favored neovascularization. Yang *et al.* have used cell based approach and transplanted differentiated neuron like cells in APP/PS1 transgenic mice. They used human mesenchymal stem cells derived from Wharton's jelly of umbilical cord and transdifferentiated into neuron-like cells (HUMSC-NCs) by tricyclodecan-9-yl-xanthogenate (D609). Transplantation of HUMSC-NCs in a transgenic APP/PS1 mouse model significantly reduced Aβ load and improved

cognitive functions via increase in microglial activation and expression of the NEP and Aβ degrading enzymes insulin-degrading enzyme (IDE). Expression of pro-inflammatory markers associated with the modulation of M2-like microglia (a type of microglia classified based on its mannose receptor and its activation by IL-4 cytokine [46]) activation was reduced whereas expression of anti-inflammatory markers, such as interleukin-4 (IL4), was found to be increased [47]. Mesenchymal stem cells derived from bone marrow of male Sprague–Dawley rats were transplanted in female rats by tail vein injection [48]. BM-MSCs were found to increase expression of nestin andcholine acetyltransferase positive cells at the injured area in the brain. Notably, these cells displayed a reduction in amyloid plaques in hippocampus.

Thus, bone marrow cells bring about their therapeutic effect by mechanisms involving anti-apoptotic activity, immunomodulation, and neurogenic properties. Furthermore, neurogenesis in the subgranular zone of dentate gyrus may act as an endogenous repair mechanism in AD via *Wnt* pathway in amyloid-related neurodegeneration associated with AD. Researchers also investigated the role of mesenchymal stem cells on hippocampal neurogenesis by co-culturing with the amyloid treated neural progenitor cells. Mesenchymal stem cells treatment to NPC significantly enhances the expression of GFAP, Ki67, HuD c, SOX2, and Nestin. Transplantation of these mesenchymal stem cells in Aβ-treated animals increased BrdU and HuD double positive cells in hippocampus at 2 and 4 weeks as compared to control and Aβ-treated alone animals [49]. This shows that MSC administration caused hippocampal neurogenesis and increased differentiation of NPC, which is modulated by *Wnt* pathway. If proven, this may provide a better therapeutic approach for treating AD patients than what is offered by anticholinesterase drugs. Zhang *et al* realized that neural stem cell transplantation could provide a better approach for the therapeutic treatment of AD and hypothesized that the transplantation of NSCs would ameliorate cognitive impairment by increased expression of synaptic proteins. Therefore, they isolated NSCs from mouse embryo at embryonic day 14 and transplanted these cells in both hippocampi of APP/PS1 transgenic mice. Indeed, there was enhancement in cognitive functions analyzed by better spatial learning and memory after 8^{th} weeks of transplantation as compared to the control group. Further, the expression of synaptophysin (SYN) and GAP-43 were found to be increased significantly. Hence, these results suggest that NPC induced cognitive improvement possibly by formation of new neural circuits [50].

Studies have also been carried out to mobilize the quiescent bone marrow stem cell population into the peripheral blood by using stimulating factors. Prakash *et al* evaluated the effect of granulocyte colony stimulating factor (GCSF) in Aβ induced memory loss in male adult Wistar rats, and they found significant escalation in behavioral performance after GCSF elevated the progenitor population and CD34 positive cells in the brain affecting neurogenesis [51]. Shetty *et al.* demonstrated the efficacy of mesenchymal stem cells (MSCs) derived from umbilical cord tissue in a Parkinson disease model [52]. They have studied the comparative therapeutic efficacy of MSCs from umbilical tissue and bone marrow as well as efficacy of undifferentiated versus differentiated cells in their model and found better efficacy of differentiated MSCs into dopaminergic phenotype when transplanted. As mesenchymal stem cells lack immunomodulatory activity, these cells provide a novel cellular approach to treat some neurological disorders. Several sources of

stem cells in combination with multiple approaches tested in pre-clinical AD models are further discussed in Table 1.

POTENTIAL ADVERSE EFFECTS OF STEM CELL THERAPY

Niche provides the regulatory molecules and suitable physicochemical environment to facilitate the cells to behave in a particular fashion [53]. These cells are exploited for therapeutic purposes, by isolating them from their niche which can pose some unexpected or undesirable outcomes such as tumorigenicity, which has a major concern. Very few studies have reported the potential adverse effect of these stem cells upon transplantation. In one of the studies, investigators evaluated the long term safety efficacy of 253G1-NSs (neural stem cells). The 253G1-NSs were transplanted to treat spinal cord injury (SCI) in SCID-NOD mice. These transplanted cells were found to have temporary improvement of motor function assessed by rota rod experiment for upto 47 days of post transplantation; however, this was followed by gradual deterioration in motor functioning [54]. It has also been shown to be involved in enhanced proliferation of grafted cells and tumor formation. The proportion of nestin positive cells have been found to be increased from 47 days and 103 post-transplantation which suggests tumor formation in the long term by the grafted cells. In an 18-years old patient with spinal cord injury at T10-T11, an olfactory mucosal cells were transplanted after three years. This led to severe back pain and paraplegia after 8 years. Further imaging revealed a mass formation of an intramedullary spinal cord [55].

The Yamanaka study of induced pluripotent stem cells opens up a possible window for untreatable diseases [56] as well as for stem cell clinical trials; even though the use of iPSCs also carries a risk for tumors formation. The generation of iPSCs involves retroviral transduction by the factors i.e. Oct3/4, Sox2, Klf4 and c-Myc [57]. The retroviral transduction of c-Myc is believed to increase a risk for tumorigenicity, hindering its clinical application. Further, the approach has also shown elimination of the c-Myc factor for iPSC generation, which is relatively safer than the earlier approach.

Therefore, an evaluation of safety efficacy of stem cells would provide us better therapeutic approach and its clinical application [58, 59].

THE PUTATIVE LINK OF BDNF AND CREB BEHIND STEM CELL MEDIATED REGENERATION

In brain BDNF and CREB (cAMP response element-binding protein) are believed to play a major role in complex memory formation, consolidation and retention [60–62]. It is also reported in both *in-vivo* and *in-vitro* studies that Aβ induced toxicity leads to downregulation of BDNF and its major regulatory molecule CREB. Hota *et al* studied the phosphorylation of CREB to investigate the molecular mechanism of bacoside action. Administration of Bacopa monniera leaf extractin hypobaric hypoxia induced rat model increased learning ability and ameliorated cognitive dysfunction [63]. Tota *et al.* investigated the effect of angiotensin II on spatial memory and BDNF expression in Sprague-Dawley male rats. Spatial memory was reduced as assessed by Morris water maze after angiotensin ICV administration, and no change was observed in BDNF expression [64]. In an *in-vitro*

study, Sharma *et al.* have investigated the role of CREB binding protein (CREB-BP) in neuronal differentiation. Their deletion construct p-CREB-BP were transfected into NT2 cells and expression profile for neuronal genes i.e. SHH, *Wnt,* Notch and their mutant counterparts were evaluated. Defects in neuronal differentiation due to aberrant interaction of CREB-BP with their transcriptional regulatory proteins were investigated by CHIP-PCR and co-immunoprecipitation. Cells that are lacking in CREB, BROMO and HAT domains were found to show more proliferation and less differentiation whereas cells expressing CREB-BP showed less proliferation and more differentiation [65]. In 2009, Verma *et al.* suggested the role of dichlorvos in memory impairment by muscarinic receptor induced signal transduction and phosphorylation of CREB. Dichlorvos belongs to the organophosphate compounds which are widely used to as insecticide and may act as cholinesterase inhibitor [66]. Low doses of dichlorvos impaired the signal transduction linked to the adenylyl cyclase pathway and reduced CREB phosphorylation, leading to neurobehavioral impairment [67].

Neurotrophic factors such as BDNF, NGF and GDNF, which have been earlier shown to rescue hypoxia induced ischemic rat brain upon intravenous transplantation of UCB cells. This indicates an intrinsic role for neurotrophic factors, rather than direct differentiation, being significant in the stem cell mediated recovery [68]. It is pertinent to note that the role of BDNF has been well described in AD literature. BDNF levels are decreased when compared to healthy controls in the postmortem brains of AD patients [69–71]. Mature BDNF and its mRNA expression have also been shown to be confined to hippocampus and parietal cortex region of the brain [72–74]. BDNF is believed to exert neuroprotective effect in several neurodegenerative diseases. This may include pathologies characterized by Aβ-induced neuronal cell death. Several studies have shown the complete reversal of neuroprotective effects driven by BDNF in neuronal culture death induced by Aβ in specific and dose-dependent manner [75–80]. BDNF induced neuroprotective effect has shown incorporation of the Trkβ receptor [76]. Moreover, specific Aβ42 induced neuronal cell death has been shown to be reversed by BDNF, in addition to other neurotrophins like IGF-1 and GDNF [75].

Since CREB is a DNA binding protein and acts as a transcription factor for several genes, including *c-fos,* tyrosine hydroxylase, several neuronal peptides, and, importantly, neurotrophin BDNF, it is possible that an association exists between the role of BDNF expression and its regulation by CREB in rescuing learning and memory deficits [81, 82]. The function of CREB in the formation of spatial memory; conversion of this memory into long term memory and in neuronal plasticity is well documented [83]. It is well known that gene expression has a major role in memory consolidation as well as long term potentiation [83, 84]. These expression profiles are possibly activated through CREB and involvement of Ca^{++}, protein kinase A (PKA) and by the activation of cAMP, but need additional studies [85–87]. The activated PKA would phosphorylate CREB protein which further regulates gene expression of several proteins [86, 88, 89]. Recently, Suzuki *et al.* have shown the effect of CREB on both short as well as long term memory. They have reported the increase in long term memory (LTM) as well as long term potential in hippocampus CA1 region in gain-of function CREB mice in which mice express dominant active CREB protein. In addition, they reported short term memory (STM) improvement in response to fear

conditioning and spatial clues, which was related with enhanced BDNF levels in these mice. Therefore, up-regulation of BDNF and CREB expression may mutually trigger enhancement of LTM and STM, suggesting that CREB mediated BDNF expression plays intrinsic role in memory consolidation and retrieval. (Fig. 2) [90].

CLINICAL STUDIES FOR TREATING NEURODE-GENERATIVE DISORDERS

Although a number of pre-clinical studies have been launched, very few clinical studies have been carried out so far. Venkataramana *et al* suggested the safety and effectiveness of autologous bone-marrow derived mesenchymal stem cells when transplanted unilaterally in PD patients. Notably, no adverse effects of these stem cells were seen, paving the way for additional studies in future [91]. In 2012, human retinal stem cells were used to treat PD patients. Authors isolated human retinal stem cells from retinal pigmented epithelium tissue from post-mortem eyes and cultured *in-vitro* to differentiate into dopaminergic neurons. These cells were then transplanted by stereotaxic operation into the post-commissural putamen of 12 PD patients. Interestingly, PET analysis showed a trend of increased dopamine release during the 6 month study [92]. In a current study of a phase I open-label clinical trial, authors evaluated the safety efficacy of intrathecal and intravenous transplantation of autologous bone marrow cells in children with cerebral palsy. Eighteen children with cerebral palsy, who had transplantation, were evaluated for motor and cognitive functions and MRI was done after the sixth month showing it was a safe procedure [93].

There are very few reports registered at www.clinicaltrials.gov of stem cells transplantation in AD patients, and their outcomes are largely unavailable. In 2011 Medipost Co Ltd. completed an open level, phase I safety and efficacy trial on Korean AD patients, but they did not post their outcome measures. Human umbilical cord blood derived MSCs were transplanted in AD patients at two different doses (3 million and 6 million) and endpoint analysis was measured by ADAS-cog scoring, PET imaging, and Aβ and tau levels in CSF [94]. Another group in China is currently recruiting AD patients in phase I/II trial in a similar study design with 30 probable AD participants, where patients are intravenously administered with 20 million human UCB-MSCs [95]. Medipost Co Ltd. has recently started a double blinded, placebo controlled, phase I/IIa trial in Korea where patients with mild to moderate AD will be subjected to repeated intraventricular administrations of UCB-MSCs and will be evaluated 24 weeks after first dose of transplantation [96].

CONCLUSION

Stem cells have promising translational significance as evident by emerging scientific data showing therapeutic benefits in several neurodegenerative disorders. The intrinsic pathways through which these cells exert their therapeutic effects still remain a challenge requiring thorough investigation. There are several studies describing the underlying pathways ranging from proliferation, differentiation, immunomodulation to cell replacement and paracrine effects at the site of neurodegeneration. Pre-clinical studies have shown variable effects depending on the types and sources of stem cells. Several studies explain this on the basis of paracrine effects either mediated by neurotrophic factors or endogenous cell proliferation. In

animal models of AD, stem cell transplantation has been shown to increase the expression of synaptic protein markers. Transplantation of mesenchymal stem cells has shown decrease in Aβ load due to microglial expression and escalation of Aβ degrading enzymes. A combinatorial approach, wherein stem cells are tagged with neurotransmitters or Aβ modifying enzymes may exhibit a substantial therapeutic outcome in AD. There is also insufficient literature to explain the actual relative contributions of exogenous cells and endogenous cells towards rescue of function after stem cell transplantation. There is absence of comparative studies involving different sources and types of stem cells i.e. undifferentiated versus differentiated cells in animal models of AD. Nevertheless, a few clinical studies have paved the way for clinical translation but such innovative treatments also carry substantial risk for tumor formation [55]. A thorough investigation is needed on the sources, types, stages, doses and routes of stem cell transplantation in AD model to validate their optimum therapeutic outcome. Moreover, the different stages of AD progression and other related pathologies may play a critical role in the outcome of the cell transplantation. Hence understanding the etiology of AD and its other pathologies is of paramount significance for successful clinical translation of stem cell related therapies [97].

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LIST OF ABBREVIATIONS

AD	Alzheimer’s disease
APP	Aβ precursor protein
Aβ	Amyloid Beta
BACE1	β-Site Amyloid Precursor Protein-Cleaving Enzyme 1
BDNF	Brain derived neurotrophic growth factor
BM-MSCs	Bone-marrow derived mesenchymal stem cells
Brdu	Bromodeoxyuridine
CHIP	Chromatin Immunoprecipitation
CREB	cAMP response element-binding protein
CREBBP	CREB binding protein
DHA	Docosahexaenoic acid
FAD	Familial Alzheimer’s Disease
GCSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acidic protein

ICV	Intracerebroventricular
IDE	Insulin-degrading enzyme
IHC	Immunohistochemistry iPSC-Induced pluripotent stem cells
LTM	Long term memory
MRI	Magnetic resonance imaging
NEP	Neprilysin
NFT	Neurofibrillary tangles
NPC	Neural precursor cells
NPC	Neural progenitor cells
NSC	Neural stem cells
MWM	Morris water maze
PD	Parkinson Disease
PET	Positron emission tomography
PPAR-γ	peroxisome proliferator-activated receptor-γ
PS1	Presilin1
RMS	Rostral migratory stream
SC	Stem Cells
STM	Short term memory
SVZ	Subventricular zone
SYN	Synaptophysin
TBI	Traumatic Brain Injury
UCB-MSC	Umbilical cord blood derived mesenchymal stem cells
VEGF	Vascular endothelial growth factor

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Fig. 1. Outline for underlying mechanism in stem cell mediated reversal of AD pathology

Some underlying hypothesis may explain functional improvements in subjects of stem cell transplantation in AD. However, current experiments point to four possible explanations. A) Paracrine effects from release of neurotrophic factors by transplanted cells. B) Immunomodulatory effects by transplanted cells. C) Replacement of diseased cells by transplanted cells. D) Proliferation of endogenous cells. It is likely that all four processes operate in a coherent and synergistic manner to produce a final salutary effect(s).

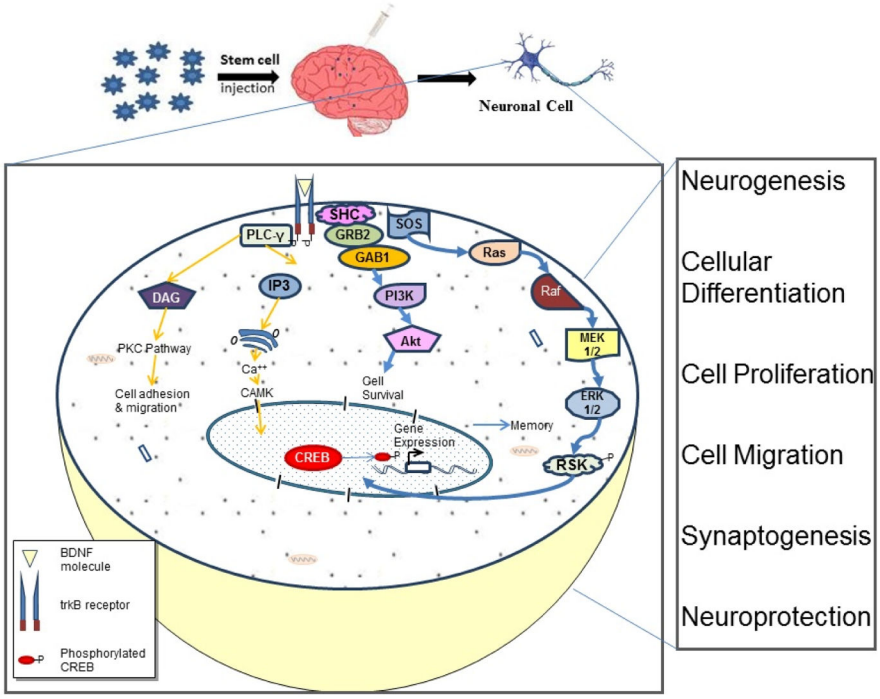

Fig. 2. Schematic showing the plausible mechanism behind stem cell mediated cognitive improvement in Alzheimer's disease

We propose that the therapeutic effect of stem cells upon transplantation into the brain is largely mediated by the paracrine effects. The increase in neurotrophic factors, such as BDNF, results in increased CREB phosphorylation which in turn activates the genes that regulate cognitive functions and memory by involving one or other phenomena, such as neuroprotection, cell proliferation, differentiation, cell migration, synaptogenesis and neurogenesis.

Table 1

Preclinical Alzheimer's studies of stem cell transplantation

No	Type/Source of Stem Cell Used	Time Points	Route of Administration of Stem Cells	Results/Outcome	Animal Model Used	References
1.	Neural stem cells	-	Intra-hippocampus	Ameliorated loss in spatial memory and learning by BDNF Increase in synaptic density	Triple transgenic mice (3xTg-AD) that express PS-1, tau and APP	[15]
2.	Neural precursor cells	Transplanted in 3 months and 24 months old rats	Cortical infarct cavity	Highlighted the cross-talk between transplanted and endogenous cells. Increased endogenous neurogenesis.	Focal cerebral ischemia in rat model	[43]
3.	Umbilical cord blood-mesenchymal stem cells	Transplanted at 29,31 and 33 weeks of age; MWM analysis at 33 week, 4 days	-	Improvement in spatial learning and memory by the microglial activation. Reduced expression of Aβ	Double transgenic mice of PS1 and APP	[44]
4.	Transdifferentiated human Wharton's jelly mesenchymal stem cells into neuron-like cells	Three weeks after transplantation MWM was performed	Bilateral hippocampus injection	Improvement in cognitive functions. Reduced Aβ load by increase in microglial activation, insulin-degrading enzyme and neprilysin expression.	AβPP/PS1 transgenic mice model	[47]
5.	Mesenchymal stem cells	End point analysis done at 2 and 4 weeks after MSC transplantation	Intra-hippocampus	Increased hippocampal neurogenesis. Differentiation of NPC by *Wnt* signaling pathway.	Amyloid β treated mice	[49]
6.	Neural stem cell	End point analysis after 8 weeks of transplantation	Intra-hippocampus	Increased expression of synaptic protein i.e. synaptophysin and GAP-43. Ameliorate cognitive impairment	APP + PS1 transgenic (Tg) mice	[50]
7.	Umbilical cord tissue derived Mesenchymal stem cell	IHC done at at 6, 12 and 24 weeks, and at 1 year post-transplantation	Substantia nigra	Studied efficacy of undifferentiated versus differentiated. Dopaminergic differentiated MSCs showed better results.	Parkinson Disease model	[52]
8.	Mesenchymal stem cell	SC transplanted after 2 hrs of injury; Behavioural assessment has been done at 1, 3, 7, 14, 21 and 28 day after TBI; IHC done after 72 hrs of TBI	Intravenously	Transplantation of MSCs showed immunomodulatory effects. Proinflammatory cytokines were reduced. Increased anti-inflammatory cytokine expression.	Traumatic brain injury mode	[98]

No	Type/Source of Stem Cell Used	Time Points	Route of Administration of Stem Cells	Results/Outcome	Animal Model Used	References
9.	Bone marrow derived mesenchymal stem cell (BM-MSC)	MWM at 2 weeks after the surgery	Intra-hippocampus	Senile plaques were reduced. Significant increased DeltaNp73 protein expression. Better performance in Morris water maze.	APP/PS1 transgenic mice	[99]
10.	Human olfactory bulb neural stem cells	NSC transplanted after 10 days of ibotenic acid administration.	Intra-hippocampus	Cells were engineered to express nerve growth factor. Enhanced cognitive abilities.	Ibotenic acid induced AD rat model	[100]
11.	Neural stem cell	NSC were transplanted in 12-months old mice; IHC performed 5 weeks post-transplantation	-	No impact on Aβ plaques. Transplanted cells showed chemotaxis towards the plques.	APP/PS1 double transgenic AD mice	[101]
12.	Placenta derived Mesenchymal stem cell		Intravenous in mouse tail	Improved cognitive function. Prevent neuronal death Reduced inflammatory cytokines. Promoted neuronal cell differentiation	Amyloid β1-42 peptide infused in mouse model	[102]
13.	Mesenchymal stem cell	Aβ administered at 6 week of age; MSC transplanted on post-operative day 1.		Reduction in Aβ levels Enhanced induction of autophagosome	Amyloid beta treated animal model	[103]
14.	T-regulatory cells educated by UC-MSC	6 months old mice were used; Behavior test performed after 2 weeks of transplantation	Intracardiac injection	Reduced microglial activation and systemic inflammation. Ameliorate cognitive function Decreased Aβ plaques deposition	Transgenic AβPPswe/PS1dE9 mice	[104]
15.	Amniotic membrane derived mesenchymal stem cell	Pathological analysis done at 1 week post-transplantation; Behavior analysis at 12 weeks post-transplantation	Intravenous injection	Reduced proinflammatory and increased anti-inflammatory cytokines. More activation of microglial cells. Improved spatial memory	Tg2576 transgenic mice model of AD	[105]
16.	Adipose derived stem cell	MWM was performed 3 months of stem cells injection	Intravenous transplantation	No immune response Migrated to brain by crossing blood brain barrier Rescued memory deficiency Upregulated VEGF and IL-10 expression	Tg2576 mice model of AD	[106]
17.	Bone marrow derived mesenchymal stem cell	End point analysis is upto 2 months	Intra-cerebral injection	Increased expression of dynamin 1 and synapsin1 Aβ plaque deposition reduces	-	[107]

No	Type/Source of Stem Cell Used	Time Points	Route of Administration of Stem Cells	Results/Outcome	Animal Model Used	References
18.	Mesenchymal stem cell derived from umbilical cord blood	Ten months old mice when stem cells were administered	Bilateral intra-hippocampal administration	Induced endogenous neprilysin expression Reduced Aβ plaques	APP/PS1 transgenic mice model	[108]
19.	Epidermal neural crest stem cell	14 days after Aβ administration stem cells were injected	CA3 region of hippocampus	Increased granule cells in hippocampus Transplanted cells expressed neuronal markers like GFAP	Amyloid-β1-40 injected AD rat model	[109]
20.	Bone marrow derived mesenchymal stem cell	Stem cell injection after 4 months of $AlCl_3$ induced AD	Tail vein injection	Reduced amyloid plaque in hippocampus. Increased choline acteyltransferase positive and nestin cells.	$AlCl_3$ induced AD female rat model	[48]
21.	VEGF overexpressing bone marrow derived mesenchymal stem cell	Cells were transplanted in 6, 9 and 12 months old animal	Lateral ventricles of brain	Reduction in behavioral deficiency Reduced amyloid plaque in hippocampus. Improved neovascularization	Double transgenic mouse model with APPswe/PS1dE9 mutations	[45]
22.	Choline acetyltransferase expressing human NSC	Learning and memory test were done at 2, 4, and 6 weeks after transplantation	CA3 region in hippocampus	Differentiated into neurons Migration of transplanted cells towards injured area	AF64A-cholinotoxin induced learning deficit rat model	[110]
23	Umbilical cord blood cells	Age of mice were 7 month old	Peripheral administration	Immunomodulation Reduced vascular amyloid β deposits	Tg2576 AD mouse model	[111]

Hype and Hopes of Stem Cell Research in Neurodegenerative Diseases

13

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Abstract

Hope from the regeneration promoting effects of stem cells have provided new insights for understanding diseases that were previously thought to have a limited prognostic improvement upon medical intervention. This is especially indicated in neurodegenerative diseases, which until the discovery and research in stem cells were thought to have minimal regenerative capabilities. This review covers various treatment modalities involving different types of stem cells, such as human embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells and neural stem cells, which have been tested for various neurodegenerative disorders such as Multiple Sclerosis, Alzheimer's disease, Parkinson's disease and Age Related Macular Degeneration.

Keywords

Animal models • Human patients • Neurodegenerative diseases • Stem cells

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Abbreviations

AHSCT	Autologous hematopoietic stem cell transplantation
AMD	Age-related macular degeneration
aNSCs	Adult neural stem cells
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
GID	Graft-induced dyskinesia
hPDLSCs	Human periodontal ligament stem cells
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MSC-NPs	Mesenchymal stem cell derived-neural progenitors
MSCs	Mesenchymal stem cells
NPC	Neural precursor cell
NSCs	Neural stem cells
OPCs	Oligodendrocyte progenitor cells
PPMS	Primary progressive MS
RPCs	Retinal progenitor cells
RPE	Retinal pigment epithelium
RRMS	Relapsing-remitting disease
S-MSCs	MSCs isolated from skin tissue
SPMS	Secondary progressive MS
sTNFR1	Soluble TNF receptor 1
TEPs	Thymic epithelial progenitors
TNF-α	Tumor necrosis factor α
VPA	Valproic acid

13.1 Introduction

Neurodegenerative diseases, such as spinal cord injuries (SCI), multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, age-related macular degeneration, etc., have serious pathology affecting persistently public health and medical problem. The self-renewing property of stem cells and their differentiation into specialized cell type(s) make them unique and have the potential to repair the damaged organs. It has been proved as a boon for bone marrow transplantations in million patients worldwide for the treatment of leukemia, anemia, or immunodeficiency.

The potential of mesenchymal stem cells (MSCs) and neuronal stem cells (NSCs) in multiple sclerosis (MS) has been promising. The use of stem cell therapy shows increased neuroprotection and decreased neuroinflammation, which decreases progression of the disease while alleviating neuronal deficits. The use of stem cells in Alzheimer's disease (AD) and age-related macular degeneration (AMD) still has to pave ahead to provide conclusive results. The use of iPSCs in animal models, however, does show a promising avenue for reducing memory deficit as well as

neuroinflammatory processes in the CNS. The major roadblock for the use of stem cells in Parkinson's disease (PD) was the development of graft-induced dyskinesia (GID) upon transplantation of stem cells. The bulk of the research is focused on reducing GID and the use of parthenogenetic stem cells in PD. There is hope for the use of stem cells in the future for PD; however, it is still a long thoroughfare ahead. The primary issue concerning the use of stem cells in age-related macular degeneration is the intricacy in replacing the retinal pigment epithelium, rods, and cones, which is challenging with stem cell therapy.

Other issues spinning around the wheel for the use of stem cell for various neurodegenerative disorders involve the prospect of neoplastic transformation of the newly transplanted stem cells into the subject. Another area of concern with stem cell therapy delves into the ethics involved in obtaining embryonic stem cells and their use as a modality of treatment. Stem cells provide bright possibilities for new treatment modalities to prevent the onset and progression of many diseases previously not treatable; however, these small pebbles in the form of research and clinical trials have to lay concrete to build a path to prove its long-term efficacy and to alleviate ethical concerns associated with it.

13.2 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder in which T cells attack the central nervous system, thus damaging neuronal axons and myelin sheaths. The disease is a major cause of non-traumatic neurological disability that affects more than 2.5 million people worldwide. According to the current estimates, more than 400,000 people in the United States have MS. There is a relatively high prevalence of MS in Europe and North America, 1 in 800, with an annual incidence of 2–10 per 100,000 individuals. In India, the prevalence is relatively less, which may be an underestimation, but it is definitely not as high as the rates found in high-prevalence temperate zones. The natural history of MS is unpredictable and has been discussed for several decades. The 80–90% of patients with MS have a relapsing-remitting disease (RRMS) course in the following 10 years after initial presentation. The disease begins with an initial attack (episode of symptom flare-ups) followed by a period of remission that could be as long as 2 years, a second attack is followed by either another period of remission or progression, and this is termed secondary progressive MS (SPMS). Eventually, remissions are of shorter durations, relapses become longer, and finally the patient enters a progressive stage. Patients with primary progressive MS (PPMS) undergo continuous worsening of neurological disability right from the disease onset with no relapse or remission and affects 10–15% of patients.

Current therapeutic strategies for MS patients are based on immunomodulation and immunosuppressive approaches that are only effective in the prevention of relapses and in the slowing down of the progression of the disease, but not completely cure. This is because of limited understanding of the pathogenic mechanisms underpinning disease progression. Therefore, therapies reversing or at least halting progression of MS are required. Stem cell transplantation has shown remarkable promise in recent years in the

treatment of various diseases. Stem cells have the ability to renew and differentiate into many different cell types of the body. There are various types of stem cells of importance in relation with MS; these are adult stem cells (ASCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). ESCs are incredibly valuable for regenerative medicine. However, they have certain limitations which include ethical concerns and the possible formation of teratoma if the cells are not properly differentiated before transplantation. Another concern is the chance of rejection of the transplanted cells. ASCs or somatic stem cells also hold much promise in the treatment of neurodegenerative disorders and demyelinating diseases including MS. In this chapter we highlight the application of different stem cells in animal models and clinical trials conducted in the treatment of MS.

13.2.1 Experimental Studies with MSCs in MS

Stem cells have the potential to differentiate into various cell types, replenish worn out or damaged tissue, and eventually recuperate lost functions [1]. Primarily two categories of stem cells are used in the treatment of experimental autoimmune encephalomyelitis (EAE); these are MSCs and NSCs. EAE is a commonly used animal model, which mimics some of the properties of MS. Mesenchymal or stromal stem cells, emanating from mesoderm, possess the capability of multipotency and self-renewal. MSCs can be obtained from the bone marrow, adipose tissue, and umbilical cord blood and can be differentiated into desired cell type. MSCs modulate the immune system. This is encouraging for their use as a therapeutic approach for MS. Both *in vitro* [2] and *in vivo* studies [3] have revealed the remarkable potential of MSCs to suppress the proliferation of T cells and preventing autoimmune attack against myelin antigens. According to the various studies, MSCs have shown the ability to promote neuroprotection as well as to modulate the immune system in EAE.

It has been shown that MSCs improve the clinical score when injected intravenously at disease onset or peak in EAE animal model and prevent immune-mediated myelin damage in the CNS. However, when the cells are administered during the chronic stage of disease, clinical symptoms reveal least improvement [4]. Similarly, intravenous administration of these cells is believed to augment the histological scores of the disease in mice induced with EAE as demonstrated by Zhang et al. [5]. The investigators also reported oligodendrocyte synthesis after MSCs were injected, which might have occurred due to the release of various neurotrophic factors by transplant. Subsequently, another group of investigators found that MSCs are endowed with neuroprotective and immunomodulatory property in EAE-induced animal models. Their findings also revealed that the degree by which MSCs co-localized with cells expressing neural markers was least indicative that MSCs had transdifferentiated into other cell types in response to disease induction [6]. Also, when MSCs are injected into SJL mice induced with EAE, there is a reduction in the production of proteolipid protein (PLP)-specific antibodies. This indicates that humoral immune response is inhibited which further ameliorates the disease

symptoms. At the same time, it was also found that there was no transdifferentiation of MSCs into neural cells. However, neural stem cells [7] and neural precursor cells derived from human embryonic stem cells [8] showed immunomodulatory and neuroprotective properties when administered in EAE-induced mice [9].

Although there are concerns over the transdifferentiation potential of MSCs, it is known that MSCs have the potential to differentiate into mature mesoderm-derived cell types such as the bone, cartilage, and fat [10]. Kopen et al. reported that MSCs cannot be transdifferentiated into nerve cells after transplantation into the central nervous system of EAE-induced mice [11]. More importantly, MSCs recruit local neural precursor cells and probably repair the endogenous cells resulting in neurogenesis and remyelination. Apparently, there is a secretion of antiapoptotic factors [12] and neurotrophins [13] in CNS, which lead to neuroprotection suggesting that these stem cells may be regarded as tolerogenic cells [14]. In addition to immunomodulatory, immunosuppressive, and neuroprotective properties exhibited by MSCs, these cells also show a vigorous antioxidant effect in mice affected by EAE [15].

A recent study showed that MSCs in combination with resveratrol is neuroprotective in EAE model of MS. The combined effect reduced the disability score and suppressed the secretion of pro-inflammatory cytokines, thereby alleviating neurological symptoms [16]. In addition, administration of MSCs isolated from skin tissue (S-MSCs) improved the disability score of EAE by inhibiting the differentiation of T helper 17 (Th17) cells. Tumor necrosis factor (TNF)-α is a key pro-inflammatory cytokine capable of promoting Th17 cell differentiation. It has been demonstrated that ample amount of soluble TNF receptor 1 (sTNFR1) are secreted by S-MSCs, which binds with TNF-α ligand. Eventually bound ligand receptor shows neutralized function, and thereby Th17 cell differentiation is inhibited. These findings suggest a novel mechanism underpinning MSC-mediated immunomodulatory function in autoimmune disorders [17]. MSCs derived from adipose tissue showed similar findings when infused intravenously in chronic EAE female rats. The number of infiltrated immune cells and axonal damage was found to be decreased significantly in treated animals with MSCs. The findings also demonstrated down-regulation of interleukin-17 expression in treated animals. Furthermore, the cells were engrafted into the brains and lymph nodes up to 25 days of post-administration. A significant finding was the increased expression of the human HLA-G gene in the brains and lymph nodes of rats treated with stem cells. It was discovered that these cells show beneficial effects when transplanted in the later irreversible period of the disease course than those introduced after stabilization of the disease [18]. MSCs derived from various other sources, such as human periodontal ligament stem cells (hPDLSCs), have also shown neuroprotective effects in EAE. The cells were injected intravenously at a dosage of 10^6 cells/150 μl in EAE mice. The findings revealed reduction in immune cell infiltration and demyelination together with immunomodulatory effects which augmented clinical disease course [19].

The neuroprotective effect of transplanted MSCs isolated from human umbilical cord blood (UCB-MSC) was investigated in EAE-induced C57BL/6 mice. The

UCB-MSCs were differentiated into neurons in vitro and transplanted in disease-induced mice. The results revealed a decrease in lymphocyte infiltration and improvement of clinical score [20]. Interestingly, adipose tissue-derived MSCs isolated from SJL mouse strain show the same features as seen in other mouse strains abrogating the notion that their application in cell therapy is strain specific. In addition, EAE induced in SJL mouse shows signs of improvement upon MSC transplantation, suggesting that they could modulate disease progression [21].

13.2.2 Experimental Studies with Adult NSCs in MS

Neural precursor cell (NPC) transplantation possesses noteworthy improvement of MS and other degenerative disorders of the CNS. Self-renewal and differentiation property into brain cells are important characteristics of adult NSCs. These cells have an ability to differentiate into nervous tissue after migration into the CNS; this prospect makes NSCs a viable candidate for cell replacement therapy. These have shown effectiveness in experimental autoimmune encephalomyelitis as well as other neurodegenerative diseases [22]. It has been seen that these stem cells possess the capability to differentiate into oligodendrocytes which are the myelin-forming cells. This replenishes injured myelin tissue and reduces inflammation of the spinal cord and brain in EAE mice [23]. It has been reported that Scutellarin, a flavonoid, is neuroprotective in a mouse model of MS. This phenolic compound prevents neurons from damage and promotes the growth and development of nervous tissue thereby alleviating several neurodegenerative diseases. In this study, adult C57BL/6 mice were subjected to cuprizone, given 8 mg/day through diet, for 6 consecutive weeks leading to demyelination of the central nervous system (CNS) of mice. Thereafter, for 10 consecutive days, the animals received scutellarin (50 mg/kg/day) treatment. The findings revealed improvement in behavioral deficits and reduced demyelination in scutellarin-treated mice. Furthermore, there was suppression of apoptosis of NSCs and their number increased, which could be differentiated into myelin-producing oligodendrocytes and neuronal lineages [24].

During the relapsing-remitting MS, the patient's brain itself has the ability to repair the damaged myelin, but this process fails many times. Studies have shown that somatic cells convert into neural progenitors and neuroblasts when there is forcible expression of some transcription factors within the brain. Dehghan et al. [25] studied the effect of forced expression of Oct4 in conjunction with valproic acid (VPA) in experimental demyelination model induced by lysolecithin. Animals treated with Oct4-expressing vector along with VPA showed signs of remyelination as some Oct4-transfected cells underwent transdifferentiation into myelinating oligodendrocytes. This was confirmed by the presence of increased number of Sox10, CNPase, and Olig2 markers. The results of this study appear to be of potential in enhancing myelin repair within adult brains.

Ravanidis et al. investigated whether transplantation of neural precursor cell effect on disease course of EAE is associated with immune modulation in the

inflamed CNS. NPCs were isolated from brains of C57BL/6 mice and were subcutaneously administered in female EAE-induced mice. There was a reduced expression of chemokines and antigen-presenting molecules in brain, which demonstrated that immune cells were modulated in inflamed CNS following NPC transplantation. Finally, NPC transplantation augmented the disease course [26].

Interestingly, MSCs suppress the activation of TH17 cells but not TH1 in EAE model, whereas, in cuprizone-mediated demyelination model, MSC administration induces remyelination. This indicates that MSCs present a variable function in diverse microenvironments [27]. In addition, multipotent adult progenitor cells also reveal immunomodulatory and neuroprotective properties in rats induced with EAE which are facilitated in response to neuroinflammatory signals [28]. Another group of investigators evaluated the therapeutic efficacy of glycan-engineered cell surface of NSCs with E-selectin ligands in EAE model. This approach resulted in the augmentation of clinical symptoms with reduced inflammation [29]. It has also been documented that MSCs do not exert regenerative outcomes in cuprizone-induced demyelination mouse model directly. Instead, peripheral immune cells and in particular T lymphocytes are needed for MSCs to exert their full effects [30].

Braun et al. demonstrated that differentiation and maturation of adult hippocampal neural stem/progenitor cells (NSPCs) into mature oligodendrocytes prevented oligodendrocyte loss, axonal impairment, and enhanced remyelination in an experimental demyelination model induced by diphtheria toxin. These results indicate the therapeutic efficacy of hippocampal NSPCs in the treatment of MS [31]. El-Akabawy et al. [32] investigated the effect of intravenously injected bone marrow-derived MSCs in nonimmune cuprizone model of MS. Their findings revealed that these cells not only modulate and suppress the immune system as observed in immune models of MS such as EAE but also are capable to facilitate remyelination and neuroprotection. Su et al. [33] reported that thymic epithelial progenitors (TEPs) can be produced by the in vitro induction of mouse embryonic stem cells (mESCs) that further develop into functional TECs in vivo. Their findings indicate that transplantation of ESC-TEPs may be a potent therapy for autoimmune diseases.

13.2.3 Experimental Studies with iPSCs in MS

iPSCs have recently emerged as a novel tool for the treatment of MS. These cells are obtained from patient's own tissue mimics; similar pathological features are seen at a molecular level and hence provide an exclusive platform to study the many facets of neurodegenerative diseases in culture (Fig. 13.1) [34]. Eventually, this can yield novel insights about disease pathology and the development of novel therapeutic strategies. Laterza et al. reported that neural precursor cells obtained from mouse iPSC-derived NPCs (miPSC-NPCs) administered intrathecally in EAE-induced mice model have the ability to form oligodendrocyte progenitor cells (OPCs) which produce oligodendrocytes and eventually clinical outcome was improved. Furthermore, reprogrammed

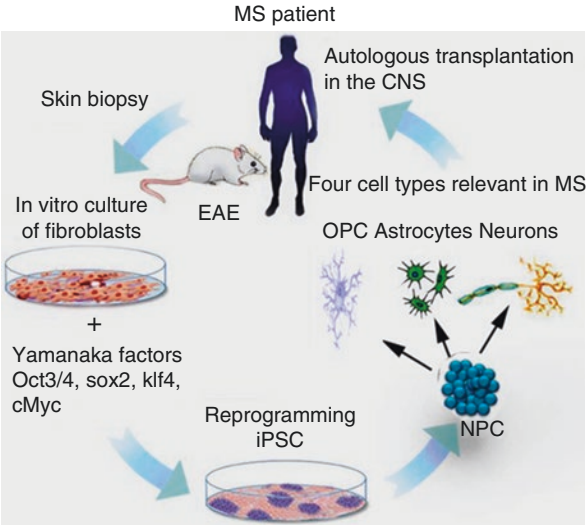

Fig. 13.1 iPSCs derivation and the scientific logic behind iPSCs-based remedy. The rudimentary idea behind iPSC-based disease treatment is that a patient's own adult cells could be reprogrammed back into iPSCs by an introduction of certain factors. These include Sox2, cMyc, Oct3/4, and Klf4 given by Yamanaka et al. in 2006. The resulting iPSCs resemble embryonic stem cells, are pluripotent, and can be differentiated into any type of cell to study disease pathology, test drugs, and develop novel therapeutic strategies. The *left panel* of the image shows the skin fibroblast cells obtained from biopsy, which can be reprogrammed into iPSCs (*bottom image*). iPSCs, in turn, can be differentiated into NPCs and eventually into OPCs, neurons, and astrocytes (*right image*), and then autologously transplanted back into the patient, at a minimal risk of immune rejection. *MS* multiple sclerosis, *NPC* neural precursor cells, *OPC* oligodendrocyte precursor cells, *iPSCs* induced pluripotent stem cells (Reproduced with permission from Ref. 34)

iPSCs have the potential to form nervous tissue precursor cells, which can further lead to the formation of neurons, astrocytes, and OPCs.

13.2.4 Clinical Trials of Stem Cells for the Treatment of MS

Stem cell therapy has been extensively used as a powerful strategy in the treatment of several diseases for a decade. Their astounding potential to differentiate into any type of cell lineage and regenerate or repair the damaged tissue makes them an attractive candidate to be used for clinical applications. Several in vivo studies in experimental models have been performed to understand the efficacy of stem cells in the treatment of MS. Although many have yielded promising results, there are limited clinical trials conducted on MS patients so far and need more research to fully translate cell-based therapies from bench to bedside. Currently, there is no approved stem cell treatment available for MS.

Uccelli et al. [35] showed that MSCs administered intravenously in EAE animal model improve the neurological disability and reduce inflammation and myelin and

axonal damage. These stem cells release soluble neuroprotective and pro-oligodendrogenic factors, which shield the tissue from getting damaged and alleviate the disease symptoms. The first small clinical trials with few MS patients have already demonstrated that transplantation of MSCs in MS and amyotrophic lateral sclerosis (ALS) patients is a safe and attainable procedure. In this study, autologous MSCs were administered both intravenously and intrathecally in 19 ALS and 15 MS patients. Each patient received a dosage of up to 60–70 × 10^6 cells per injection and was examined for 6–28 months. The only major side effect noted was mild meningeal irritation such as transient fever and headache in those given an intrathecal injection of MSCs. The findings revealed significant improvement in clinical outcomes and induction of immunomodulatory effects post-MSC transplantation [36]. Furthermore, experts in MS and stem cell field established a treatment protocol on the use of MSCs for the treatment of MS. It was tested whether transplantation of MSCs caused any risk in MS patients who had failed to treatment with conventional immunomodulatory drugs. When comparing the nature of the stem cells derived from MS patients and healthy donors, it was found that they exhibited similar properties. Other reports demonstrated that the intrathecal route of MSC injection in ten PPMS patients [37] and seven MS patients [38] is a risk-free method except for those who intrathecally received an increased dosage of 100 × 10^6 cells. Ten SPMS patients received an intravenous dose of 1–2 × 10^6 MSCs per body weight in a phase II open-label clinical trial [39]. The patients were kept under surveillance for any adverse effects for 4 h after receiving the dose and were observed for the same over a period of 3 and 6 months after treatment. There were no major significant side effects reported except in 10% of the patients who received intravenous injection. These include type I hypersensitivity reactions, i.e., pruritus, rashes, or fever. Improvement in evoked potentials as well as visual acuity was observed when MSCs were transplanted in these patients. Additionally, cell transplantation increased the optic nerve area, which further demonstrated safety of MSCs in SPMS patients. Harris et al. conducted a phase I open-label clinical trial with 20 PPMS patients and presented their preliminary result at ACTRIMS 2014 [40]. These investigators administered autologous MSC-derived neural progenitors (MSC-NPs) to PPMS patients via intrathecal route. Over a 3-month interval time, cells were injected in three doses of up to ten million per injection. Initially results showed safety outcomes in the five study subjects. An open-label clinical trial was conducted by Bonab et al., in 2012, which included 25 patients with progressive MS. Patients were injected with autologous MSCs intrathecally once and were followed up for a year. There were no major long-term side effects reported in these patients. Short-term side effects were observed which consisted of nausea, vomiting, weakness in the lower limbs, low-grade fever, and headache. The disability score of some patients improved indicating that MSCs may stabilize the progressive disease course during the first year following transplantation, without any serious side effects [41]. A recently conducted randomized placebo-controlled phase II clinical trial also showed promising results with nine patients receiving a dosage between 1–2 × 10^6 MSCs/kg body weight which was given intravenously [42]. A phase Ib, multicenter, double-blind, randomized placebo-controlled study was conducted on RRMS or SPMS patients. Patients received

two low-dose infusions of mesenchymal-like cells isolated from human placenta (PDA-001) (150×10^6 cells) or placebo, in an interval of 1 week. Thereafter, patients received either a high-dose of PDA-001 (600×10^6 cells) or the patient would receive the placebo. MRI of the brain was performed every month. Ten patients with RRMS and six with SPMS were randomly assigned to treatment. One of the patients on high-dose PDA-001 had increased disability score as well as increased T2 and gadolinium lesions during an MS flare occurring 5 months after receiving treatment with PDA-001. This was the only case with an increase in disability; the rest of the patients had no increase in disability. Most patients had stable or decreasing disability. Side effects experienced on high-dose PDA-001 were headache, fatigue, urinary tract infection, etc. PDA-001 infusions in general were safe and well tolerated by patients with RRMS and SPMS patients. There wasn't any paradoxical worsening seen in lesion counts with either dose [43].

Burt et al. determined whether neurological disabilities or other clinical outcomes in RRMS and SPMS patients were alleviated by peripheral blood stem cell transplantation. The findings revealed improvements in patients with neurological disability, relapse rate, neurologic rating scale scores, multiple sclerosis functional composite (MSFC) scores, and total quality of life score. Furthermore, the volume of T2 lesions was found to be decreased in these patients [44]. In a multicenter, phase II, randomized clinical trial, beneficial effects of autologous hematopoietic stem cell transplantation (AHSCT) in intensively immunosuppressed patients with relapsing-remitting (RRMS) or secondary progressive MS (SPMS) were observed as compared to mitoxantrone (MTX)-treated patients. The total number of new T2 lesions as well as Gd^+ lesions and relapse rate was found to be reduced in patients transplanted with stem cells [45]. Recently, Kyrcz-Krzemien et al. [46] showed that autologous hematopoietic stem cell mobilization is a safe and efficacious strategy in the treatment of patients suffering with MS. The study also reported adverse effects observed in 8 patients out of 39 MS patients recruited.

13.3 Alzheimer's Disease

Alzheimer's disease (AD) is seen to be most prevalent among older individuals and results in progressive dementia and cognitive impairment. AD is a characteristic of widespread neural degeneration resulting in damage to the brain cells culminating to impaired memories (progressive dementia) and decreased cognitive functioning. Microscopically, AD is demonstrated by the presence of plaques (beta-amyloid) and neurofibrillary entanglements (tao proteins). These plaques have beta-amyloid deposition commonly seen in the neuronal tissue of the brain and in the cerebral vessels resulting in atrophy of the cortex and hippocampal region of brain. The most common clinical symptom associated with Alzheimer's is progressive memory loss. Currently, treating AD is a challenging task as current pharmacological modalities only aim in providing symptomatic relief rather than providing with a permanent cure. However, in recent years various research studies have been carried out using stem cell strategies in finding a cure to these neurodegenerative diseases like enhance

the production of brain neurons (neurogenesis), provide neuroprotective agents to improve the function of brain neural tissue, and find various modalities to destroy beta plaques and halt progression of disease. Currently, two pharmacological approaches are used for treating AD. One is prevention of degradation of acetylcholine at synapse by giving acetylcholinesterase inhibitors like donepezil and galantamine, and second is memantine therapy, which protects against neuronal death due to endotoxicity [47].

13.3.1 Stem Cell Studies for AD in Humans and Animals

13.3.1.1 MSC Therapy in AD

MSCs can be obtained from various tissues like bone marrow, umbilical cord blood, and adipose tissue, which are multipotent in nature. Although there are various protocols proving the ability of these mesenchymal cells to differentiate into specialized neurons in vitro, concerns have been raised for their effectiveness in vivo. Though their neurogenic differentiation properties are still questionable, nevertheless, they are believed to be main cell type for curing neurodegenerative disorders such as Parkinson's diseases, multiple sclerosis, and AD. As such, MSCs seem to be relevant in regeneration and replacement of lost neural cells; however, it is debated that MSCs might prove beneficial in halting the progression of AD by impacting the pathological aspect of the disease, thus proving that MSC-based therapy can not only regenerate damaged neuronal tissue but also prevent the progression of the disease. Hence, most of the in vivo research has undertaken the latter approach in finding a cure to AD [48].

Lee et al. transplanted human umbilical cord blood-derived MSCs (hUCB-MSCs) into double-transgenic mice having amyloid precursor proteins (APP) as well as presenilin 1. They found significant improvement in spatial learning and memory in these models. Apart from the symptomatic improvement, amyloid-β peptide deposition, β-secretase 1 levels, and tau hyperphosphorylation were drastically reduced in these test subjects. These findings were suggestive that hUCB-MSCs provide with prolonged neuroprotective effect as a result of a feed-forward loop which alternatively activates microglial neuroinflammation, hence decreasing the disease pathophysiology and reversing cognitive damage associated with AD mice [49].

13.3.1.2 Experimental Studies with NSCs and Gene Therapy

In a phase 1 study conducted by Mark et al. [50], ex vivo NGF gene delivery was done in eight individuals of mild AD, characterized by implantation of genetically modified autologous fibroblasts into the forebrain. Patients were followed up for 22 months and evaluated with MMSE and Alzheimer's disease assessment scale. There was vast improvement in cognitive activities and increase in 18-fluorodeoxyglucose showing improvement. Hence, this suggests the role of genetically modified differentiated stem cells that can be beneficial in treating AD.

Stimulation of the brain for progressive neuronal regeneration may prove to be useful in treating the neurodegenerative disorders and halt the pathological

progression of the disease thereby increasing the synaptic activity. Lilja et al. [51] investigated the impact of transplanting hNSCs in mice models and the additive effect on improving efficacy of various drugs. Under this study, 6 to 9 months old AD Tg2576 mice were treated with the amyloid-modulatory and neurotrophic drug (+)-phenserine or with the partial α7 nicotinic receptor (nAChR) agonist JN403, and prior to this they were transplanted with hNSCs in bilateral intra-hippocampal regions. Results showed that transplanted hNSCs enhanced endogenous neurogenesis and prevented further cognitive deterioration in Tg2576 mice, while simultaneous treatments with neurotrophic (+)-phenserine or JN403 provided counter therapeutic effects.

Agger et al. showed the effect of hNSCs in increasing neurogenesis and proved their efficacy and safety in treating AD. The researchers found that human NSCs have the ability to rapidly divide and differentiate into immature glial cells and improve synapsis there by aiding growth and improving cognition in mice models. These results show extreme potential in the use of stem cell therapy and its application in clinical trials in order to find a definitive cure in AD [52]. In another study, hippocampus of two transgenic models of AD (3×Tg-AD and Thy1-APP) mice were transplanted unilaterally with neprilysin secreting modified neural stem cells. After 3 months, AD pathology, neprilysin expression, and stem cell engraftment were assessed. Stem cell-mediated delivery of NEP provided marked and significant reductions in Aβ pathology and increased synaptic density in both the models. It was analyzed that genetically modified NSCs improve not only synaptic activity but also delay the progression of the disease by preventing amyloid deposits in the hippocampal region [53].

Above studies showed the impact of stem cell transplantation in enhancing the efficacy of drugs as well as neurogenesis which is triggered as a result of stem cell grafting; but none of these assessed the effect of clearance of plaques and neurofibrillary tangle on cognition. Blurton et al. [54] demonstrated the role of NSCs in improving cognition without altering the beta-amyloid plaques. He showed that cognitive improvement correlated directly with brain-derived neurotrophic factor which is responsible for neurogenesis and increasing synaptic activity. This study can help in correlating dementia to the reduction in neurogenesis as a result of toxic damage to neural cells because of prolonged effect by tau proteins and amyloid deposits. Stem cell therapy has been found not only to help in focusing improvement of pharmacological basis in treating AD but also finding effective ways in decreasing the damage caused as a result of amyloid deposits and enhancing neurogenesis by increasing certain nerve growth factors, i.e., BDNF as well as replacement of cholinergic neurons to enhance cognitive capability of AD models [54].

13.3.1.3 Experimental Studies with iPSCs

Understanding the pathological basis of AD was limited due to lack of similarity between the animal models and actual disease itself. To overcome this problem, reprogrammed primary cells from the patients were used to form iPSCs. The primary fibroblasts were taken from the patients of familial Alzheimer's disease, sporadic AD, and control. The purified and characterized neurons from

differentiated cultures were subjected to injection by fetal brain messenger RNA. As a result of this, the electrophysiological activity of all the cells was within normal range.

Compared to the controls, extracted neuronal cells from amyloid-β precursor protein gene and sporadic Alzheimer's disease showed increased amounts of pathological markers as amyloid beta, phospho-tau, and active glycogen synthase kinase. Large RAB5-positive early endosomes were found in neurons of SAD and APP as compared to controls. GSK-3β and phospho-tau (Thr 231) levels were significantly reduced in purified neuron after being treated with beta-secretase inhibitor, but this was not seen with gamma-secretase inhibitors. The results show a direct correlation between APP proteolytic processing but do not show any direct link between amyloid-beta and its impact on the phosphorylation of tau proteins and activation of GSK-3 beta in human neurons [55]. In another study, the intact brain of mice or rats grafted with adult tissue along with its insertion into host parenchyma showed differentiation into multiple functional neuronal cells. The transplanted stem cells migrated toward damaged regions and promoted neurogenesis. It has been observed in the animal models of AD that transplanted neural precursor cells (NPCs) have better survival owing to their migration and differentiation into cholinergic neurons, astrocytes, and oligodendrocytes, thereby curing memory and learning problems. The recent development of the AD model using iPSCs has helped in elucidating various cell types and finding newer potential therapeutics [56].

Ross et al. [57] suggested that reprogrammed primary somatic cells of patients into iPSCs may help to cure the neurodegenerative diseases. In this article, different approaches were followed to generate iPSCs for the treatment of neurodegenerative diseases. Another study demonstrated that iPSCs help to develop newer models by carrying certain mutation encoding for amyloid precursor proteins (APP). These models provide with better understanding of the pathogenesis of AD and hence prove to be beneficial in research advancements. The generation of neurons carrying various FAD mutations or SAD genomes may provide a unique human neuronal system and aid in the evaluation of various therapeutic interventions for treating AD [58].

13.3.1.4 Clinical Trials at Threshold

Recently the US FDA has approved phase II clinical trials to be conducted on ischemia-tolerant MSCs in treating AD. Similar to these various projects are undertaken all over the world to find the basis of stem cell therapy for curing the disease especially in Asia and Europe. Although ethical and safety concerns are still there, stepwise approach with human stem cell-based translational research in vitro and in vivo may prove to be a cornerstone in finding the complete pathological analysis of the disease and finding a possible permanent cure in treating AD [59].

13.3.1.5 Future of Stem Cells in AD

AD is a complex neurodegenerative disorder having wide range of permutations and combinations for its pathology and its possible impact on the cognitive decline. Presently, the transgenic mice models are prominent in advancing the research.

However, they do not match up to the overall similarity with the rapid progression of the disease seen in patients.

Although recent animal models have revealed the efficacy and the impact of stem cell transplantation in the regeneration of the damaged neurons, its role in treating long-term progressive nature of Alzheimer's disease remains elusive especially with relation to teratoma formation. With newer advancements in the field of somatic gene therapies, iPSCs may be crucial in treating Alzheimer's disease.

13.4 Parkinson's Disease

Parkinson's disease (PD) is a complex multifactorial disease that affects approximately seven million people globally. There is increasing trend of prevalence rate of the disease with age. PD shows the characteristic feature of selective destruction of dopaminergic neurons in the pars compacta region of the substantia nigra (SNc) [60]. There is no cure of the disease, and current strategies to treat the patients are mainly focused on symptomatic relief. The pharmacological treatment modalities include the use of oral tablets of L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine receptor agonist as well as deep brain stimulation (DBS) [61]. Each of these treatments comes with serious side effects; therefore, there is a need for the safe and secure regenerative strategies for successful treatment of the PD patients without any side effect. Cell-based therapy for neurodegenerative disease like PD has potential to revolutionize the future therapeutics. Till date, most successful cell replacement studies involve transplantation of fetal ventral mesencephalic (VM) cells in striatum of Parkinson's patients [62]. The motor symptoms were found to be improved, demonstrating increase in F-DOPA uptake by the patients. However, 15% of these patients developed graft-induced dyskinesias (GIDs) [62]. These results put the strategy on hold until further research reveals that dopamine replacement therapy is specifically effective for younger or less severely affected patients, and marked improvement is noticed from 2 to 4 years posttransplantation.

There are numerous preclinical studies conducted in the last two decades to establish the use of human NCSs, ESCs, and MSCs obtained from different sources and iPSCs as potential candidates [63]. ESCs provide virtually limitless supply of dopaminergic progenitors, which makes the treatment of PD become promising. Various studies demonstrated a significant role of these cells in generating functional recovery and improvement in PD symptoms when delivered to striatum of experimental PD models [64]. However, ethical issues, immune rejection, and teratoma formation have limited their clinical applications. More studies are needed to optimize the differentiation and transplantation protocols to make the progress toward clinical trials.

As discussed above, MSCs exhibit many properties to be an ideal stem cell candidate for clinical studies. It is possible to expand them rather simply on a large scale for convenient clinical application. In PD animal models, MSCs are partially shown to restore dopaminergic pathway [65]. In a human clinical trial, where autologous BMSCs were transplanted in Parkinson's patients, even 36 months post therapy, these patients displayed a degree of improvement in Parkinson's symptoms without any tumor

formation or other side effects [66]. This appears as a restorative therapy of choice in PD, but more clinical studies are required to confirm the safety and efficacy of the therapy. iPSCs from adult human somatic cells have revolutionized a new method to generate disease-specific pluripotent stem cells from the individual's own cells. iPSCs obtained from the somatic cells of PD patients to produce dopaminergic neurons can be transplanted into the patient's brain. Xu et al. [67] in his recent publication summarized various studies in which iPSCs were used for modeling PD with gene mutations. Another study showed that iPSCs derived DA neurons from PD patients carrying *Parkin* mutation exhibiting an increase in DA release as well as a reduction in DA uptake. Additionally, elevated level of reactive oxygen species (ROS) in these neurons was found due to mitochondrial dysfunction. This further indicates the importance of *Parkin* in DA transmission and in suppression of DA oxidation. Because of their unmatched implications, iPSCs hold tremendous potential for future cell replacement therapy in PD patients in order to move forward for clinical trials.

13.4.1 Stem Cell Trials for PD

In the early 1990s, open-label clinical trials were performed using dopaminergic neurons containing fetal ventral mesencephalic tissue which revealed significant clinical benefit after transplanting the tissue into the striatum of PD patients [68]. Not only there was significant variation of results among patients, two subsequent double-blind randomized trials using ventral mesencephalic tissue did not succeed in showing any robust improvement and showed graft-induced dyskinesia (GIDs) in the patients [68]. Further studies were conducted to shed some light on the underlying mechanisms controlling GIDs, improved methods to minimize the risk of GIDs, and subsequently led to a new European trial called TRANSEURO which used fetal ventral mesencephalic tissue (www.transeuro.org.uk). TRANSEURO was designed to form a basis and a benchmark for future stem cell-based transplantation trials in PD through a better understanding of PD patients, standardizing methodologies, and transplantation techniques and adequate clinical end points that match the time course of biological repairs. In March 2013, the California Institute for Regenerative Medicine in their workshop on cell therapies for PD discussed on having a more refined approach and in having an appropriate patient population prior to conducting new clinical trials using stem cell-based therapies for PD [69]. In December 2015, International Stem Cell Corporation (ISCO), based in California, announced a phase I/IIa trial using a parthenogenetic stem cell source in PD patients. This trial has raised expectations in the PD community [70].

13.4.2 Future of Stem Cells for PD

Each stem cell transplantation technique has its own unique advantages and limitations. ESCs and iPSCs seem to be the easiest to manipulate to generate a large number of DA neurons in vitro. However, the more advantageous iPSCs may be

used for autologous transplantation; they still have their own disadvantages. Major research efforts need to be focused on efficacy, methodology of transplanting cells, tumorigenicity, and other safety issues to ensure that future trials can be undertaken with greater confidence. Most recent reports on using parthenogenetic stem cells (created by providing chemical stimulus to oocytes (eggs) to begin division) in PD patients are the ray of hope in treating PD symptoms. The oocytes do not undergo fertilization and thus no viable embryo is created or destroyed. This provides a more stable ethical footing and, when combined with the advantage of immunomatching, makes these stem cells show great potential as a future source for cell-based therapy. The future is hopeful, but still there is long way to go.

13.5 Age-Related Macular Degeneration

Age-related macular degeneration (AMD), a degenerative disease of the retina, is the leading cause of blindness. Its main pathology comprises the photoreceptor death, which ends in vision loss [71]. There is no permanent treatment for this condition; however, anti-vascular endothelial growth factor (VEGF) is helpful in controlling the wet AMD to some extent [72]. The replacement of damaged cells by embryonic stem cell-derived photoreceptors and retinal pigment epithelium (RPE) promises on the therapeutic effects in this disease. Studies have been conducted on different cell types to produce RPE, photoreceptors, and retinal progenitor cells (RPCs) [73]. Stem cells are providing a major way for scientists to recognize how diverse cells in the retina functions together. It has led to finding of different ways to change cones and rods with the RPE cells. In the last few years, extensive progress has been made in advancing the goal of RPE correction in AMD retina.

13.5.1 Which Stem Cells to Be Used?

The key question in using the stem cells is which one to use. Replacing cones and rods is a tough task; these cells are responsible to make connections with nerve fibers, which send signal to the optic nerve. However, RPE cells may be easier to transfer because they do not need to form connection with nerve fibers. These new cells could replace the older degenerated RPE cells and could support them. It is also easy to make identical stem cells from RPE cells, which reduce the problems of uniformity of cells to transplant. To grow the rods, cones, and RPE cells, some investigators are now using iPSCs, which can be reprogrammed in a laboratory. Some are using ESCs, and others are using RPE-specific stem cells from adult RPE [74]. In retinal lineage differentiation, ESCs are considered as an attractive source of cells due to unlimited expansion and pluripotency in vitro [75]. In animal models of retinal degeneration, ESC-derived cells have shown realistic approach to rescue the visual function [76]. Recently, in human clinical trials, ESC-derived retinal progenitor cells were used to treat 18 retinal degenerated patients out of whom visual acuities improved in ten patients [77]. In the mouse model of retinal degeneration, the photoreceptors were repaired by transplanting RPCs from newborn mouse

retina [78]. The promising sources of stem cells to cure retinal diseases are UC-derived stem cells, ESCs, fetal stem cells, bone marrow-derived HSCs, and MSCs.

13.5.2 Stem Cell Trials for AMD

In retinal degeneration diseases, there are several phase I clinical trials that are in progress. Schwartz et al. [77] conducted a clinical trial on dry atrophic AMD and Stargardt's macular degeneration. About 50,000–150,000 RPE cells were injected behind the retina in nine patients with Stargardt's macular degeneration and dry AMD eyes. Results from this study showed possible multipotent stem cell progeny of the transplant along with long-term safety. The first report on the rescue of visual function was due to transplantation of cynomolgus monkey ESC-derived RPE into RCS rats, an established model for AMD [79]. Recently, enzymatically dissociated technique was used to separate human fetal NSCs from brain tissue (16–20 weeks gestation) on the basis of CD133 expression. These cells were expanded in nerve tissue culture medium; the dissociated fetal stem cells were administered in the subretinal space of the RCS rats. It was observed that the transplant distributes throughout the retinal area [80]. In another study, endogenous HSCs were found to migrate in the subretinal space of the damaged retina in mouse, potentially for supporting tissue repair [81].

In a mouse model, shortly after inducing injury to the retina, tail vein injection of allogeneic bone marrow-derived HSCs was carried out [82]. These cells proliferated and migrated to the retina and expressed RPE65, which indicates that the engraftment responded correctly to the new niche; however, the expression of RPE65 alone is not sufficient for the RPE function. Multipotent umbilical cord tissue-derived cells were found to be involved in vision improvement in 80 RCS rats as compared to controls [83]. In RPE disease, bone marrow MSCs also showed promise as a possible modality for the therapy. Arnhold et al. [84] injected 5×10^4 cells in the retina of RCS rats and showed improvement in the RPE morphology. Histological analysis showed that cell injection leads to the development of three to six layers of photoreceptors, whereas there was only one layer in sham and uninjected control rats [85]. Considering that RPE is the main cell type that is affected in AMD, many endeavors have been made to replace them, particularly in the macula. Various sources of RPE grafts have been used in these procedures including autologous sheets of fetal RPE, grafts of free RPE choroid, and cell suspension of peripheral RPE [86]. In cell suspension method, the main limitation was found to be low incorporation of the graft in diseased Bruch's membrane.

13.5.3 Future of Stem Cells in AMD

At first it is necessary to ensure that the treatment is safe, following proof-of-principle one can plan for the long-term study. The diseases for which stem cells therapy has been found encouraging are still very few. However, where conventional medication

does not offer any hope, stem cell therapy could be considered as an option. So far no successful treatments are available for humans using ES cells. In severe cases of retinal degeneration, stem cell therapy has the potential to become a standard means for the treatment; however, it is still a long way to go before each to bedside. Fetal brain-derived stem cells are able to form several RPE functions, but the use of these cells is restricted due to ethical issues and unavailability. The main challenge is to develop ethically accepted therapy, which will help in restoring the vision without immunological rejection.

Conclusions

In less than a decade, the stem cell research started as a stepping stone, but gradually it has taken massive dives and has paved through the rocky path from laboratory to clinical application. In this chapter, the author has recapitulated recent approaches of investigations on neurodegenerative disorders using various stem cells so as to explore how disease onset and progression can be prevented. Stem cells have always been a burning topic with debates and controversies. Many scientists are debating over the likeliness of ESCs and iPSCs, whether iPSCs can satisfactorily take the place of ESCs in translational research. Due to ethical restrains of using ESCs, iPSCs are considered a better alternative with more promising results. Depending upon the disease of interest, the medical history of the donor can be obtained in case of iPSCs, which is not possible with ESCs. Yet, the challenges stand in the way of accessibility of the patient history so as to ensure privacy and then use iPSCs for research and treatment. With the creation of more disease-associated iPSCs (like haploidentical cell bank) for both genders and sharing of information to the public databases, these will help in screening the drugs which is the need of an hour. Though MSCs and NSCs are promising for the treatment of MS, a lot of impediments need to be resolved. Furthermore, iPSCs obtained from patients' own tissue also represent a novel method in MS treatment. The method and mechanisms by which transplanted stem cells are engrafted to the destined injured region, their fate in vivo in different clinical subtypes of MS, dosage, route of administration, their biological safety, efficacy, and shelf life post-transplantation are still poorly understood. So-called immature cells, which are capable of undergoing differentiation to form specific stem cells, are transplanted into target tissues, leading to mobilization of the endogenous stem cells in the tissue-specific region. This has paved its way for future developments in treating degenerative diseases of the CNS. Although it may seem unlikely in complete replacement of the damaged neurons in the brain, nevertheless advancements in the research on animal models have provided a glimpse of hope and happiness with loads of positive results revealing that partial replacement of damaged neurons is definitely possible [87]. A pioneering mechanism called bystander mechanism by Gianvito Martino and Stefano Pluchino explained that how stem cell therapy along with regeneration of neural cells also decreases the inflammation in these degenerative disorders [88]. Stem cells have also shown promising results in the mouse model transplanted with neural multipotent stem cells thereby not only facilitating neurogenesis but also providing neuroprotection by immunomodulatory

mechanisms [89]. Concomitantly, different types of stem cells are used for establishing effective therapeutic interventions; apparently, it still has limitless potential to meet the demands in the field of personalized medicine. Rather, we are still in our infancy stage to treat these neurodegenerative diseases. Everyone working within the field of stem cells is meticulously studying to make this dream into reality. Though both scientists and clinicians are optimistically trying to circumvent the problem of neurodegenerative diseases by the use of stem cells, a few issues can turn the other way which is difficult to anticipate. No one can answer all questions that come on the way in stem cell research, yet we hope stem cell therapy will come into reality with more clear horizons that will address all questions including the safety measures.

Conflict of interest: The authors declare that they have no competing interests.

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Research report

Effect of human umbilical cord blood derived lineage negative stem cells transplanted in amyloid-β induced cognitive impaired mice

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HIGHLIGHTS

- Umbilical cord blood derived Lin −ve stem cells alleviate amyloid-β induced behavioral deficits.
- Lin −ve cells showed a time and dose specific spatial memory improvement in mice.
- These cells mediate up-regulation of BDNF and CREB in brain.
- Concomitant downregulation of Fas-L expression were also observed.

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ABSTRACT

Alzheimer's disease (AD) is pathologically characterized by extracellular deposition of insoluble amyloid-β (Aβ) plaques and intracellular tangles made up of phosphorylated tau in brain. Several therapeutic approaches are being carried out in animal AD models for testing their safety and efficacy in altering disease pathology and behavioral deficits. Very few studies have examined the effect of human umbilical cord blood (hUCB) derived stem cells in degenerative disease models despite growing number of cord blood banks worldwide. Here we have examined the therapeutic efficacy of hUCB derived lineage negative (Lin −ve) stem cells in alleviating behavioral and neuropathological deficits in a mouse model of cognitive impairment induced by bilateral intrahippocampal injection of Aβ-42. Lin −ve cells were transplanted at two doses (50,000 and 100,000) at the site of injury and examined at 10 and 60 days post transplantation for rescue of memory deficits. These cells were found to ameliorate cognitive impairment in 50,000—60 days and 100,000—10 days groups whereas, 50,000—10 days and 100,000—60 days groups could not exert any significant improvement. Further, mice showing spatial memory improvement were mediated by up-regulation of BDNF, CREB and also by concomitant down regulation of Fas-L in their brain. The transplanted cells were found in the host tissue and survived up to 60 days without expressing markers of neuronal differentiation or reducing Aβ burden in mouse brain. We suggest that these undifferentiated cells could exert neuroprotective effects either through inhibiting apoptosis and/or trophic effects in the brain.

1. Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases of central nervous system (CNS). In 2013 the Alzheimer's association estimated 5.2 million people suffering from AD in USA alone with an alarming rate of diagnosing one case every 68 s. This number is expected to rise to 13.8 million by 2050, with a rate of nearly a million people newly diagnosed for AD each year [1]. In India, the number of people suffering from AD and other dementia is estimated to approximate 3.7 million and this number is expected to double by the year 2030 [2]. With such prevalence, AD is threatening to be epidemic worldwide. The current treatment strategies can only provide symptomatic relieves without altering

Abbreviations: AD, Alzheimer's disease; AP, anterioposterior; Aβ, amyloid-β; BDNF, brain derived neurotrophic factor; CFDA–SE, carboxyfluorescein diacetate–succinimidyl ester; CNS, central nervous system; CREB, cAMP response element binding protein; CTCF, corrected total cell fluorescence; DV, dorsoventral; HSC, hematopoietic stem cell; hUCB, human umbilical cord blood; IAEC, Institute Animals Ethical Committee; IP, intraperitoneal; MACS, magnetic associated cell sorter; ML, mediolateral; MSCs, mesenchymal stem cells; MWM, Morris water maze; NFT, neurofibrilary tangles; PBS, phosphate buffer saline; RO, reverse osmois; RT, room temperature.

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underlying disease pathologies. The acetylcholinesterase inhibitors and NMDA receptor antagonists are currently approved drugs for AD but none of them are efficient in delaying or halting the disease progression [3].

AD is pathologically characterized by intracellular neurofibrilary tangles (NFT) and extracellular β-amyloid (Aβ) plaques, leading to selective neuronal loss in diseased brain. There is progressive memory loss and cognitive decline as the disease progresses [4]. In AD brain autopsies it is demonstrated that synaptic plasticity and neuronal hypertrophy are imbalanced leading to disease pathology whereas a compensatory mechanism sets in rescue for normal cognitive aging [5]. At present, there are no definitive consensus preventive measures established in management of AD. There are no promising outcome obtained from several AD trials in last two decades leading to explore other causative mechanisms instead of targeting Aβ and tau pathologies alone [6]. The putative role of neuroinflammatory cytokines [7], cytoskeletal associated proteins [8] and exocytic lysosomal enzymes [9] are under extensive investigations to alleviate the pathological burden of the disease.

There have been multiple approaches carried out to establish animal models of AD in preclinical setting [10]. Aβ induced selective neurotoxicity is well demonstrated in experimental rodent models. Tang et al. demonstrated that fibrillar form of Aβ(1–40), when administered in rat hippocampus, formed insoluble aggregates showing Congo red positivity and induced neurotoxicity in the DG and CA3 regions [11]. It is also reported that Aβ induced toxicity downregulates one of the important neurotrophins, such as, brain derived neurotrophic factor (BDNF) and its major regulatory molecule, cAMP response element-binding protein (CREB) in both in-vitro and in-vivo studies [12,13].

Cell based preclinical therapies in AD have been limited owing to the presumption that the pathology in brain is not as well localized as in case of PD or ALS. Hence, the recent developments in early diagnosis and treatment strategies of AD have raised an unparallel requirement to screen a suitable drug, alternative extracts or cell based therapeutics. Stem cell transplantation (SCT) therapy is attracting attention for chronic diseases of ageing especially those that result from a defined and localized deficit not characterized in AD. However, despite the hope held by cord banks, the human umbilical cord blood (hUCB) derived stem cells have not been adequately studied. A limited number of studies with disease models of stroke [14], AD [15] and spinal cord injury [16] have been conducted even though it has been argued that stem cells from hUCB banks can provide a reliable source for replacing damaged neurons and restoring their functional activity [17]. There are potential studies demonstrating therapeutic effects of neural stem cells by offering pathological and functional recovery in AD models. Although there are few studies supporting the neuronal replacements by transplanted stem cells as a therapeutic mechanism but larger number of studies have reported that this neuroprotection offered by transplanted cells are mediated by their neurotrophic effects [18]. It has been reported that neural stem cells (NSCs) derived from embryonic stem cells, when transplanted at the site of neural degeneration, can facilitate neuronal differentiation, leading to functional recovery from motor and cognitive impairments in a mouse model of AD [19]. In another study, transplantation of NSCs derived from postnatal brain in a triple transgenic AD model does not alter underlying Aβ or tau pathology, instead, exerts cognitive improvement mediated by neurotrophins such as BDNF [20]. A recent study also demonstrated the therapeutic effect of NSCs in AD mice by enhanced expression of cognitive related proteins and neurotrophins without altering the Aβ pathology [21].

Although, very few studies have been reported demonstrating the therapeutic role of hUCB derived stem cells in AD. Largely, mesenchymal stem cell (MSCs) population from hUCB has been tested in AD models. Lee et al. has reported that hUCB–MSCs play potential role in reducing Aβ deposition, tau hyperphosphorylation and BACE1 activity through microglial immunomodulation in APP/PS1 mice [22]. Darlington et al. demonstrated the therapeutic effects of hUCB derived mononucleated cells in PS/APP transgenic mice. Multiple low dose infusion of these cells in peripheral circulation between 6 to 12 months of age has led to cognitive improvement in a battery of spatial memory testing paradigm followed by reduced load of Aβ plaques and microgliosis [23]. It is reported that autologous T regulatory cells purified from transgenic AD mice when co-cultured with human umbilical cord derived mesenchymal stem cells in-vitro and transplanted in the systemic circulation in these AD mice, they could exert a protective effect on AD pathology by decreasing microglial activation and levels of systemic inflammatory factors [24]. Above studies reveal that stem cells in hUCB has significant clinical potential but not adequately tested in AD pathology. Hence our study is a timely intervention to evaluate the therapeutic role of hUCB derived lineage negative (Lin −ve) cells in AD treatment.

Lin −ve cells, the purified subset of cells without expressing surface antigens associated with lineage commitment are reported to have unrecognized heterogeneity within the hematopoietic stem cell (HSC) population [25–27] but not adequately studied thereafter. Such primitive population of cells are deprived of any mature hematopoietic markers such as, CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and CD235a (glycophorin A) [28] and found to be present in only 0.1% population of mono-nucleated cells enriched from hUCB [29] These are also found in peripheral blood and bone marrow cell in a very limited fraction [30,31]. These cells were shown to have the potential to differentiate into granulocyte-macrophage, erythroid and megakaryocytic colony forming units when cultured in-vitro [32]. Unrestricted somatic stem cells from hUCB, when induced with dopaminergic regulatory factor in-vitro, can be differentiated into dopaminergic neurons expressing tyrosin hydroxylase [33]. Limited studies show that Lin −ve cells are capable of integrating and differentiating in different tissues of the host organs when transplanted in the peritoneal cavity of goats [34]. Unfortunately, there are not many in-vivo studies to demonstrate their efficacy in disease models. We had earlier shown that Lin −ve stem cells derived from mouse bone marrow, when transplanted via intravenous route, survived for 8 weeks and repaired the laser induced lesions in mouse retina without undergoing differentiation [27]. Similarly, this study further advances our current knowledge of Lin −ve cells by testing the role of such primitive cells derived from hUCB. We show that these cells have potential to rescue memory loss mediated by pathways relevant to cell survival not necessarily linked to differentiation.

2. Materials and methods

2.1. Animals

All the experiments were conducted in GLP compliant laboratory supported by quality assurance program, using periodically calibrated instruments and molecular biology grade chemicals. Each experimental step was recorded and archived in appropriate formats and samples were coded and stored in dedicated storage locations ensuring back traceability of both data and samples. All animals were obtained from Institute's central animal facility vide Registration No. 47/1999/CPCSEA and all the experiments were carried out by trained animal experimenter following the guidelines approved by the Institute Animals Ethical Committee (Approval no. 46/IAEC/217). Appropriate measures were taken to minimize the pain or discomfort subjected to the animals used in the experiments. Sex (male) and age (4–6 weeks) matched, inbred Swiss albino mice (20–35 g) were used for all the experiments. Mice

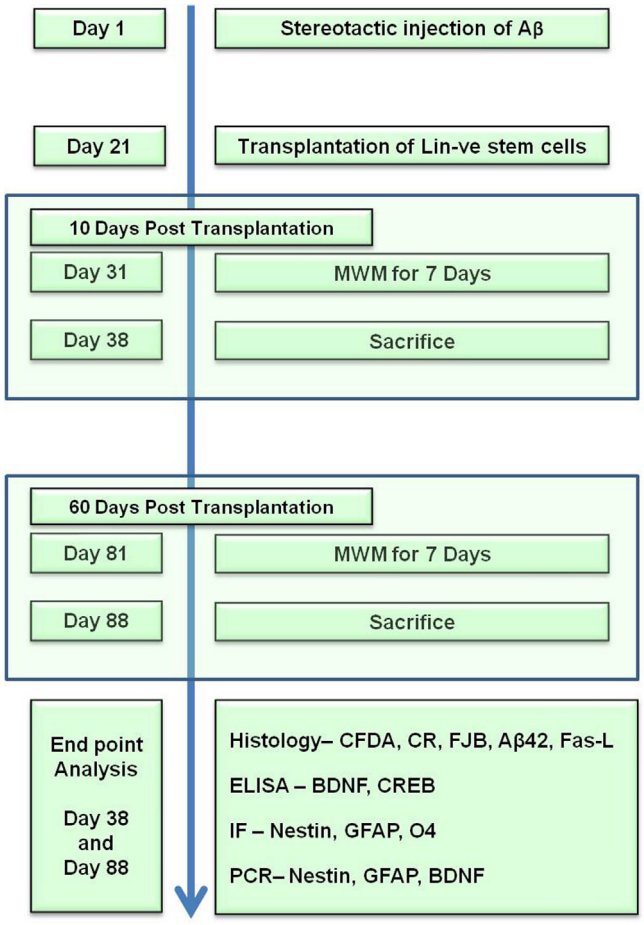

Fig. 1. Day wise plan explaining time course of the treatment and analysis.

were kept in the central animal house with rooms regulated for humidity (45–55%) and temperature (20–23 °C) and kept on a 12 h light/dark cycle. Chow diet (protein 20–24%, fat 4–5%, fiber 4–5%, Ca 1–1.5%, P 0.5% and vitamin supplements 0.5%) and RO drinking water were available ad libitum. Six mice were kept in a transparent polypropene cage (38 cm × 24 cm × 16 cm), using sterilized corn cob as bedding material. The experiments were carried out in between 9 AM and 6 PM in a semi-soundproof working laboratory (Fig. 1).

Before recruiting animals they were tested in Morris water maze (MWM) for their swimming ability. Animals showing normal behavioral pattern, with standard vision and motor activities were included in the study. They were allowed to freely swim in the MWM pool for 3 min to verify their general exploratory behavior to escape from water. The rota rod tests were performed to exclude the animals with irregular muscle coordination. Only those mice which could sustain 3 min of locomotory behavior on rota rod, at a speed of 15 rpm, were included in the study. There were 12 groups formulated for the study and 10 mice were included in each group (Table 1). All the mice underwent stereotactic surgery at two time points, namely Aβ delivery at day 1 and cell transplantation at day 21. PBS was injected in control groups in place of Aβ or cells. All the mice were tested in MWM after either 10 days or 60 days post transplantation of stem cells. Mice were sacrificed post MWM analysis by I.P. injection of an over dose of anesthesia (xylazine–ketamine cocktail) in accordance with the approved guidelines of IAEC and brains were stored at −80 °C for immunohistological, biochemical and molecular analysis (Fig. 2). Measures were taken to minimize the use of animals for experiments. $n = 6–8$ mice were recruited for MWM analysis and further 2 brain samples for each group were used for IHC, ELISA and PCR analysis.

2.2. Intra-hippocampal injection of aggregated Amyloid-β peptide

Hippocampus injury was induced by stereotaxic injection of aggregated form of Amyloid-β (1–42) peptide (Sigma, USA). The peptide was made by dissolving it in 1× phosphate buffered solution (PBS) and incubated at 37 °C overnight for aggregate formation. Mice were anesthetized using xylazine (10 mg/kg)–ketamine (100 mg/kg) cocktail and mounted in a stereotaxic apparatus. Bilateral craniotomies were done for the injection by exposing the skull and drilling the injection points using a 26G needle according to the Bregma scale following the stereotaxic coordinates [35]. Bregma coordinates used for the hippocampal delivery was anterioposterior (AP) 2 mm, mediolateral (ML) ±2 mm and dorsoventral (DV) 2.5 mm. 800 pMol of aggregated form of Aβ solution (Stock conc. 250 μg/ml) in 5 μl of PBS was injected in a controlled rate of 1–2 μl/min using a microinjector. The needle of the microsyringe was kept unmoved for 5 min after the solution was delivered in the hippocampus for and then slowly removed from the brain by unscrewing the stereotaxic arm of the injection [36]. PBS was injected bilaterally for sham-operated groups.

2.3. Enrichment of Lin −ve stem cells for transplantation

Human UCB samples were collected from the umbilical cords of newborns delivered by pregnant women (aged between 20 and 35 years) at gestation period of ≥28 weeks after proper informed consent was taken in accordance with ethical guidelines approved by Institutional Committee on the Stem Cell Research and Therapy (IC-SCRT) (Approval no. IC-SCRT/10/DTM-3272). hUCB samples were processed by density gradient centrifugation, using histopaque (Sigma, USA), for enrichment of mono-nucleated cell population (MNCs). The Lin −ve stem cells were isolated from such MNC population using a magnetic associated cell sorter (MACS) (Miltenyi Biotech, Germany) with human Lin −ve isolation kit (Miltenyi Biotech, Germany). Finally, Lin −ve cells were analyzed using an automated cell counter (Millipore, USA) for their absolute counts and diameters. These cells were labeled with fluorescent CFDA–SE dye (amine-reactive carboxyfluorescein succinimidyl ester) (Invitrogen, USA) for post-transplantation analysis. Briefly, cell pellet was suspended in 1× PBS containing CFDA (10 μM). Cells were incubated at 37 °C for 15 min accompanied with mild shaking. These cells were further suspended in pre-warmed (37 °C) 1× PBS for another 30 min to ensure complete reaction. Finally, theses cells were washed and suspended in 1× PBS for transplantation. The CFDA labeled cells were also validated through flowcytometric analysis to ensure complete staining.

2.4. Flowcytometry analysis of Lin −ve stem cells

The Lin −ve cells isolated from UCB—were characterized by flowcytometry for the presence of stem cell markers. CD45, CD34 and CD117 marker profile was analyzed in all the three populations of UCB viz., MNCs, Lin +ve and Lin −ve cells. Approximately one million cells were suspended in 100 μl FACS or MACS buffer (PBS–BSA–azide solution). FC blocking reagent (20 μl for up to 10^7 cells) (Miltenyi Biotech, USA) was added in each tube and incubated for 30 min at RT. Antibody-conjugated with fluorochrome (Conc: 20 μl/1 × 10^6 cells) (BD Bioscience, India) was added and incubated for 1 h at RT. Further, 1 ml of FACS or MACS buffer (PBS–BSA–azide solution) was added and washed 2 times with the buffer to remove unbound antibodies in the solution. The cells were suspended in 300 μl of buffer and processed in flowcytometer (within 2–6 h of

Table 1
Details of experimental groups.

Group	Stereotaxic injection at day 1	Stereotaxic injection at day 21	Post-trnaplantation analysis at day 31/81
PBS–PBS—10 d	5 μl PBS	5 μl PBS	Day 31: analyzed in MWM after 10 days of second injection
PBS–PBS—60 d	5 μl PBS	5 μl PBS	Day 81: analyzed in MWM after 60 days of second injection
PBS-50,000—10 d	5 μl PBS	50,000 Lin −ve stem cells (5 μl)	Day 31: analyzed in MWM after 10 days of cell transplantation
PBS-50,000—60 d	5 μl PBS	50,000 Lin −ve stem cells (5 μl)	Day 81: analyzed in MWM after 60 days of cell transplantation
PBS-100,000—10 d	5 μl PBS	100,000 Lin −ve stem cells (5 μl)	Day 31: analyzed in MWM after 10 days of cell transplantation
PBS-100,000—60 d	5 μl PBS	100,000 Lin −ve stem cells (5 μl)	Day 81: analyzed in MWM after 60 days of cell transplantation
Aβ-PBS—10 d	800 pM Aβ-42 (5 μl)	5 μl PBS	Day 31: analyzed in MWM after 10 days of second injection
Aβ-PBS—60 d	800 pM Aβ-42 (5 μl)	5 μl PBS	Day 81: analyzed in MWM after 60 days of second injection
Aβ-50,000—10 d	800 pM Aβ-42 (5 μl)	50,000 Lin −ve stem cells (5 μl)	Day 31: analyzed in MWM after 10 days of cell transplantation
Aβ-50,000—60 d	800 pM Aβ-42 (5 μl)	50,000 Lin −ve stem cells (5 μl)	Day 81: analyzed in MWM after 60 days of cell transplantation
Aβ-100,000—10 d	800 pM Aβ-42 (5 μl)	100,000 Lin −ve stem cells (5 μl)	Day 31: analyzed in MWM after 10 days of cell transplantation
Aβ-100,000—60 d	800 pM Aβ-42 (5 μl)	100,000 Lin −ve stem cells (5 μl)	Day 81: analyzed in MWM after 60 days of cell transplantation

antibody labeling). If these were not analyzed immediately, cells were fixed with 300 μl of fixation buffer solution (BD Bioscience, USA) and stored at 4 °C till processed in flowcytometer.

2.5. Transplantation of Lin −ve stem cells at the site of Aβ injection

Cell transplantation was carried out using a stereotaxis apparatus and same protocol as was used for Aβ injection. CFDA–SE labeled Lin −ve stem cells were suspended in PBS in two different cell doses i.e. 50,000 or 100,000 cells and were stereotactically transplanted in the respective mice after 21 days of Aβ injury. The cells were delivered bilaterally at the site of the Aβ lesions in the hippocampus at the speed of 1–2 μl/min with the auto-injector. Vehicle treated groups were given bilateral injection of PBS.

2.6. Evaluation of spatial memory in Morris water maze

The loss of spatial memory following Aβ injection and also the effect of cell transplantation in these animals was tested in MWM. The MWM pool (diameter 2 m, height 45 cm) was filled with water (22–24 °C) and nontoxic black poster color (Camel, India) was added to make it opaque. Seven days protocol was followed for this experiment. Mice were subjected to acquisition trials for first 6 days, followed by retrieval trial on 7th day. Each animal was given 4 trials a day with a resting phase of 20 min in between two trials for individual mouse. The starting position of each mouse was changed from Q1 → Q2 → Q3 → Q4 for 4 trials in a day. The position of the hidden platform (submerged 1 cm below the water level) was kept constant for the entire period of acquisition trials and it was removed from the pool during retrieval trials. All the activities inside the MWM pool were digitally recorded by an automated video tracking system using Anymaze software (Stoelting Co, USA). Escape latency time (ELT) was measured, as an index of acquisition, by estimating the time taken by the mice to reach the hidden platform. The swimming path for all the mice was mapped for each trial comprising of 120 s. If the animals were not able to reach the platform within the stipulated time, they were gently guided to the hidden platform using a wooden stick. On 7th Day (retrieval) the mice were freely allowed to swim for 120 s in the pool and the time spent by each mouse in the target quadrant as well as the path used while exploration was measured as a degree of retrieval of acquired memories.

2.7. Histological studies

8–12 μm coronal brain sections were taken on poly-L-lysine coated glass slides using a Cryotome (Leica, USA) and tissue embedding media (Leica, USA). Sections were stored at −20 °C until further processing for histological and immunofluorescent analysis. Images were analyzed for quantitation of immunefluorescence in ImageJ software by normalizing the background fluorescence from total fluorescence. Images have been edited using Adobe photoshop to enhance visibility in compliance with publishing ethics. Integrated density (INT DEN) for all the images and their respective backgrounds (2–3 randomly chosen areas/image) were measured and their corrected total cell fluorescence (CTCF) were analyzed using the formula: CTCF = [Integrated Density − (Area of selected cell × Mean fluorescence of background readings)].

2.7.1. Congo red staining

Brain sections were stained by Alcoholic Congo red (HiMedia, India) solution for identification of Aβ plaques in the mouse brain. Brain sections were fixed with histochoice fixing solution (Sigma, USA) for 5 min at room temperature (RT) and then washed with water. Sections were then subjected to hydration in 90%, 70% and 50% ethanol, respectively, followed by washing in water. Nuclei were stained with haematoxylin for 3 min at room temperature. Sections were thoroughly washed in water and stained with 1% alcoholic Congo red solution for about 30 min at room temperature. The slides were immersed in 70% alcohol for a few seconds to remove excess stain and then dipped in xylene before mounting them with fluorosave mounting medium (Calbiochem, USA).

2.7.2. Immunofluorescent staining

Fluorescent-tagged antibodies viz., MAP2 (1:50, Santa Cruz, USA) for microtubule proteins in host neurons, Nestin (1:100, Santa Cruz, USA) for neuronal progenitors, GFAP (1:100; Santa Cruz, USA) for astrocytes, O4 (1:50; Sigma USA) for oligodendrocytes, Aβ42 (1:100, Novus Biological, USA) for amyloid plaques and Fas-L (1:100; Chemicon, USA) for apoptotic cells co-stained with DAPI nuclear stain (1:1000; Life Technologies, USA) were used to analyze the histopathological changes in brains upon cell transplantation. The slides were incubated overnight at 4 °C with respective primary antibodies. After removing the solutions from slides they were incubated with respective secondary antibodies (1:200; Jackson, USA) for 1 h at RT. The sections were washed with PBS for 3 times and counter-stained with DAPI for 5 min at RT. The slides were rinsed with PBS for 3 times and mounted with fluorosave mounting medium before examining under the fluorescent microscope.

2.8. Estimation of neurotrophic factors in mouse brain homogenate

Brain homogenates from different groups (were subjected to quantitative expression of neurotrophin such as brain derived neurotrophic factor (BDNF) and its regulatory molecule cAMP-response element binding protein (CREB). Whole brains were homogenized using freshly prepared lysis buffer composed of 1% Triton-X, 10% Glycerol, 150 nM NaCl, 50 nM HEPES buffer, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. These factors

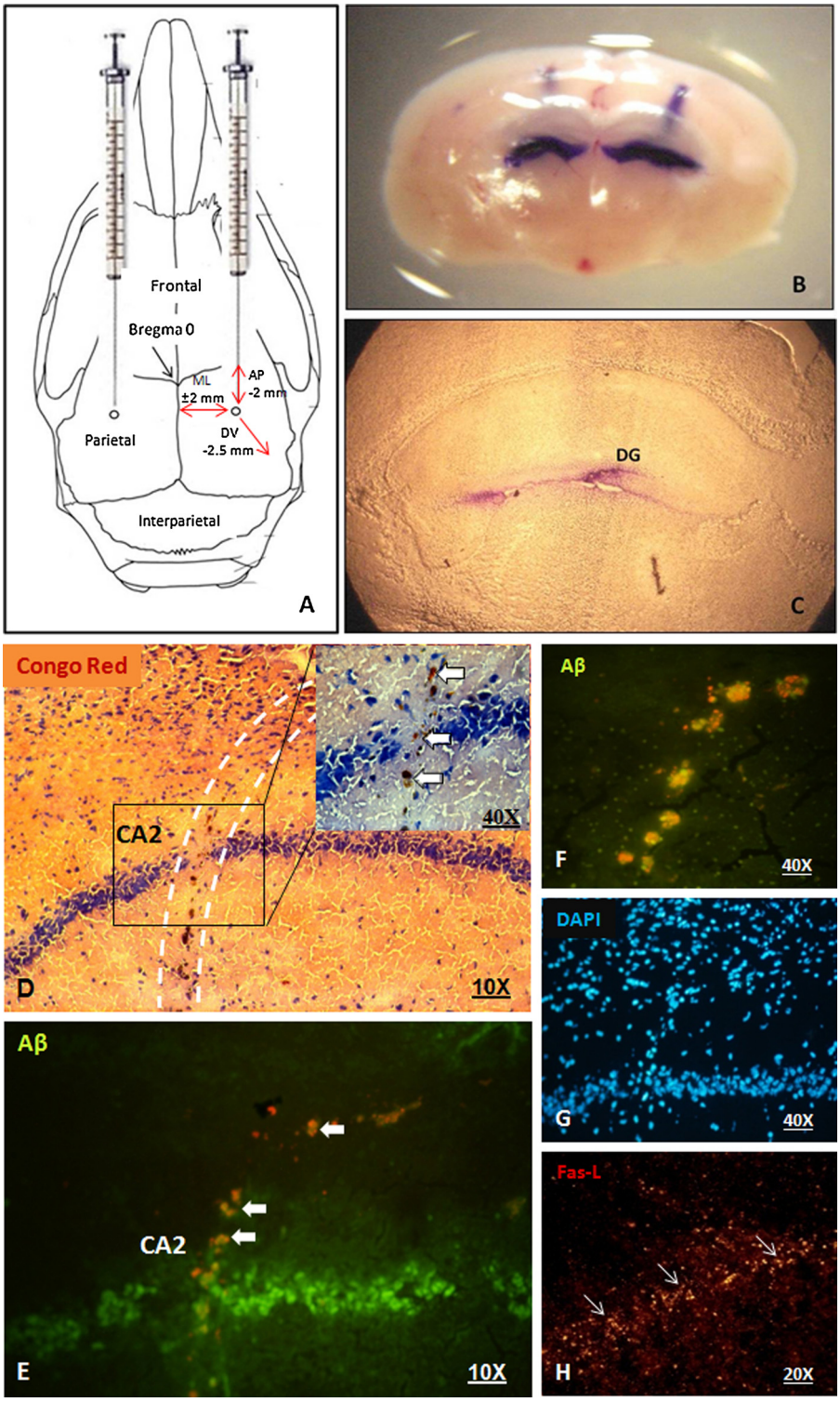

Fig. 2. *Intra-hippocampal micro-injection and AD like pathology in mouse brain.* (A) Coordinates for hippocampus showing site of injections indicated by circle. Arrow indicates Bregma 0. (B) Gross section of mouse brain showing diffused crystal violet dye through-out the hippocampal area with a prominent needle track under dissection microscope. (C) Cryosection of the brain showing the deposition of dye in dentate gyrus (DG) region of the hippocampus. (D) Arrows show appearance of Congo red positive Aβ aggregates in the CA2 region of mouse hippocampus. Dotted line shows deposition of Aβ along the tract of injection. The CA2 layer was also found to be dissociated in this section due to Aβ deposition. (E) Immunofluorescent staining for Aβ42 antibody confirms Aβ42 positive plaques in these sections. (F) Immunofluorescent staining for Aβ42 at higher magnification. (G) Corresponding DAPI image of section F showing CA2 region of hippocampus. (H) Fas-L positive cells were present around the Aβ plaques confirming their neurodegenerative effects in mouse brain.

were estimated by ELISA at 450 nm using protocol prescribed by the manufacturer of ELISA kits (Cusa Biotech, China). All the ELISA values were normalized to their respective total protein values estimated by Bradford method.

2.9. Quantitative real time PCR

Molecular expression of different cell markers and neurotrophic factors were analyzed using quantitative PCR (qPCR) (Applied

Table 2
Primer sequence of target genes for qPCR.

Target gene	Target length (bp)	Primer sequence (5′ → 3′)	Annealing temperature (°C)
BDNF	131	F: TGGCTGACACTTTTGAGCAC R: CAAAGGCACTTGACTGCTGA	59
GFAP	63	F: ACAGACTTTCTCCAACCTCCAG R: CCTTCTGACACGGATTTGGT	63
Nestin	235	F: AACTGGCACACCTCAAGATGT R: TCAAGGGTATTAGGCAAGGGG	63
β-Actin	228	F: AGCCATGTACGTAGCCATCC R: CTCTCAGCTGTGGTGGTGAA	59–63

Table 3
Amplification conditions for qPCR.

Stage	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Temperature (°C)	95	95	59 (BDNF) 63 (GFAP) 63 (Nestin)	72	72
Duration (min)	10	1	1	1	10
Cycles	1	35			1

Biosystem, USA). The expression of BDNF, Nestin and GFAP was estimated according to their relative fold change in mRNA expression as compared to control samples. Total RNA was isolated from the brain tissues by Qiazol RNA extraction kit (Qiagen, USA). cDNA was synthesized from their respective RNA using cDNA synthesis kit (Thermo Scientific, USA) and was subjected to PCR amplification using standard primers. This was normalized to corresponding β-actin expression. The quantitative expression by qPCR confirmed the results obtained from immunofluorescent and biochemical observations. The primer sequence of target genes and PCR details are listed in Tables 2 and 3, respectively.

2.10. Statistical analysis

All the brain and RNA samples were blinded to the experimenter to remove any experimenter bias. All results were expressed as mean ± S.E.M. Data was statistically analyzed using 16.0 version of SPSS. In MWM, the acquisition and retrieval data was analyzed by repeated-measures ANOVA with acquisition days and retrieval trials as a measure of repeated observations. Further, LSD test was used for post-hoc analysis. For ELISA and qPCR observations were measured by repeated measure ANOVA with follow-up comparisons using LSD test. $p < 0.05$ was considered as statistically significant in the results obtained from statistical analysis.

3. Results

3.1. Standardization of Bregma coordinates for intra-hippocampal micro-injection

Crystal violet dye was injected in the hippocampus of mouse brain to standardize the Bregma coordinates for mouse hippocampus. The coordinates for hippocampus were standardized as anterioposterior (AP) 2 mm, mediolateral (ML) ±2 mm and dorsoventral (DV) 2.5 mm (Fig. 2A). The mouse was sacrificed immediately after injection and brain was removed for histological analysis. Gross section of the brain under dissection microscope showed diffuse crystal violet dye throughout the hippocampal region with a prominent needle track (Fig. 2B). Brain was also embedded in tissue freezing medium and 10–15 μm cryosections were obtained. Brain sections examined under microscope showed a streak of dye deposited in the dentate gyrus region of mouse hippocampus (Fig. 2C).

3.2. Deposition of Aβ aggregates in the mouse brain after 21 days of intra-hippocampal injection

Brain sections were stained with 1% alcoholic Congo red dye to identify the Aβ aggregates. Congo red has a high affinity towards insoluble Aβ plaques. The stained sections showed presence of Congo red positive Aβ aggregates in the mouse hippocampus. The deposits appeared brownish red in color and were found to be aggregated along the needle tract of the injection. CA2 layer of the hippocampus was found to be disrupted by Aβ deposition visible by H&E staining (Fig. 2D). Immunofluorescent staining for Aβ42 antibody also confirmed the presence of Aβ aggregates in these sections followed by dissociation of CA2 layers in the hippocampus (Fig. 2E–G). Immunostaining for Fas-L showed presence of apoptotic cells at the site of Aβ injury (Fig. 2H).

3.3. Characterization of Lin −ve stem cells isolated from human UCB

Lin −ve stem cells isolated from hUCB by MACS using Lin −ve isolation kit were characterized by automated cell counter to estimate the absolute number and size of these cells. About 100 μl of cell suspension was loaded into the cell counter using a 60 μm sensor. A histogram was generated by the counter showing distribution of the cells based on their diameter. The average diameter of cells was found to be 8.73 μm with a range of 6–14 μm (Fig. 3A and B). These cells were labeled with CFDA dye in order to track them after 60 days of transplantation. These CFDA labeled cells were found to be 86.7% positive for green fluorescence when analyzed in a flowcytometer (Fig. 3D and E). They were also found to express green fluorescence when observed under the microscope (Fig. 3C). These cells were smaller in size compared to Lin +ve cells (average diameter 11.40) and appear as a separate bunch in FSC/SSC plot in flowcytometer.

MACS enriched Lin −ve cells were characterized by FACS using antibodies for hematopoietic progenitors such as, CD45, CD34 and CD117 antigens. These cells were found to express 38.33% of CD34 and 43.13% of CD117 markers as compared to very low expression (<1%) among MNCs and Lin +ve cells ($p < 0.001$, $F = 16.051$ and

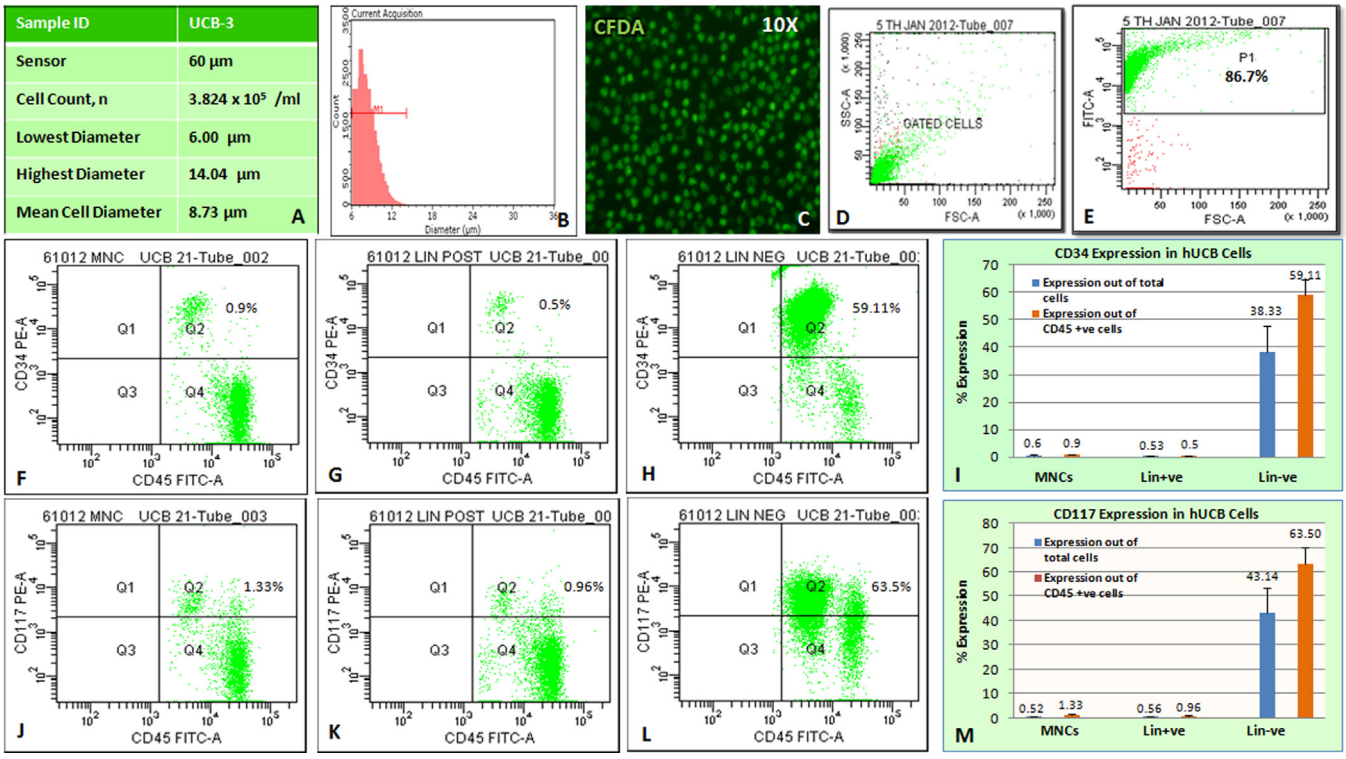

Fig. 3. *Characterization of Lin −ve stem cells from human UCB.* (A) Table showing characteristic features of analyzed Lin −ve cells isolated from hUCB in the automated cell counter showing average diameter of the cells (8.73 µm) and cell count. (B) Histogram showing distribution of cells based on their diameter (µm) varying from 6 to 14 µm. (C) CFDA labeled Lin −ve stem cells showing green fluorescence under the microscope. (D and E) CFDA labeled cells showing 86.7% positivity (P1) for green channel in flowcytometric analysis. Further the UCB samples (were analyzed for CD45, CD34 and Cd117 expression. (F)–(H) Expression of CD34 in CD45 positive cells was found to be (F) 0.9% in MNCs, (G) 0.5% in Lin +ve cells and (H) 59.11% in Lin −ve cells. (I) Comparative expression of CD34 in total gated cells and CD45 positive cells. (J)–(L) Expression of CD117 in CD45 positive cells was found to be (J) 1.33% in MNCs, (K) 0.96% in Lin +ve cells and (L) 63.5% in Lin −ve cells. (M) Comparative expression of CD117 in total gated cells and CD45 positive cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

df = 2 for CD34 expression; $p < 0.001$, $F = 17.24$ and df = 2 for CD117 expression). The CD34 and CD117 expression was found to be significantly higher (59.11% and 63.5%, respectively) in Lin −ve cells when analyzed in CD45 positive cells ($p < 0.001$, $F = 101.008$ and df = 2 for CD34 expression; $p < 0.001$, $F = 87.189$ and df = 2 for CD117 expression) (Fig. 3F–M).

3.4. Aβ induced cognitive impairment was rescued by Lin −ve stem cell transplantation

After 21 days of Aβ injection the mice were subjected to either Lin −ve stem cell transplantation or vehicle treatment. After 10 days or 60 days post transplantation all the mice were evaluated in MWM for their spatial memory performance. Aβ injured mice showed significant cognitive impairment in both 10 and 60 days groups when compared to PBS injected control groups. There was significant delay in escape latency time (ELT) in acquisition trials from day 1 to 6 in Aβ-PBS—10 d and Aβ-PBS—60 d groups. The average ELT on day 6 for Aβ-PBS—10 d group was 89.44 s when compared to 29.48 s in PBS–PBS—10 d group (Fig. 4A). The ELT on day 6 was found to be 107.18 s in Aβ-PBS—60 d group as compared to 29.10 s noted in PBS–PBS—10 d group (Fig. 4B). These mice, when tested for retrieval on day 7, showed similar pattern of swimming, compared to learned control mice, which spent maximum time in target quadrant in search of the platform (Fig. 4C). The search error index also revealed increased average distance of the mice from the platform zone indicating higher search error due to cognitive impairment in Aβ injured mice (Fig. 4D). The swimming track recorded by the video tracking system showed thigmotactic swimming pattern in these mice even after 6 days of acquisition trials (Fig. 4E).

When Lin −ve stem cells were transplanted in these mice, there was an improvement in spatial memory performance with specific cell dose. Two cell doses i.e. 50,000 or 100,000 cells were transplanted at 10 or 60 days post transplantation and all the mice were evaluated in MWM. Aβ-50,000—60 d group showed best recovery of impaired spatial memory in the mice induced by Aβ delivery. The ELT of these mice was significantly reduced in acquisition trials (50.28 s on day 6) as compared to Aβ-PBS—60 d group (107.18 s on day 6) (Fig. 4B). There was no improvement found in Aβ-50,000—10 d group. In Aβ-100,000—10 d group, mice showed significant recovery from day 1 (89.43 s) to day 6 (53.73 s) but it was not found to be significantly improved when compared to Aβ-PBS—10 d mice (Fig. 4A). The retrieval trials on day 7 revealed that both Aβ-50,000—60 d group and Aβ-100,000—10 d group spent maximum time in target quadrant (Q1) compared to Aβ-PBS—10 d group. There was no significant difference among other cell transplanted groups in term of retrieval index (Fig. 4B). The search error on day 7 was correspondingly found to be significantly reduced in both Aβ-50,000—60 d group and Aβ-100,000—10 d group compared to Aβ injured mice (Fig. 4D) (df = 11; $p < 0.001$, $F = 13.627$ for ELT; $p < 0.001$, $F = 3.679$ for quadrant time; $p < 0.001$, $F = 4.15$ for search error).

3.5. Localization and characterization of transplanted cells in mouse brain

All the mice were sacrificed after completing MWM trials and the brains were analyzed to investigate the fate of transplanted

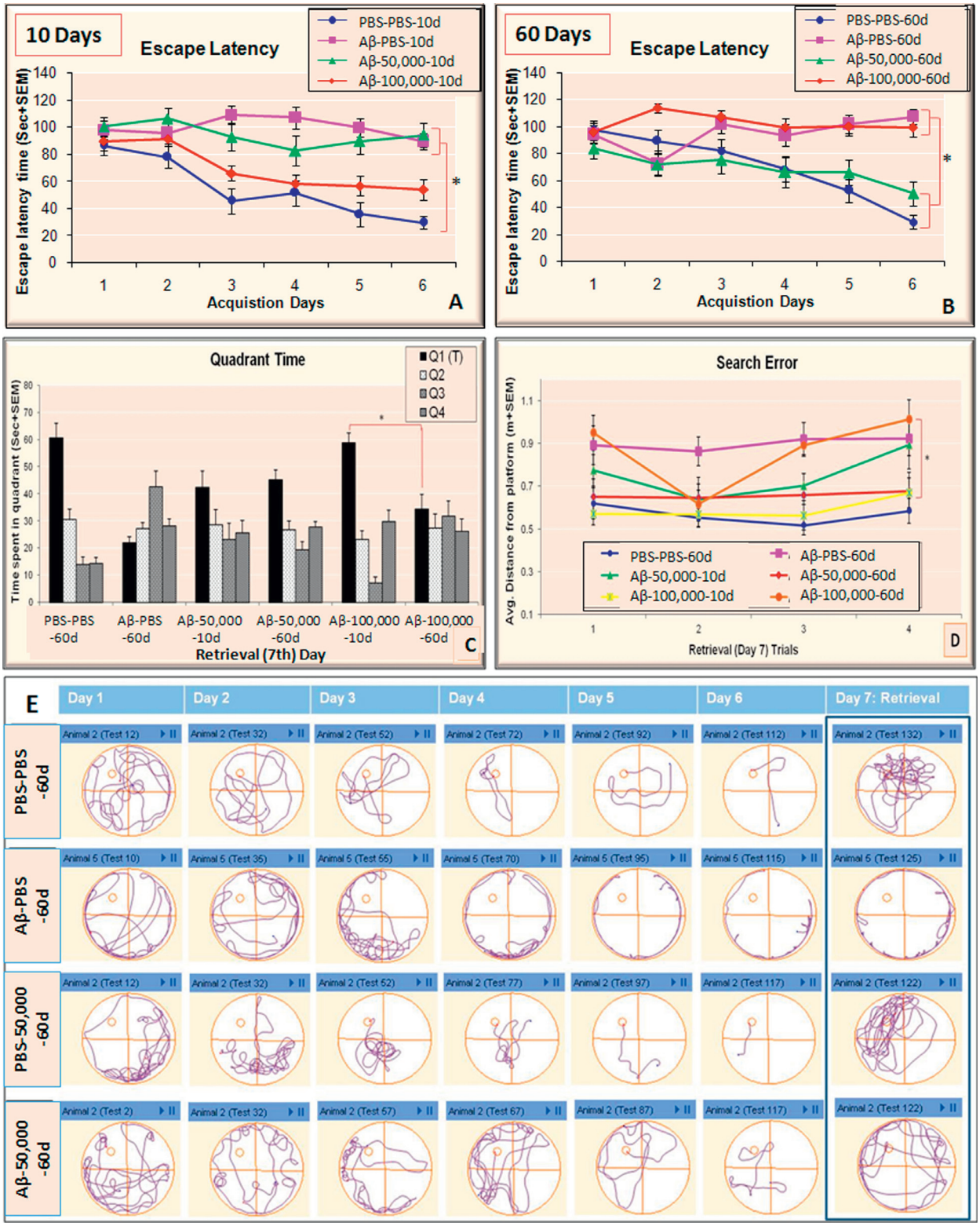

Fig. 4. *Morris water maze analysis of mice with Aβ injury and Lin −ve stem cell transplantation.* (A) Acquisition trials in 10 day group show impaired learning in Aβ-PBS—10 d mice marked by significantly higher escape latency time (ELT) as compared to PBS–PBS—10 d mice. There was no improvement in Aβ-50,000—10 d group. In Aβ-100,000—10 d group, mice showed significant recovery from day 1 to day 6 but it was not significantly improved when compared to Aβ injury alone. (B) Aβ induced cognitive impairment was evident in Aβ-PBS—60 d group. Aβ-50,000—60 d mice showed significant improvement in ELT, whereas there was no improvement shown by Aβ-100,000—60 d group. (C) In retrieval trials time spent in the target quadrant (Q1) was found to be significantly higher in Aβ-100,000—10 d groups. Aβ-50,000—10 d group also spent maximum time in Q1 but it was not significantly higher compared to Aβ injured group. (D) Reduced search error was observed in Aβ-50,000—60 d and Aβ-100,000—10 d, compared to their other cell dose groups showing time and dose dependent recovery of spatial memory. All results expressed as mean ± S.E.M. Data statistically analyzed using SPSS 16.0 by repeated-measures ANOVA with acquisition days as repeated measure. Other comparisons by LSD test. * $p < 0.001$. (E) Representative swimming track plots revealed that Aβ-PBS—60 d mice showed thigmotactic swimming pattern without decreasing their swimming track even after 6 days of acquisition. Length of swimming track was significantly reduced in Aβ-50,000—60 d group from day 1 to day 6. On day 7, when platform was removed, these mice spent maximum time in target quadrant similar to control PBS–PBS—60 d mice, showing the cognitive improvement after 60 days of 50,000 Lin −ve stem cells transplantation.

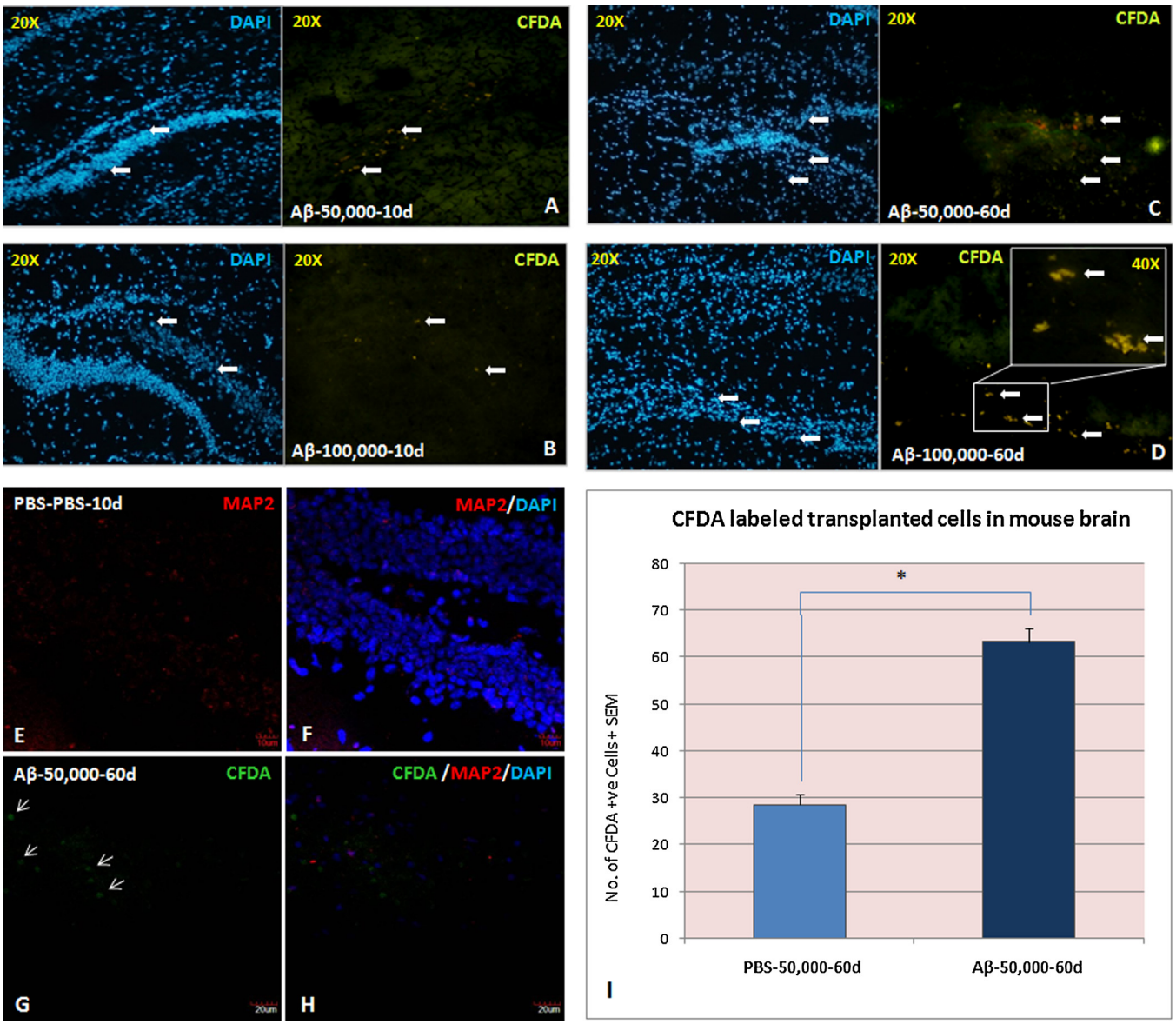

Fig. 5. *CFDA labeled transplanted Lin −ve stem cells in hippocampal sections of different stem cell transplanted groups.* (A) Aβ-50,000—10 d brain section showed presence of CFDA labeled Lin −ve cells in the dentate gyrus (DG) region. B) CFDA +ve cells were also identified in Aβ-100,000—10 d mouse hippocampus. (C) Aβ-50,000—10 d brain showed maximum numbers of CFDA +ve cells in DG layer and they were found to be incorporated along with the host cells. (D) In Aβ-100,000—60 d brain these cells were found to be aggregated in clusters of cells without being incorporated in the host tissue. All the corresponding sections (blue) left to individual group shows DAPI stained nucleuses. (E) MAP2 positive mouse neuronal cells (red) were identified in hippocampal layers in PBS–PBS—10 d (control) brain section. (F) Corresponding merged image with DAPI showing nuclear stain along with MAP2 markers. (G) CFDA +ve transplanted cells (green) were identified in Aβ-50,000—10 d mouse hippocampus. (H) Co-staining of MAP2 positive host neurons (red) and CFDA +ve transplanted cells (green) were identified in the brain section. (I) Quantitative analysis of CFDA +ve transplanted cells in brain sections. All results expressed as mean ± S.E.M. Data statistically analyzed using SPSS 16.0 by *T* test. * $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Lin −ve cells and their pattern of distribution in the brain tissue. A significant number of CFDA labeled Lin −ve cells were identified in Aβ-50,000—60 d mouse brains in comparison to PBS-50,000—60 d mice (Fig. 5I). The confocal imaging revealed that these CFDA labeled transplanted cells were found to be co-localized with the host cells (MAP2 +ve) in the layers of hippocampus (Fig. 5G and H). Transplanted cells were found to be aggregated in Aβ-100,000—60 d mouse brains, without being incorporated in the host hippocampus (Fig. 5D). Immunolocalization of different neuronal markers viz., Nestin, GFAP and O4 and their molecular expression analyzed by RT-PCR, in cell transplanted brains revealed that these cells were not differentiated even after 60 days of transplantation (data not shown). The results also showed that a large number of transplanted cells tend to form aggregates in the long term, in a diseased brain.

3.6. Down-regulation of apoptotic marker Fas-L in cell transplanted brain

To analyze the molecular basis of behavioral improvement noted in MWM, the mouse brain sections from different groups were immunostained for Fas-L apoptotic expression. Fas-L expression was found to be down regulated in Aβ-50,000—60 d mouse brain, showing remarkable reversal of attenuated Fas-L when

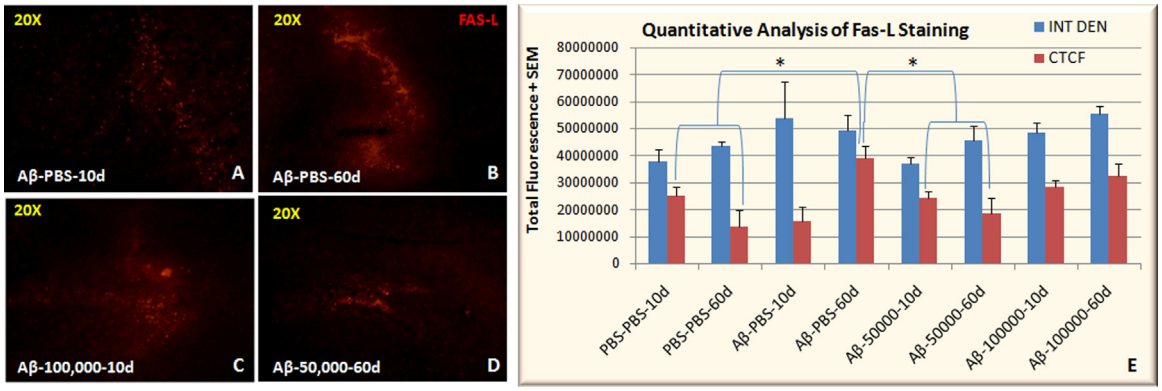

Fig. 6. *Expression of Fas-L in brain sections of representative Aβ injured and stem cell transplanted groups.* (A) Expression of Fas-L in Aβ-PBS—10 d group showing presence of apoptotic cells at the site of injection. (B) Higher Fas-L +ve apoptotic cells were observed in Aβ-PBS—60 d group. (C) This expression was not changed in Aβ-100,000—10 d group. (D) The expression was found to be significantly down-regulated in Aβ-50,000—60 d time point when compared to only Aβ injured mice. (E) Quantitative expression of total cell fluorescence from different groups showing down-regulated Fas-L expression in Aβ-50,000—60 d group. *Y* axis measures mean total fluorescence (INT DEN) and normalized fluorescence (CTCF) from multiple sections. Data was analyzed in ImageJ software using SPSS 16.0 by univariate ANOVA. Follow-up comparisons were made using LSD test. * $p \leq 0.05$, $F = 3.766$ for CTCF. INT DEN: integrated density; CTCF: corrected total cell fluorescence.

compared with Aβ-PBS—60 d brain sections. This expression was found to be relatively higher in Aβ-100,000—10 d group (Fig. 6).

3.7. Up-regulation of CREB and BDNF after transplantation of hUCB Lin −ve cells

Whole brain tissue from all the experimental groups was used for biochemical estimation of CREB and BDNF. The expression was estimated in all the brain homogenates using ELISA kits for CREB and BDNF and all the values were normalized to their corresponding total protein concentrations. The CREB expression was found to be significantly up-regulated in Aβ-50,000—60 d mouse brains as compared to Aβ-100,000—10 d or Aβ-100,000—60 d groups (Fig. 7A). BDNF expression was found to be unaltered in all the brain homogenates among different Aβ injured and cell transplanted groups (Fig. 7B) (df = 11; $p < 0.05$, $F = 3.077$ for CREB; $p = 0.67$, $F = 2.478$ for BDNF). Interestingly, when BDNF expression was analyzed at mRNA level, the molecular analysis showed its up-regulation in the Aβ-50,000—60 d mouse brains when compared to other stem cell transplanted groups (Fig. 7C) (df = 11, $p = 0.623$, $F = 0.831$).

4. Discussion

We have examined the effect of intra-hippocampal delivery of Aβ on mouse neuronal degeneration as evaluated by MWM analysis, immunostaining, molecular and biochemical expression. Transplantation of Lin −ve stem cells in these mice showed significant reversal of impaired spatial memory in a specific cell dose group. Aβ-50,000—60 d group showed best recovery from memory loss as compared to other groups with different cell doses and time intervals. The transplanted cells were found to be co-localized with the host tissue without being differentiated to neuronal lineage and also found to down-regulate the Fas-L mediated neural toxicity induced by Aβ delivery. Further, biochemical and molecular studies revealed that CREB and BDNF were also significantly up-regulated in Aβ-50,000—60 d mouse brains when compared to other cell transplanted groups.

The transgenic models of AD may not always carry the true pathology of disease as the target gene is induced under forceful mutation. Even the transgenic mouse models for AD do not exhibit the most common sporadic nature of the disease, rather represent rarer familial mutations. Overall, no animal model is able to reproduce disease onset, progression, and relapse, reminiscent of human AD. Hence we have chosen the Aβ induced neurotoxicity model which is the most common pathological features in AD. There are several groups demonstrating the toxic effect of different form of Aβ aggregates in mice leading to selective neuronal loss and cognitive impairment [37,38]. de Oliveira et al. have reported localized neuroinflammation, oxidative stress and neuronal loss followed by memory impairment in C57BL/6 mice upon acute intracerebroventricular injection of aggregated form of Aβ-40 peptide in the hippocampus [37].

Cell survival mechanisms are key to understand the pathophysiology for several neurodegenerative diseases. Therefore, we have validated the role of neurotrophins and apoptotic factors in short-term and long-term memories which are mediated by chemical changes inside the cells. These changes in neurons lead to secretion of a series of neurotransmitting factors (like acetyl choline, glutamate or Ca^{2+} etc.) in pre-synaptic spaces. These molecules act as messenger molecules which bind to the post-synaptic receptors of the neurons. Any disruption in neurotransmitter generation at pre synaptic neuron, binding to their receptors at post-synaptic neuron or degeneration of neurons can hamper the signal transmission from target tissue to the CNS or vice versa. Such attenuated signal transmission can retard the cognitive activity of an individual or experimental animal and may depend on BDNF, CREB or Fas-L. Impaired cognitive function being the common hallmark for AD is often ascribed to degeneration of neurons which may be induced by protein aggregates in extra-cellular matrix (ECM) like tau protein, Aβ-amyloid protein or oxidative stress. There are several strategies which have been applied to validate and screen potential therapeutic agents for the treatment of AD targeting disease pathology instead of symptomatic relief alone. In last two decades different cell replacement therapies were extensively tested on animal models in order to attenuate pathophysiological and behavioral burden of AD. Our study demonstrates, for the first time, that Lin −ve stem cells, one of the primitive population found in hUCB, can ameliorate AD related cognitive impairment and Aβ induced neuronal degeneration in a mouse model.

The increased immunoreactivity for Fas-L noted in Aβ injected brains suggests enhanced neuronal cell death accounting for learning deficits. Recently, it has been reported that Aβ induced neuronal apoptosis is mediated by increased ROS production [39]. Postmortem study in AD patients also revealed increased Fas expression and Fas-L immunoreactivity on reactive astrocytes in frontal and hippocampus region, especially in tangle-bearing and non-tangle-bearing neurons [40]. The 50,000 cell dose mediated downregulation of Fas-L after 60 days of transplantation in Aβ

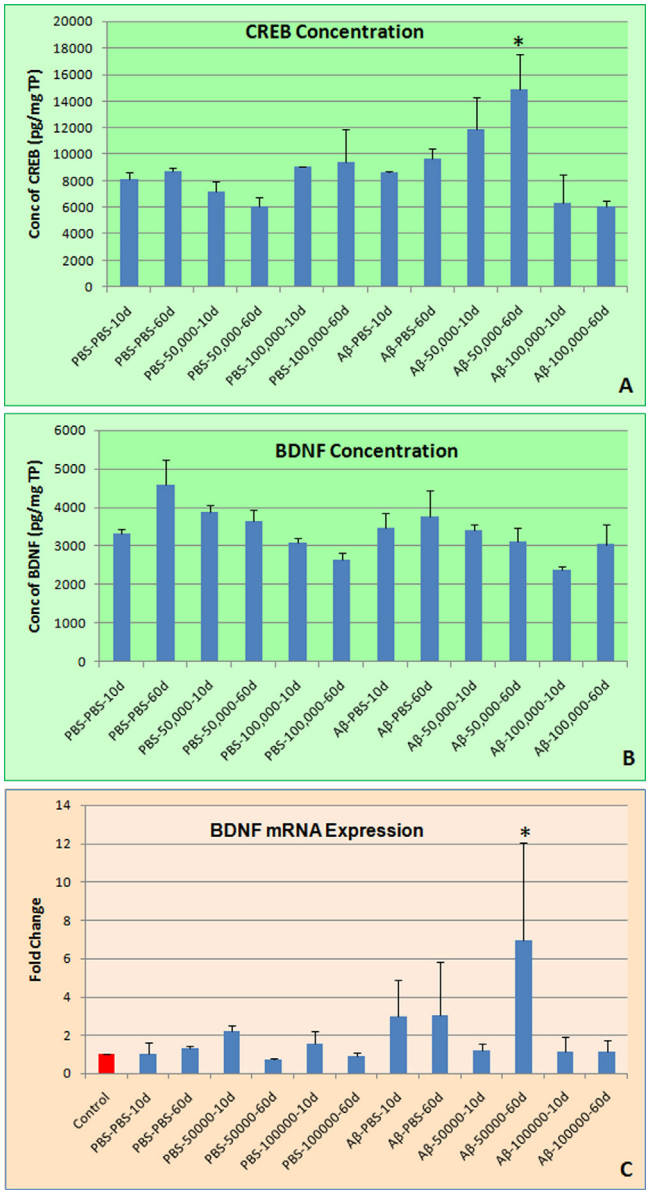

Fig. 7. *Biochemical (A and B) and molecular expression (C) of BDNF and CREB in Aβ injured and stem cell transplanted groups.* (A) CREB level was found to be significantly up-regulated in brain homogenates of Aβ-50,000—60 d group when compared to Aβ-100,000—10 d or Aβ-100,000—60 d groups. $p < 0.05$, $F = 3.077$. (B) No significant difference in BDNF level was found in brain homogenates among different cell dose groups. $p = 0.67$, $F = 2.478$. Absorbance measured in triplicate at 450 nm. All the values were normalized with their respective total protein concentration. (C) Significant increase in fold expression of BDNF mRNA in Aβ-50,000—60 d group was found as compared to other cell dose groups. $p = 0.623$ ($p < 0.05$ between Aβ-50,000—60 d and other Aβ-cell transplanted groups), $F = 0.831$. All the values were normalized with their respective β-actin expression. Data was analyzed using SPSS 16.0 by repeated measures ANOVA. Follow-up comparisons were made using LSD test.

injured brain suggests that these transplanted cells offer neuroprotection to host cells undergoing apoptosis augmenting reversal of cognitive impairment. In a similar study, intra-hippocampal transplantation of bone marrow derived mesenchymal stem cells, when transplanted in Aβ induced AD model, showed cognitive improvement by inhibiting oxidative stress induced apoptosis in mouse hippocampus [41]. In intracerebroventricular transplantation studies it is also evident that needle injury due to stereotaxic injection may induce damage of neuronal cells and also attract the transplanted cells along the needle tract surrounding the site of injury [11]. Therefore, it is pertinent to mention that needle induced cell death should be also considered in pathological outcomes of these cell transplantation studies. Hence we have included two control groups viz., PBS injected and Stem cell injected group in our study without exposing them to Aβ injury. These mice did not show any significant behavioral changes due to needle injury in MWM when compared to absolute healthy control (data compared with control group from our previously published study) [42]. In our study the Aβ load was found to be unaltered in all the stem cell transplanted groups even after 60 days. It is postulated that these transplanted cells do not play a redundant role in cell survival either mediated by alleviation of ROS production or other neuromodulatory mechanisms which are independent of Aβ dissolution. It is possible that sequestration of soluble Aβ results in improved cognitive decline. In this context it is compelling to refer to a study which showed that Aβ immunization in a transgenic AD model led to amelioration of memory deficits without altering the Aβ burden proposing that this must have been mediated by sequestration of soluble forms of Aβ peptides, something we did not examine in this study [43].

The role of CREB, however, remains well documented in the formation of spatial memory and its consolidation into long term memory [44]. It is one of the important cellular transcription factors regulating the expression of several genes like, c-fos, tyrosine hydroxylase, several neuronal peptides and importantly, neurotrophin BDNF which is involved in the mammalian memory mechanisms [45]. These gene expression profiles are activated through CREB, mediated by Ca^{2+}, protein kinase A (PKA) and activation of cAMP [46–48]. Studies show that up-regulation of CREB enhances both short-term and long-term memory in mice [49]. Therefore, enhanced CREB expression in 50,000 cell transplanted group suggests its non redundant role in behavioral improvement confirming it as an important target for cell replacement therapy. The role of BDNF is also well described in AD literature. Several studies have demonstrated the neuroprotective effect of BDNF in Aβ induced neurotoxicity in cultured neurons [50–53]. Enhanced BDNF expression was found to be protective against apoptotic cells in APP/PS1 double transgenic mouse model of AD [54]. Even in the postmortem brains of AD patients, BDNF levels were found to be decreased when compared to the healthy controls [55–58]. From these findings, the therapeutic role of BDNF in rescuing the spatial memory in Aβ injured mice becomes worth further investigation. As CREB is a known factor for regulation of BDNF expression in mammalian brain, it is, therefore, possible that up-regulation of BDNF and CREB activity cooperatively enhances learning and retrieval [59–61].

Our results reveal that Aβ injury alone did not alter the levels of BDNF and CREB in mouse brains but in Aβ-50,000—60 d brains their levels were found to be upregulated when compared to PBS injected control brains. Interestingly, this upregulation in BDNF and CREB was not found in stem cell transplanted brain without Aβ injury (PBS-50,000—10 d and PBS-50,000—60 d groups). Our data illustrates that Aβ injury alone could not exert any effect on the expression level of BDNF and CREB but there could be a possibility that this injury stimulates the transplanted cells in a manner to upregulate BDNF and CREB in cell transplanted brains. Further, these cells could not exert similar effect when transplanted alone in mouse brains without any injury. Hence it is relevant to mention that injury play a critical role to facilitate some paracrine effects induced by the transplanted stem cells in a disease brain. Moreover, it is reported earlier that decreased levels of neurotrophins after traumatic brain injury in rat go back to their normal level (compared to sham control) after 24 h of injury in their cortical neurons [62]. As we estimate the levels of BDNF and CREB after 31 and 81 days of injury, it could be possible that the early effects of Aβ injury on these neurotrophins might have stabilized over time.

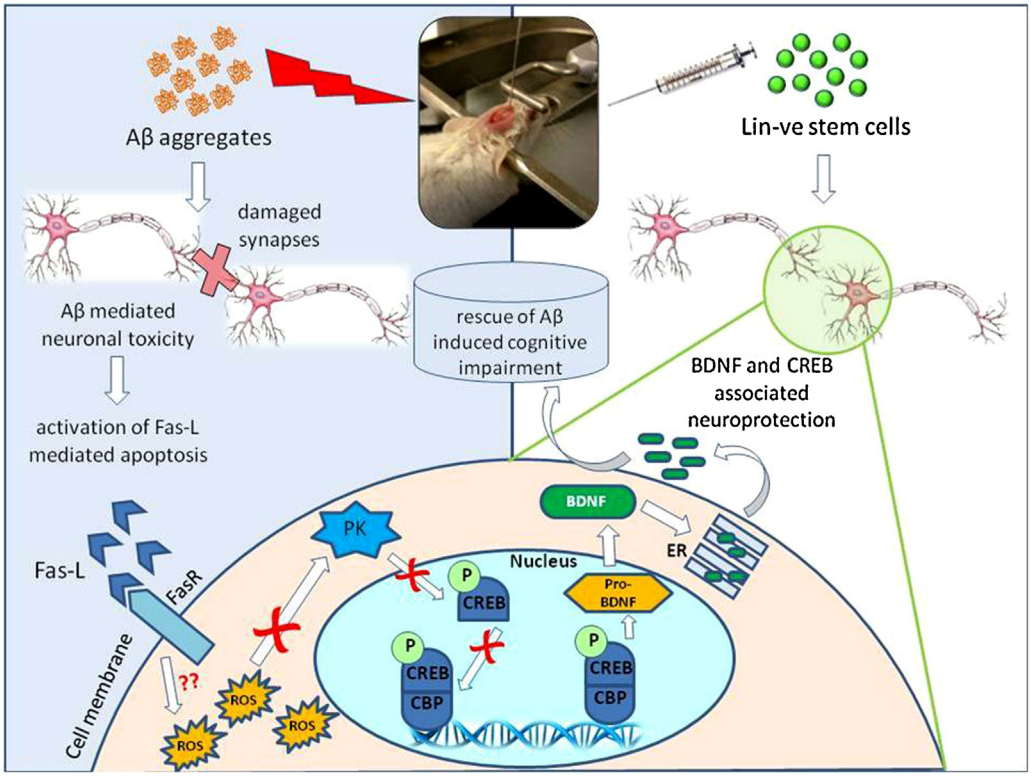

Fig. 8. Proposed mechanism of action in Lin −ve stem cell mediated reversal of Aβ induced neuronal cell death and cognitive impairment.

On the other hand, in water maze task, lower dose of cells (50,000) was ineffective at 10 days but effective at 60 days post-transplantation, whereas this effect was found to be reversed in higher dose (100,000). The inability of 50,000 cells at 10 days and 100,000 cells at 60 days to improve spatial memory could be accounted for unaltered expression of BDNF and CREB levels. Why these doses fail to restore lost memories raises additional research questions which we are unable to explain. However, it can be concluded that the optimal effect of stem cells remain highly dependent on dose and time of transplantation which needs to be carefully factored in planning additional experimental studies. In this respect it is pertinent to cite Darsalia et al. study which showed the effect of number and time of transplanted cells which triggers their survival in stroke induced rat brains. This is mediated by differential cell migration magnitude, neuronal differentiation and cell proliferation in the grafted region. The higher number of transplanted cells did not show greater cell survival or neuronal differentiation [63]. We propose that lower dose of cells could not sufficiently mount a therapeutic effect at an early time point which a higher dose of cells could achieve whereas higher number of transplanted cells possibly form aggregates at later time point which is not the case in lower cell dose. Likewise, higher cell dose at later time point possibly provoke immunomodulatory response, as these cells are derived from human origin, rendering them ineffective in the rescue of Aβ induced neurodegeneration, which the lower dose of cells are protected from.

Regardless of the absence of convincing evidence underlying the mechanisms of rescue of memory deficits, the study unambiguously reveals that Lin −ve stem cells, consisting of endothelial progenitor cells, isolated from hUCB have potential to reverse Aβ induced cognitive impairment through a neuroprotective mechanism mediated by CREB and BDNF (Fig. 8). Furthermore, our study also strengthens the need to explore other sub sets of hUCB besides examining the preclinical effects of cryo-preserved cord blood. Additional approaches may include investigating the effect of other doses, routes and cell type comparisons with other non hUCB cell types with varying severity of injuries. Future studies may focus not only on associated functional molecular links of hippocampal BDNF and CREB, resulting from hUCB transplantation but also the effects of their supplementation on cognitive improvement, using knock out animals and si RNA approach.

Author contributions

Writing and editing of manuscript was done by Avijit Banik and Akshay Anand. Conceptualization of the project was done by Akshay Anand and data acquisition was done by Avijit Banik. UCB samples were taken in cooperation with Jasvinder Kalra and the work was co-supervised by Sudesh Prabhakar.

Conflict of interest statement

The authors declare no competing financial interests and compliance to ICMJE guidelines. The corresponding author declares that he had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis as well as the decision to submit for publication.

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An Enriched Population of CD45, CD34 and CD117 Stem Cells in Human Umbilical Cord Blood for Potential Therapeutic Regenerative Strategies

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Abstract: Human umbilical cord blood (hUCB) is a primitive source of stem cells and is being banked worldwide for the purpose of future clinical use. Lineage negative (Lin-ve) stem cells are one of the primitive populations in hUCB which are capable of self-renewal and differentiation into multiple lineages. These cells have not been adequately investigated for their stem cell characteristics. We have analyzed the immunophenotypic expression of CD45, CD34 and CD117 markers on three different cell populations in hUCB viz., mononucleated cells (MNCs), Lineage positive (Lin+ve) and Lin-ve cells. The CD34 and CD117 expression was found to be 60 fold more in the Lin-ve population as compared to MNCs and Lin+ve cells. This expression was further enhanced in CD45+Lin-ve fraction. The CD34+ and CD117+ Lin-ve cells showed 99% positivity for CD45. Our data suggest that CD34+ CD117+ Lin-ve cells are reasonably enriched stem cell population in hUCB which can be clinically used for transplantation and other therapeutic purposes.

Keywords: CD117, CD34, Haematopoietic, Lineage, Stem cell, Umbilical cord blood.

INTRODUCTION

The first report of successful transplantation of human umbilical cord blood (UCB) cells in a child with Fanconi anemia in 1988 has shaped the field of UCB research and subsequent clinical applications [1]. The vast clinical potential of UCB derived stem cells has paved the way for UCB banking services worldwide, where UCB cells are cryopreserved for future therapeutic applications. In 1991 the first public UCB bank was established in New York. Since then there have been more than 150 UCB banks operating worldwide with a repository of more than 500,000 stored UCB units and over 25,000 samples used for successful transplantations. In India, presently, 3 public banks and 7 private banks are functional and store over 45,000 UCB units, promising unverified therapies to Indians. This industry is slowly growing into a powerful form of experimental therapies converting India into a favourable market place being one of the world's largest populations [2, 3]. It is, therefore, imperative that UCB cells are studied not only to test their clinical efficacy but also to perform comprehensive cellular characterization in order to understand the molecular phenotypes in UCB cell population.

Umbilical Cord Blood (UCB) derived stem cells represent the intermediate stage, because they are neither embryonic, isolated after nine months of fertilization, nor of adult origin. These cells are abundant in number due to the ever increasing birth rate in the developing world and also the easy non-invasive method of collection. Clinical significance of UCB derived stem cells has increased enormously in the last few decades since its first application to successfully reconstitute the hematopoietic system of a 5 years old child suffering from Fanconi Anemia [1]. Since then UCB derived stem cells were extensively tested in several human disorders and their corresponding animal models such as leukemia [4], ischemic stroke [5], spinal cord injury [6], amyotrophic lateral sclerosis [7], Alzheimer's disease [8] as well as other neurological disorders [9] but their comprehensive characterization was lacking.

UCB is similar to the blood being composed of RBCs, WBCs, platelets, plasma etc. The mononuclear cell fraction (MNCs) in UCB contains WBCs, the progenitor cells and the stem cells of various lineages including hematopoetic, mesenchymal and endothelial cells [10, 11]. The differentiation capacity of these UCB derived cells into neuronal and other lineages is widely investigated. The mesenchymal lineages in hUCB are capable of differentiating into nerve cells *in-vivo* in a spinal cord injury model [12]. Human umbilical vein endothelial cells (HUVECs) were found to be differentiated into skin substitute when cultured with derml fibroblast [13]. The differentiation capacity of human umbilical cord derived mesenchymal stem cells was also demonstrated in-vivo when they were shown to be differentiated into epithelial cells [14]. Even the mammalian ciliary epithelium derived cells are capable of *in-vitro* proliferation in the presence of vascular endothelial growth factor (VEGF) showing their apposition with endothelial cells [15]. There is also a rare fraction of primitive stem cells termed as very small embryonic-like stem cells identified in human UCB. Although these cells are unable to self renew in culture condition, they are well defined for embryonic like pluripotency [16]. Unrestricted Somatic Stem Cells (USSC) is another rare population of the UCB, which can be induced

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into cells representing all the three germinal layers [17]. The most primitive and naïve population in UCB consists of lineage negative (lin-ve) cells which are not committed to any of the lineages and do not express any of the lineage specific or mature hematopoietic lineage markers such as CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and CD235a (glycophorin A) [18]. These cells are found to be discrete in UCB comprising only 0.1% population of UCB MNCs [19]. Presence of these primitive cell populations is also evaluated in peripheral blood [20] as well as in bone marrow cell fractions [21]. The lin-ve fraction in mouse bone marrow possesses higher percentage of CD34 and CD117 positive cells showing that most of these cells express stem cell progenitor markers [22].

It is widely believed that the hematopoietic as well the endothelial cells originate from the same precursor termed as hemangioblast and hence feature similar characteristics. CD45, being the common leukocyte marker or hematopoietic progenitor marker has been part of raging debate over its expression pattern on hematopoietic and endothelial cells populations [23, 24]. Ciraci et al. added further to this confusion when they showed that CD34-Lin-CD45-CD133- cells from human peripheral blood could successfully differentiate into both hematopoietic and endothelial lineage *in-vitro* and *in-vivo* in NOD/SCID mice [25]. Since a long time CD34 expression has been considered as the most reliable positive selection marker for the hematopoetic stem and progenitor cells [19] and the endothelial progenitor cells [18]. Later, it was proposed that the cells expressing the CD133 marker are more primitive and therefore suited to transplantation. A $CD133^+$ population has been analysed and was found not to express CD34. These cells which, lack the CD34 surface glycoprotein, were further analyzed and found to contain the most primitive Lin-ve cells. These lin-ve and $CD34^-$ cells manifest sustained and long term haematopoiesis in the immune-compromised animal models [26]. However, the $CD34^+$ and lin-ve population in UCB was found to have extensive proliferating capacity in culture condition [27] and also successful engraftment and differentiation in multiple organs upon transplantation in mammal [28]. CD117 (c-Kit), another hematopoietic progenitor marker, plays a critical role in stem cell survival, proliferation, and their differentiation. It is found to be expressed on stem cells from both UCB and bone marrow [29]. These $CD117^+$ cells in Lin^- population from bone marrow have shown differentiation properties towards lymphoid lineage in culture condition [30]. Another study showed that bone marrow derived CD117+ cells could successfully differentiate into osteoclasts and other immune cells *in-vitro* [31]. Even the therapeutic role of these cells was evaluated in pneumonectomised mice to show their mobilization and recruitment efficiency in faster wound healing [32]. Unfortunately, these cells are not adequately validated in UCB population for their characteristic features as well as therapeutic potentials. Overall, there is no current consensus about the number of pure stem cells present in the lin-ve population from UCB.

Hence, in this study we attempted to quantitate the stem cell population in human UCB samples. We enriched the lin-ve population from full term UCB samples (n=8) by immunomagnetic cell sorting and further evaluated the presence of CD45 (nucleated hematopoietic cell marker), CD34 and CD117 (c-Kit) (stem cell markers) through standard flowcytometry protocol. Three different cell populations from UCB such as (i) density gradient separated mononucleated cells (MNCs), (ii) Lin positive cells (Lin+ve) and (iii) Lin negative cells (Lin-ve) were compared for the expression of these cell markers.

METHODOLOGY:

Collection of Umbilical Cord Blood

UCB samples were collected from the full term deliveries after proper informed consents were obtained from the parents under approval of the ethical guidelines by the Institutional Committee for Stem Cell Research and Therapy (Approval No. IC-SCRT/10/DTM-3272). Eight pregnant women were randomly selected for the study. They were aged between 20-35 years, at gestation period of ≥ 28 weeks. All the donors were screened negative for Hepatitis B, Syphilis, HIV and urinary tract infection and congenital malformation in the new born. The blood samples were collected immediately post delivery from the umbilical cord and placental vessels using a 21G syringe and transferred into a sterile tube containing EDTA (Sigma-Aldrich). The blood samples were thoroughly mixed with EDTA and transported in ice to the processing laboratory.

Isolation of mononucleated cells (MNCs)

MNCs were isolated from UCB by density gradient separation method. Equal volume of UCB was carefully layered on top of the histopaque (Sigma-Aldrich) solution without mixing both of them and centrifuged at 3000 rpm at room temperature in a swing bucket centrifuge (Remi, India). MNCs were separated at the interface of histopaque and plasma while RBCs and platelets were deposited at the bottom of the tube. MNCs were carefully removed using a micropipette without disturbing other layers and washed twice with 1X PBS.

Isolation of Lin negative (Lin-ve) cells

Lin-ve cells were isolated from density gradient separated MNC population in a magnetic associated cell sorter (MACS) (Miltenyi Biotech, Germany) using human Lin-ve isolation kit (Miltenyi Biotech, Germany). The MNCs were incubated with a cocktail of biotin conjugated Lin+ve markers such as CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123 and CD235a (glycophorin A). Further, these were incubated with Anti-biotin microbeads and passed through the MACS column fixed in the magnetic field. The cells positive for Lin+ve markers were trapped in the MACS column. Only the Lin-ve cells could pass through the column and collected at the bottom. Finally, these cells were washed and suspended in 1X PBS for further analysis.

Isolation of Lin Positive (Lin+ve) Cells

The trapped Lin+ve cell fractions were isolated from the MACS column after removing it from the magnetic field.

Table 1. List of antibodies used for flow-cytometric characterization of hUCB cells. The antibodies, their specific clones used, fluorochrome attached and working concentration are listed in the table.

Antibodies Used	Clone	Fluorochrome	Make	Product No.	Stock Concentration	Working Concentration
CD45, Mouse anti-Human	HI30	FITC	BD Pharmingen	555482	NA	20 µl/ 10^6 Cells
CD34, Mouse anti-Human	581	PE	BD Pharmingen	555822	NA	20 µl/ 10^6 Cells
CD117, Mouse anti-Human	104D2	PE	BD Pharmingen	340529	10 µg/ml	20 µl/ 10^6 Cells

Table 2. Detailed depiction of CD34 and CD117 expressions in individual UCB samples. The expression of CD34+ and CD117+ cells in SSC/FSC gated cells and CD45+ cells are individually listed in 3 different cell population (MNCs, Lin+ve and Lin-ve) from 8 different UCB samples.

Samples	MNCs				Lin+ve				Lin-ve			
	% Expression of CD34		% Expression of CD117		% Expression of CD34		% Expression of CD117		% Expression of CD34		% Expression of CD117	
	Out of Total Cells	Out of CD45+ Cells	Out of Total Cells	Out of CD45+ Cells	Out of Total Cells	Out of CD45+ Cells	Out of Total Cells	Out of CD45+ Cells	Out of Total Cells	Out of CD45+ Cells	Out of Total Cells	Out of CD45+ Cells
UCB-1	0.9	1	0.4	0.5	0.8	0.7	0.1	0.1	28.9	42.2	32.5	50.2
UCB-2	0.4	0.4	0.5	1.2	0.2	0.5	0.6	1.1	13	44.6	20.4	58.8
UCB-3	0.8	1.5	1.1	2.9	1.4	0.3	1.7	1.8	80.3	85.9	84.5	87.5
UCB-4	0.9	1	0.9	0.9	0.3	0.3	0.6	0.5	85.5	84.1	85	70.9
UCB-5	0.4	1.1	0.1	0.1	0.3	1.2	0.1	0.1	52.9	77	63.8	85.1
UCB-6	0.4	1.2	0.7	1.6	0.3	0.4	0.9	0.7	61	85.1	71.7	89
UCB-7	0.3	1.1	0.5	1.6	1	0.2	0.6	0.9	61	63.8	77.7	83.5
UCB-8	2.1	1.2	0.4	2.2	1.2	0.8	0.6	2	65.4	64.5	71.3	80.5
Average	0.77	1.06	0.57	1.37	0.68	0.55	0.65	0.9	56	68.4	63.3	75.6
SE	0.20	0.11	0.11	0.32	0.16	0.11	0.17	0.25	8.65	6.26	8.50	5.08

A volume of 1X PBS was forced through the column using a plunger and the Lin+ve cells were collected in a tube at the bottom of the column. Finally, these cells were washed and suspended in 1X PBS for further analysis.

Analysis of Cell Number and Size

All the three cell populations viz., MNCs, Lin+ve and Lin-ve cells were analyzed using an automated cell counter (Millipore, USA) for their absolute counts and diameters. Cells were suspended in 100 µl PBS and drawn into the counter through 60 µm sensor tip. Data were analyzed using Scepter 2.0 software (Milipore, USA).

Flowcytometry Analysis

All the three cell population were analyzed in flowcytometry for the presence of stem cell markers viz., CD45, CD34 and CD117. Approximately one million of each cell population was suspended in 100 µl FACS buffer (PBS-BSA-Azide solution). Cells were initially incubated with Fc blocking reagent (20 µl for upto 10^7 cells) (Miltenyi Biotech, USA) for 30 min at RT. Then they were incubated with fluorochrome conjugated Antibodies (Conc: 20 µl/10^6 cells) (BD Pharmingen, USA) for 1 hour at RT. Further, all the tubes were washed twice with FACS buffer and finally resuspended in 300 µL of buffer and processed in FACSCalibur (BD Bioscience, USA) within 2-6 hrs of antibody labelling. Details of antibodies used for flowcytometry are elaborated in Table **1**.

Statistical Analysis

All the results were expressed as mean±S.E.M. Data were statistically analyzed using SPSS 16.0. One-way ANOVA was used to compare the expression levels of different markers in all the three cell populations. LSD test was used for post-hoc analysis. P values were considered ≤ 0.05 as statistically significant.

Sample ID	UCB 3 Lin-ve Cells	UCB 3 Lin+ve Cells
Sensor	60 µm	60 µm
Current	320 µA	320 µA
Test Time	11.05 sec	9.44 sec
Cell Count	3.824 x 10^5 /ml	5.165 x 10^5 /ml
Lowest Diameter	6.00 µm	6.00 µm
Highest Diameter	14.04 µm	14.96 µm
Mean cell diameter	8.73 µm	11.40 µm

A

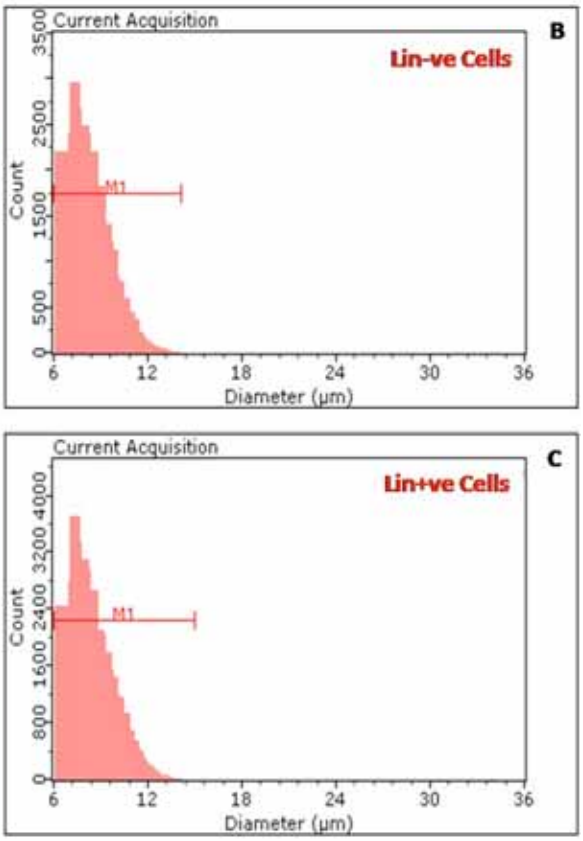

Fig. (1). Cell counter analysis of Lin-ve and Lin+ve cells isolated from one of the UCB samples. A) Table shows characteristic features of analyzed Lin-ve and Lin+ve cells isolated from hUCB showing average diameter, range of diameter and their cell count. The Lin-ve cells were found to be smaller in size (diameter: 8.73 µm) compared to Lin+ve cells (diameter: 11.4 µm). B) Histogram shows distribution of Lin-ve cells from 6-14 µm based on their diameter. C) Histogram shows distribution of Lin+ve cells from 6-15 µm based on their diameter.

RESULTS

Lin-ve Cells are Smaller in Size Compared to Lin+ve Cells

The cells analyzed in automated cell counter revealed that Lin-ve cells are smaller in size (mean cell diameter 8.73 µm) as compared to the Lin+ve counterpart (mean cell diameter 11.40 µm) (Fig. **1A**). Although Lin-ve cell diameter varies from 6-14 µm, yet most of the cells were found to be ranging towards reduced diameter in the histogram (Fig. **1B**). For Lin+ve cells, most of them were found to be distributed towards higher diameter (Fig. **1C**).

Lin-ve Cells Express Higher Percentage of CD34 Markers

Three different cell populations in hUCB viz., MNCs, Lin+ve and Lin-ve cells were analyzed in flowcytometer to examine the expression of stem cell markers on them. CD34, one of the important early hematopoietic lineage markers, was found to be expressed in very low percentage (<1%) both in MNCs as well as Lin+ve fraction. This expression was found to be increased by 60 fold in the Lin-ve population (56 ± 8.65%) (Fig. **2**). When the CD34 expression was evaluated in CD45+ gated cells from all the three cell fractions, the percentage expression was found to be enhanced by 68.4 ± 6.26% in Lin-ve cells. The CD45+/CD34+ expression in MNCs (1.06 ± 0.11%) and Lin+ve cells (0.55 ± 0.11%) remained similar to gated cells alone (Fig. **3**).

Lin-ve Cells Express Higher Percentage of CD117 Markers

When these cells were further analyzed for the expression of CD117, a stem cell marker on early hematopoietic progenitors, we observed that Lin-ve cells expressed higher proportion of CD117 on their surface as compared to MNCs and Lin+ve cells (Fig. **4**, **5**). This expression was found to be 63.3 ± 8.5% among the gated Lin-ve cells (Fig. **4D**) and further increased to 75.6 ± 5.06% in the CD45+ Lin-ve cells (Fig. **5D**), whereas a very small fraction of cells (0.5-1.5%) was found to be positive for CD117 in MNCs and Lin+ve cells (Fig. **4D**, **5D**).

CD34+ and CD117+ UCB Cells Show Positivity for CD45 Markers

We also evaluated the presence of CD45+ cells in CD34 and CD117 positive population in one of the UBC samples. When analyzed in flowcytometer, almost all the CD34+ and CD117+ cells showed positivity for CD45 marker in all the three cell populations (Fig. **6**). This expression was found to

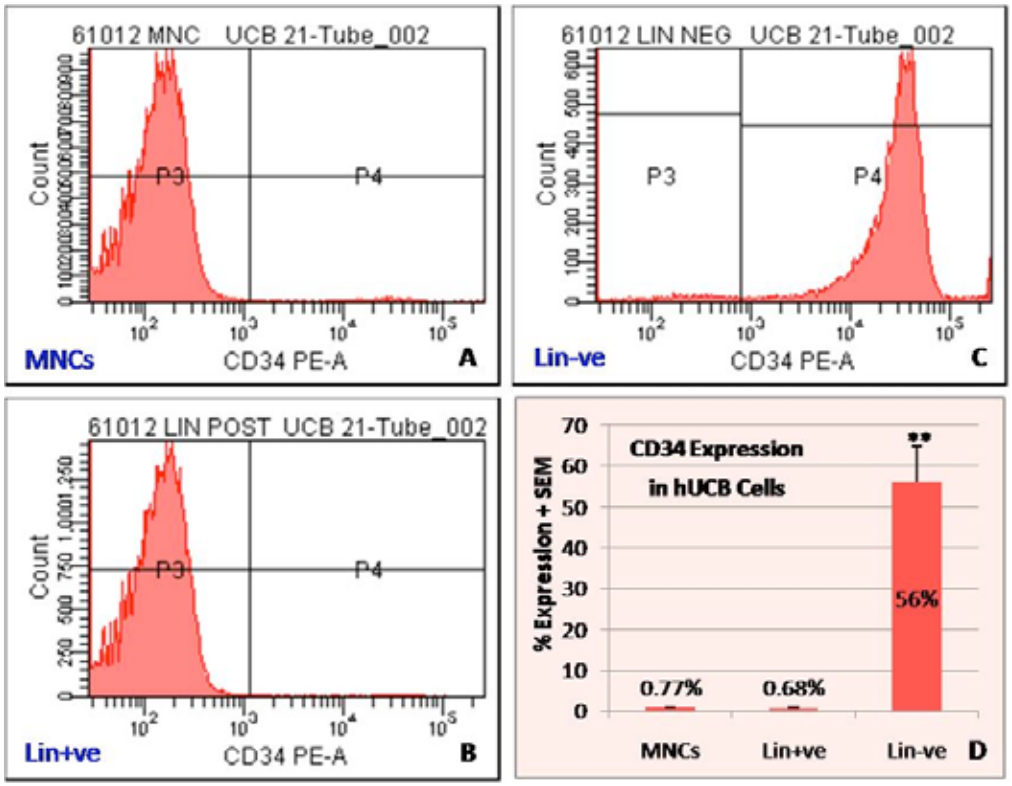

Fig. (2). Expression of CD34 in three different cell populations in hUCB. A,B,C) Histograms show that CD34 expression was significantly increased in Lin-ve cells as compared to Lin+ve and MNC population. D) This percentage was found to be 56% in Lin-ve population, 0.77% in MNCs and 0.68% in Lin+ve cells. Data analyzed by SPSS 16.0 using one-way ANOVA and LSD test was used for post-hoc analysis. n = 8, **p <0.001.

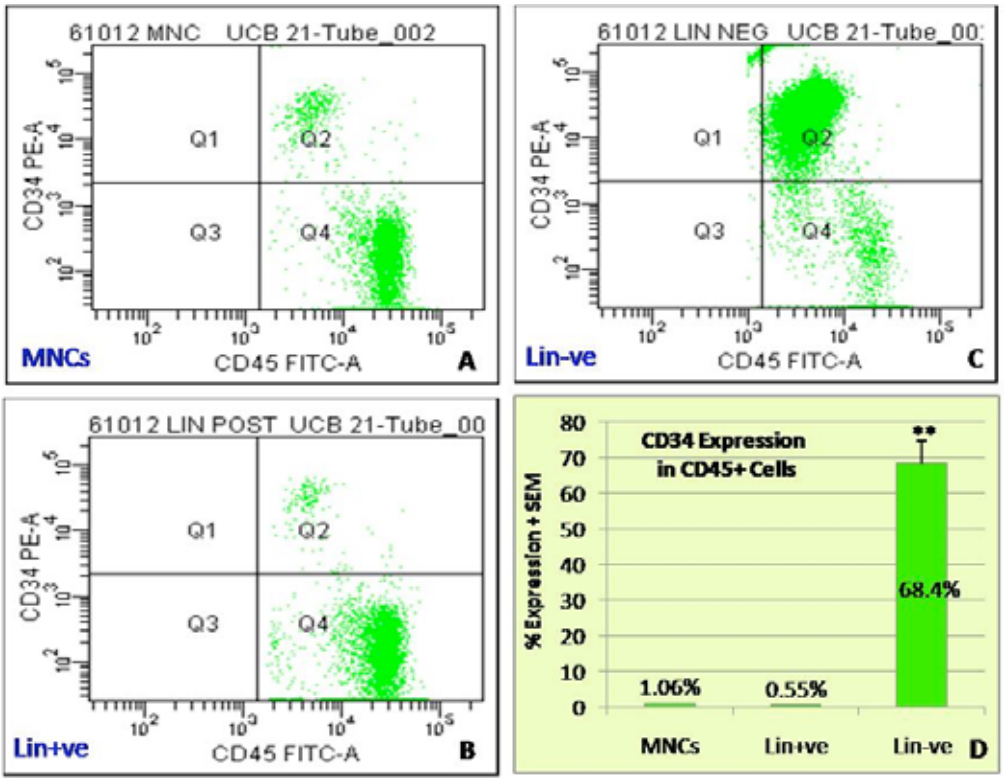

Fig. (3). Expression of CD34 in CD45+ cells in three different populations in hUCB. A,B,C) Dot plots show significantly higher percentage of CD34 expression out of CD45+ cells in Lin-ve cells as compared to Lin+ve and MNC population. D) This percentage was found to be 68.4% in Lin-ve population, 1.06% in MNCs and 0.55% in Lin+ve cells. Data analyzed by SPSS 16.0 using one-way ANOVA and LSD test was used for post-hoc analysis. n = 8, **p <0.001.

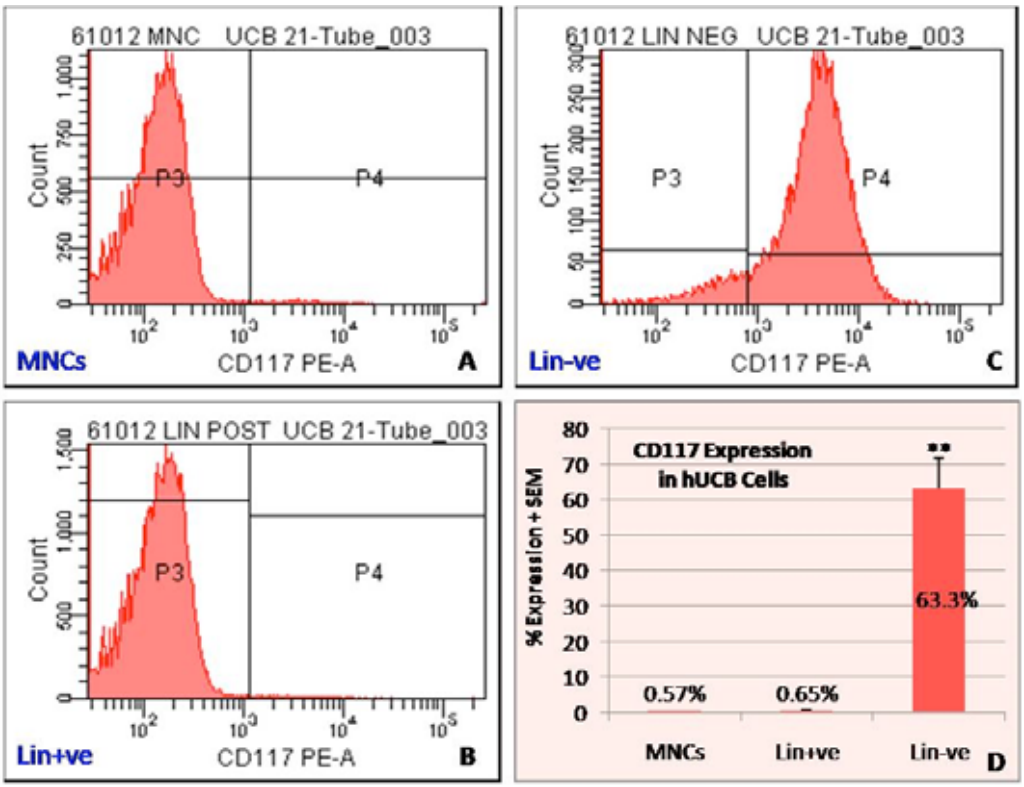

Fig. (4). Expression of CD117 in three different cell populations in hUCB. A,B,C) Histograms show that CD117 expression was significantly increased in Lin-ve cells as compared to Lin+ve and MNC population. D) This percentage was found to be 63.3% in Lin-ve population, 0.57% in MNCs and 0.65% in Lin+ve cells. Data analyzed by SPSS 16.0 using one-way ANOVA and LSD test was used for post-hoc analysis. n = 8, **p <0.001.

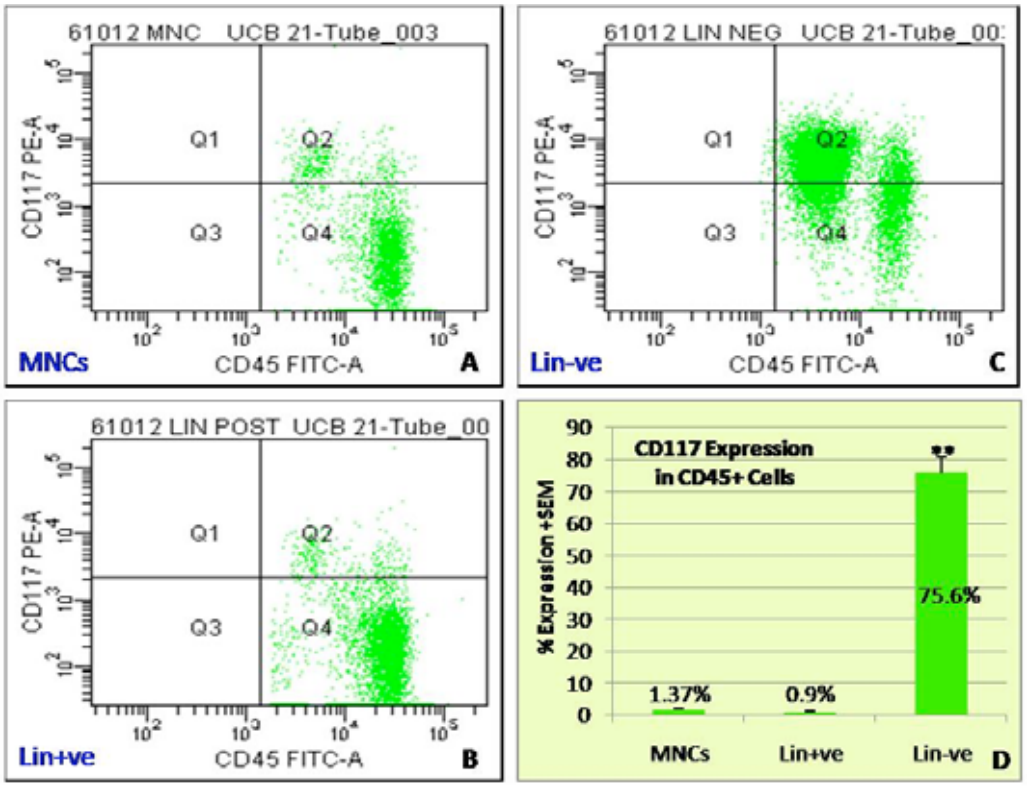

Fig. (5). Expression of CD117 in CD45+ cells in three different cell populations in hUCB. A,B,C) Dot plots show significantly higher percentage of CD34 expression out of CD45+ cells in Lin-ve cells as compared to Lin+ve and MNC population. D) This percentage was found to be 75.6% in Lin-ve population, 1.37% in MNCs and 0.9% in Lin+ve cells. Data analyzed by SPSS 16.0 using one-way ANOVA and LSD test was used for post-hoc analysis. n = 8, **p <0.001.

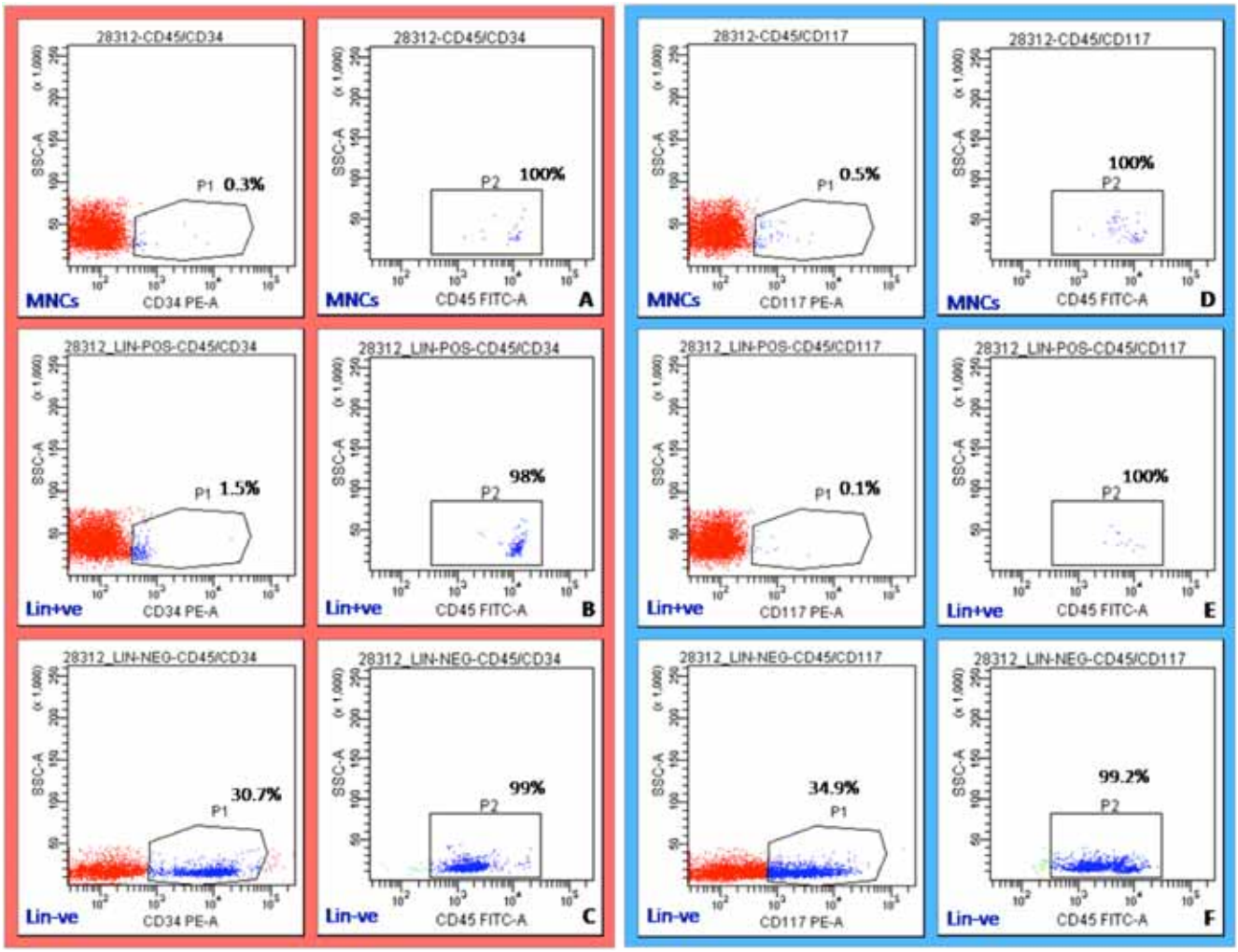

Fig. (6). CD45 expression in CD34+ and CD117+ cells. A,B,C) CD45 expression in CD34+ cells in MNCs, Lin+ve and Lin-ve populations was found to be 100%, 98% and 99% respectively. D,E,F) CD45 expression in CD117+ cells in MNCs, Lin+ve and Lin-ve populations was found to be 100%, 100% and 99.2% respectively.

be 100% for MNCs in both CD34 and CD117 positive cells (Fig. **6A**, **D**). The CD34+ and CD117+ Lin+ve cells showed 98% and 100% positivity for CD45 respectively (Fig. **6B**, **E**). In Lin-ve cells, these proportions were found to be 99% and 99.2% for CD34+ and CD117+ cells, respectively (Fig. **6C**, **F**).

DISCUSSION

Human UCB derived stem cells hold enormous therapeutic potential as cell replacement strategy for neurodegenerative disorders. The hematopoietic stem cells isolated from hUCB are used for a range of haematological disorders. The CD34 counts are routinely projected as universal standard for UCB transplantation studies. The CD34 is also believed to represent the absolute stem cell population in a sample of UCB before storing it in cord blood banks. Hence, the stem cell marker analysis is an important step for characterization of UCB samples before proposing clinical applications.

Lin-ve cells present in hUCB provide a potential source of multipotent stem cells which can be tested for their efficacy in cell based therapy not only for haematological disorders but also many neurodegenerative disorders. These cells are the most primitive population in UCB excluding committed cells and hence considered capable of regenerating or differentiating into a desired cell type when transplanted into a diseased model. Koike-Kiriyama et al. demonstrated the differentiation capacity of hUCB derived Lin-ve cells into neuronal lineage when transplanted in the subretinal layer of SCID mice. Two weeks post transplantation, these cells expressed a series of neuronal markers such as nestin, MAP2, beta-III tubulin and rhodopsin [33]. Hence, these cells possess therapeutic potential necessitating thorough characterization for the presence and distribution of several stem cell markers on them.

In this study, we have demonstrated that the Lin-ve cells are a smaller fraction of cells in hUCB. When analyzed in an automated cell counter, these cells showed much smaller diameter than the Lin+ve cells. Because Lin-ve cells are non-committed, undifferentiated and are in early stage of haematopoietic lineage, they are smaller in size as compared to the Lin+ve committed cells. Interestingly, a rare population of cells with very small size isolated from hUCB, termed as very small embryonic-like (VSEL) stem cells also

express pluripotent markers such as Oct-4 and Nanog exerting regenerating effects [34].

Our data showed striking reproducibility in Lin-ve cells expressing significantly higher percentage of CD34 and CD117 markers when compared to MNCs and Lin+ve cells. The Lin-ve population possesses 56±8.65% CD34+ cells and 63.3±8.50% CD117+ cells. This number was further increased to 68.4±6.26% and 75.6±5.08% respectively when analyzed in gated CD45+ Lin-ve cells. CD45 is a protein tyrosine phosphatase transmembrane molecule expressed by most of the haematopoietic progenitors and differentiated cells. The increased expression of CD34 and CD117 in CD45+Lin-ve cells than in the Lin-ve cells alone could be possibly due to the presence of a small fraction of Lin+ve cells in Lin-ve population. As Lin-ve cells are enriched by negative selection using MACS, these cells could have retained a small number of Lin+ve cells which could not be ascertained by FACS. This was further validated in our UCB cells when CD34+ and CD117+ Lin-ve cells showed almost 100% positivity for CD45. It was indeed evident in a recent article where Alvarez-Gonzalez et al demonstrated that Lin-CD45- UCB cells could not retain their *in-vitro* self-renewal capacity notwithstanding their pluripotent embryonic characteristics [16]. Therefore, it is suggested that CD45+Lin-ve cells represent a superior population in UCB cells possessing true characteristics of stem cells.

CD34 expression varies in UCB-MNCs from one sample to another and at times it remains as less as 0.1%. About 10^5 of CD34+ cells/kg of body weight is optimally used for transplantation which may fall short from a single UCB unit [35,36]. Hence, Lin-ve population with significantly higher (60 fold) proportion of CD34+ cells can be a good substitute to MNCs used for transplantation or other cell based therapeutics. CD117 (c-Kit) is another marker abundantly expressed on the hematopoietic progenitor cells. This cell surface antigen is expressed more on myeloid progenitor cells as compared to lymphoid progenitors. In general, this cell surface marker binds to stem cell factor and mediates activation of tyrosine kinase which is one of the important steps involved in cell division, proliferation and differentiation. CD117 can be used in tandem with CD34 to obtain pure stem cell population in UCB samples, which is not widely investigated either in transplantation studies or in the samples stored in UCB banks for future applications. It is also evident that there is a positive correlation between the expression of CD34 and CD117 stem cell markers both in UCB and mobilized blood [29]. Hence, we propose that CD34+ CD117+ Lin-ve population in hUCB represents one of the primitive stem cell sources with presumably superior efficacy in regeneration and should be tested for transplantation in disease models or limited clinical trials.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. The corresponding author declares that he had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis as well as the decision to submit for publication.

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PROSPECTS
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Preserving Neural Retina Through Re-Emerging Herbal Interventions

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ABSTRACT

Eye related diseases such as glaucoma, diabetic retinopathy, cataract, conjunctivitis are very common worldwide. With the current scenario India will be among the top five countries in the number of glaucoma cases. Limited discovery of successful drugs for the treatment of such diseases led scientists to look towards the use of conventional sources for treatment. Herbal extracts from Ayurveda have remained an important part of treatment regime in many parts of world even today. For this reason, local herbs possessing curative properties are still being used by local inhabitants due to its anti-inflammatory and antioxidant properties. Because retinal damage involves alterations in oxidative enzymes, blood flow changes and increase in apoptotic signals, herbal extracts are being tested for their ability to moderate antioxidant machinery and trigger neuroprotective pathways. The present review summarizes some of such herbal extracts which have been tested for their neuroprotective role in eye related diseases. The active components that exert neuroprotective effects have also been discussed along with possible mechanisms of action. J. Cell. Biochem. 115: 1659–1668, 2014.

KEY WORDS: HERBAL EXTRACT; COMPLEMENTARY MEDICINE; OCULAR; OXIDATIVE STRESS; ANTIOXIDANTS

Diseases related with retinal ganglion cell (RGC) disruption are widely prevalent. Glaucoma is the second major cause of blindness after cataract, among eye disorders, and the most common cause of irreversible blindness. By 2020, India will become second in glaucoma incidence and, therefore, there is an urgent need to improve therapeutic approaches to retinal ganglion cell damage that can be applied not only nationwide but also worldwide [Shahsuvarya, 2012]. There is evidence that dying neural cells create a toxic internal milieu which can affect healthy cells surrounding it. The essence of neuroprotection involves protection afforded to these healthy cells from damage. Glaucoma, being an irreversibly blinding disease, could benefit immensely from this strategy if it could be made to work.

RGCs are neurons present in retina. While the cell bodies of these entities are located in the retina, the axons extend along the optic nerve which connects to the visual center of brain. RGC loss is a characteristic feature of various optic neuropathies. The ultimate fate of any eye disease is RGCs death regardless of the mechanisms of damage. These could be modeled as artificially induced ischemia either due to middle cerebral artery occlusion [Shakakibara et al., 2008] (MCAO) or carotid artery occlusion (CCAO) (Shri and Bora, 2008), neurotrophin deprivation, glial activation, excitotoxicity, oxidative stress [Hyun et al., 2013], episcleral vein cauterization [Dan et al., 2011] etc. Retinal ischemia is the prominent form of injury that can artificially induce RGC death. As confirmed from previous studies ischemia results in reduced flow of blood to retina and elevated levels of free radicals such as superoxide anion, hydroxyl radical, and hydrogen peroxide, along with a progressive depletion in endogenous antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), or antioxidants, glutathione (GSH) (Makris and Rossiter, 2001). Under normal conditions, free radical generation due to various metabolic

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processes is balanced by antioxidant enzymes. Oxidative stress occurs when this balance between production of endogenous antioxidative defense systems and reactive oxygen species (ROS) is disturbed (Kumari and Augusti, 2007). Excessive free radicals cause oxidative stress, damage lipid, protein and DNA, and ultimately result in cell death. Therefore, ischemia results in deprivation of oxygen to tissue and metabolic substrates ultimately affecting waste recycling. These processes lead to homeostatic imbalance exacerbating injury. Retinal ischemia has been studied extensively because it has been proposed to be involved in number of optic neuropathies such as anterior ischemic optic neuropathy (AION), glaucoma, retinal and choroidal vessel occlusions, retinopathy of prematurity (ROP), diabetic retinopathy, and traumatic optic neuropathy (Fig. 1).

CURRENT THERAPY AND ITS LIMITATION

The process of RGC degeneration is multivariate and can occur with or without increase in IOP. Various IOP independent factors such as ischemia, deprivation of one or more trophic factors [Quigley et al., 2000], excitoxicity [Cherecheanu et al., 2013], and oxidative stress [Chrysostomou et al., 2013] could lead to this damage. The current treatments available for various optic neuropathies which results due to RGCs degeneration are not adequate and thus alternate therapies can constitute re-emergent approaches from traditional knowledge resources for damage prevention. Moreover, commercial drugs available for diseases related to RGC damage like, timolol and letanoprost, for glaucoma exhibits toxic outcomes [Pisella et al., 2004]. Studies carried out to examine metabolic properties of Timolol have shown CYP2D6 as one of the principal enzymes involved. Mainly expressed in the liver, CYP2D6 has a very low expression in eye. Thus, after topical administration of Timolol, adverse effects may follow [Volotinen et al., 2011]. Even the systemic drugs used to treat other disorders have been critically reviewed by Santaella and Fraunfelder (2007) and they have found serious ocular side effects associated with these drugs. Therefore, it is imperative that an alternative treatment strategy from among re-emerging herbal interventions derived from natural resources be explored for diseases of retina. In this context, this review discusses the studies emanating from use of *Allium cepa* and other natural products from plant sources.

ALTERNATIVE THERAPEUTIC APPROACHES

According to National Institute of Health's (NIH) National Center for Complementary and Alternative Medicine (NCCAM), Complementary and alternative medicine (CAM) is a group of different medical related systems and practices which are commonly used by people around the globe, yet do not constitute the conventional approach in practice of medicine. The alternative approaches which can address the diseases of eye are needed for validation against the protection of ocular diseases. For example, various dietary supplements are known to have a direct or indirect role in the signaling pathways to protect the retina [Kiser and Dagnelie, 2008] but have not been tested widely.

Institution of complementary and alternative intervention is more frequent among developing nations and are now being regarded as re-emerging medical intervention in the face of slow pace of worldwide drug discovery. Rhee et al. (2001) reported that use of CAM among patients is approximately 5% (54 out of 1,000 patients). They also categorize the complementary and alternative therapies for glaucoma treatment into major nine branches: herbal extracts,

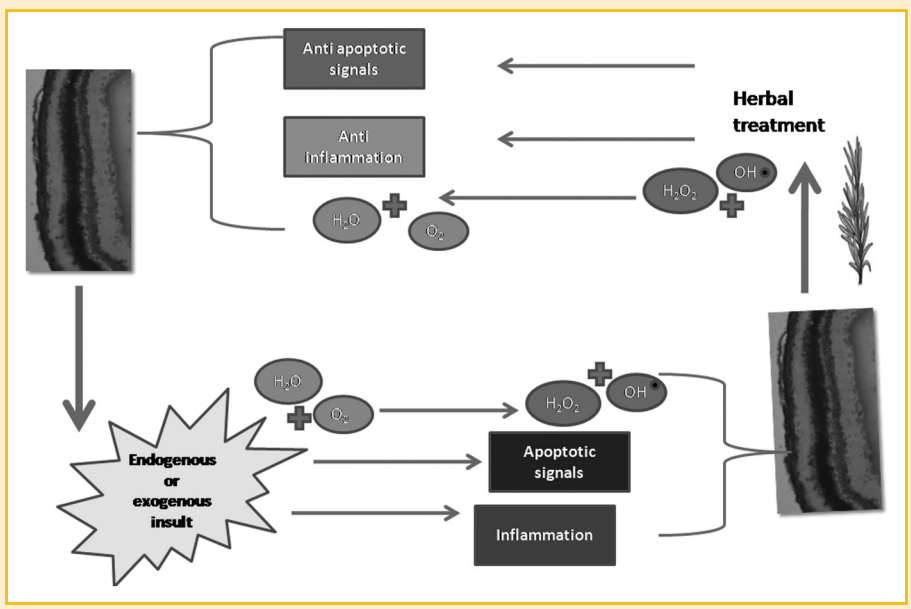

Fig. 1. Graphical abstract.

acupuncture, homeopathy, meditation, Vitamin supplementation, therapeutic touch, faith healing, exercise, and dietary modification.

Natural compounds such as grape seed extracts [Ritch, 2007], α- lipoic acid [Liu et al., 2012a], Vitamin E [Majumdar and Srirangam, 2010], curcumin [Alwan et al., 2012] have been tested from time to time to screen their neuroprotective role in retinal degenerative diseases of the brain. In contrast to singular herb centric (Chang and So, 2008) or singular disease centric [Mi et al., 2013] reviews, we have undertaken to present a critical analysis of those herbal extracts which have re-emerged as potentially efficacious agents in ocular disorders with minimal side effects. Present literature has some articles discussing different herbal interventions in ocular diseases (Wilkinson and Fraunfelder, 2011); however, the molecular mechanisms associated with neuroprotection were not emphasized in-depth. Therefore, the review summarizes the various herbal remedies and the convergent molecular mechanisms central to retinal degeneration protection.

HERBAL REMEDIES

Herbal extracts are being used from time immemorial for the treatment of various diseases. Studies have been carried out to screen the potential of natural extracts in preventing retinal degeneration.

ALLIUM CEPA

Allium cepa is a common component of our daily diet and has been widely studied for its therapeutic effectiveness due to its role as antidiabetic, antibiotic, anticancer, antiatherogenic, etc. [Helen et al., 2000]. Onions are rich in flavonoids (quercetin, myricetin, kaempferol) and organosulphur compounds (thiosulphimates and cepaenes). The antioxidant activity of *Allium cepa* is attributed to these key elements (Bhanot and Shri, 2010). Quercetin present in the onion is believed to inhibit the caspase-3-activity.The neuroprotective effect of quercetin was shown to act via wnt/-catenin pathway. Because of such an extensive study on use of *Allium cepa* in a variety of diseases and its normal uptake in body it provides an attractive agent to be tested against retinal ischemia. Besides, there are studies that showed the neuroprotective effect of *Allium cepa* in neurodegenerative and other related diseases, but it has not been adequately evaluated in models of retinal ischemia (Table I).

Antioxidant effect of *Allium cepa* has already been established in previous studies (Makris and Rossiter, 2001). Kumari and Augusti (2007) have shown that *S*-methyl cysteine sulfoxide isolated from *Allium cepa* reduced the concentration of cholesterol and phospholipids in rat fed on high cholesterol diet. Antioxidant effects were further confirmed by Campos et al. (2003). Alcoholic extracts of *Allium cepa* have been reported to have wound healing activities as shown by Shenoy et al. (2009). Our earlier studies have shown that methanolic extracts of *Allium cepa* provides neuroprotection against brain ischemia-reperfusion injury and a dose of 100 mg/kg B.W. and 200 mg/kg B.W. was found to be effectively reducing cerebral infarct volume (Shri and Bora, 2008) (Fig. 2).

CURCUMIN

Curcumin is a bioactive component of turmeric which is commonly used spice in India. Retinal pigment epithelium (RPE) is an important layer of retina which provides nutrition to other retinal layer and is

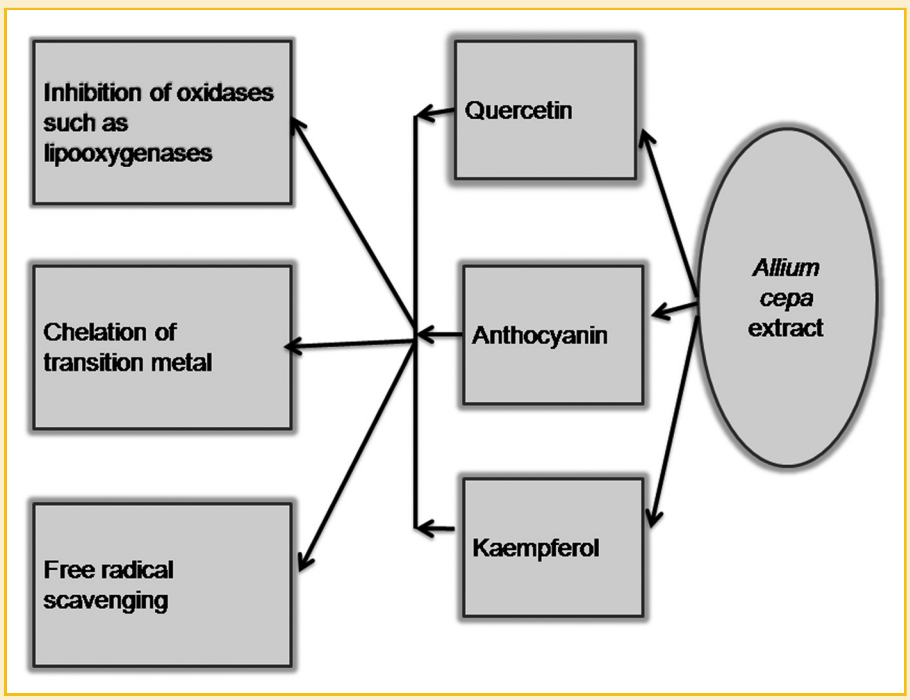

Fig. 2. Proposed mechanism for antioxidant activity of *Allium cepa* extract.

TABLE I. *Allium cepa* Extracts in Various Studies

S. no.	Model animal	Purpose	Reference
1	Gerbil	Cerebral ischemia	Hwang et al., 2009
2	Rat	Antidepressant	Shakakibara et al., 2010
3	Mice	Brain ischemia	Shri and Bora, 2008; Hyun et al., 2013
4	The brain microvascular endothelial cells(BMVECs) and astrocytes	In vitro neuroprotection against neuronal apoptosis	Dan et al., 2011
5	Mice	Diabetic neuropathy	Bhanot and Shri, 2010

thus indispensible for proper growth and survival of retina. RPE damage due to oxidative stress is one of the various factors responsible for pathogenesis leading to Age related macular degeneration. Woo et al. (2012) studied the effect of curcumin in RPE cells and they found that curcumin induces elevated expression of heme oxygenase 1 enzyme which provide defense against oxidative stress. Similar protective effect was reported against ischemia/reperfusion injury and photoreceptor degeneration. Wang et al. (2011) studied the neuroprotective effect against neurovascular damage and hypothesized that this result can be attributed to inhibitory effect of curcumin on NF-kB and STAT-3 responsible for degeneration. P23H-rhodopsin transgenic mouse, model for photoreceptor damage, when supplemented with curcumin also showed improved retinal structure and presents curcumin as potential therapy for diseases of photoreceptor degeneration [Vasireddy et al., 2011]. Anti-inflammatory role of curcumin has been established in endotoxin induced uveitis model in rabbits, where pretreatment with *C. longa* along with *B. aristata* has shown reduced levels of inflammatory mediators and aqueous humor a common symptom of uveitis [Gupta et al., 2008]. Curcumin exhibits its neuroprotective action through regulating NF-κB, AKT pathways influencing the inflammatory responses to various retinal injuries. Studies have shown that curcumin protection against light induced photoreceptor degeneration is mediated through reduction of expression levels of pro-inflammatory genes such as *Timp1*, *Icam1*, *Mmp3*, *Ccl2*. Besides, curcumin also reduces the expression level of genes related to oxidative stress (Ho-1) and apoptosis (Fos l1). Thus, regulation of genes related to inflammatory pathway support the immunomodulatory role of Curcumin [Mandal et al., 2009].

GINKO BILOBA

The ginkgo tree is a native of remote mountains of China, Japan, and Korea. Leaves and seeds of the tree generally have medicinal value which is believed to be due to presence of flavanoids and terpenoids [Mesbah et al., 2005]. Ginko biloba extract (GBE) extract EGb 761 has flavanoids kempferol, quercetin, and isorhamnet [Ritch, 2000]. The polyphenolic flavonoids possess antioxidant properties and the terpenoids inhibit platelet activating factor [Mozaffarieh et al., 2008].

In vitro studies have supported the neuroprotective nature of GBE against retinal cells. Studies on retinal pigment epithelium cells have shown increased expression of HIF-1and vascular endothelial growth factor (VEGF) in presence of GBE [Oh et al., 2013] while retinal explants culture reported increased survival of retinal ganglion cells by protecting cells from caspase activated apoptosis [Wang et al., 2012]. EGb 761, when administered to rats in which cataract was induced by selenium, prevented progression of disease. The chemical stress was ameliorated by ROS scavenging that inhibits the oxidation of proteins [Thiagarajan et al., 2002].

Pretreatment with GBE was shown to reduce the loss of retinal ganglion cells in chronic glaucoma induced in rat [Hirooka et al., 2004]. Herbal mixture containing *Ginko biloba* as one of the component supported the inhibition of caspase activity by extract in optic nerve transaction model in hamsters [Cheung et al., 2004]. GBE has also been found effective against dexamethasone induced ocular hypertension. The extract reduced the elevated IOP in rabbits and protected trabecular meshwork cells from adverse effect of dexamethasone. Further, cultured human trabecular cells, pretreated with GBE before dexamethasone showed reduced anti-Fas ligand-induced apoptosis [Jia et al., 2008]. GBE was also found helpful in survival of RGC cells after optic nerve crush in rats. Intra-gastral applications of extract applied after optic nerve crush protected RGC cells from damage and survival rate was found to be dosage dependent [Ma et al., 2009]. Additional studies by Juarez et al. (2008) have reported *G. biloba* to be effective against retinopathy of prematurity induced in both mouse and rat, adding credence to the potential of alternative therapies for diseases of retina.

VACCINIUM MYRTILLUS (BILBERRY)

Bilberry is a herb with very high anthocyanin content. Anthocyanins provide bilberry extract ability to combat oxidative stress as shown by study on cataract [Yamakoshi et al., 2002]. Anthocyanosides have positively charged oxygen which scavenges negatively charged electrons readily [Bagchi et al., 2006]. Antioxidant properties were further confirmed by increased level of MDA, GSH, and SOD in mouse model of endotoxin induced uveitis (UIV). Mouse treated with lipopolysaccharide (LPS) showed elevated level of nitric oxide (NO) which was reduced by bilberry extract [Yao et al., 2010]. In another study on UIV model, pretreatment with bilberry extract resulted in improved vision by preventing photoreceptor function impairement. Treatment with bilberry extract also increases the electroretinogram response in treated group. The underlying mechanism that prevented this retinal degeneration is believed to include prevention of STAT3 activation thereby reducing inflammation-related rhodopsin damage. Besides anti-inflammatory effect, antioxidative property exhibited by extract is also believed to help in ameliorating the ROS and damage to retina [Miyake et al., 2012].

In vitro studies have provided evidence for protective role of bilberry extract against RGCs. RGC-5 cells when treated with sydnonimine hydrochloride, known to elevate levels of peroxynitrite, have shown activation of ROS and neurotoxicity. Bilberry

treatment inhibited the neurotoxic effect induced by the compound. The neuroprotective effect of extract was further tested in mouse model of chemically induced retinal damage. Upon *N*-methyl-D-aspartic acid (NMDA) treatment ganglion cell layer damage was ameliorated by bilberry extract [Matsunaga et al., 2009].

LYCIUM BARBARUM POLYSACCHARIDE

Lycium barbarium also known as wolfberry is a medicinal plant with fruit and leaves of the plant having high medicinal value. Polysaccharides present in these plant parts are known to have antioxidant, anti-aging, and anti-tumor properties [Liu et al., 2012b].

The neuroprotective effects of *Lycium barbarum* have been tested in in vitro studies on cortical neuronal cell cultures [Ho et al., 2010]. Li et al. (2011) showed that pre-treatment with an extract from wolfberries (*Lycium barbarum* polysaccharides, LBP) could effectively protect the retinal layers from neuronal death, apoptosis, disruption of blood retinal barrier and oxidative stress in retinal I/R injury. Further, in another study it was also postulated that LBP could prevent damage to RGCs from hypertension-induced ischemic injury [Mi et al., 2012]. In vivo studies to screen the potential effect of extract on protecting retinal ganglion cells from elevated ocular pressure were carried out by Chan et al. (2007) in rats by using laser photocoagulator elevated IOP model and the results suggested *Lycium barbarum* extract to be protective against such damage.

CROCETIN

Crocetin is biochemically an aglycone of crocin and usually found in stigma of saffron and gardenia fruit. In vitro and in vivo studies on crocetin have revealed a promising therapeutic role of the carotenoid in retinal damage. RGC-5 cells treated with hydrogen peroxide or tunicamycin rescued cell damage with the treatment of 3 μM concentration of crocetin. Photoreceptor damage in mice model by light exposure was also counteracted by the crocetin administration. Protective effect of crocetin is believed to be due to inhibition of caspase 3 and caspase 9 which are responsible for cell death by apoptosis [Yamauchi et al., 2011]. Ohno et al. (2012) further evaluated the crocetin role in NMDA damaged retina and reported that oral administration improved the ERG responses in treated mice as compared to controls. The protective effects of crocetin (found in the saffron crocus, *Crocus starus* and in gardenia fruit *Gardenia jasminoides)* against the retinal ischemia induced by ligation of the pterygopalatine artery (PPA) and the external carotid artery (ECA) was also evaluated by Ishizuka et al. (2013) and found to be neuroprotective. Further studies can shed more light on its mechanism of action.

ASTAXANTHIN

Astaxanthin is a carotenoid that has characteristic oxidation properties. In vitro and in vivo studies carried out by Nakajima et al. (2008) have shown low level of oxidation in astaxanthin treated groups. The adverse effect of NMDA treatment in mice i.e. thinness of ganglion cell layer was overcome by astaxanthin treatment. Also in RGC culture studies cell viability after treating RGC-5 cell line with hydrogen peroxide was more in astaxanthin treated group. Astaxanthin, when given orally to the rats, with induced elevated ocular pressure, showed reduced retinal injury as compared to the control ones. Electrophysiological analysis of visual evoked potential (VEP) revealed that astaxanthin administration decreased the VEP in experimental animal when compared to controls [Cort et al., 2010]. In another study on cultured retinal ganglion cells, astaxanthin, a carotenoid pigment, was found to inhibit the neurotoxicity induced by H2O2 or serum deprivation. The attenuation of retinal cell damage by astaxanthin is possibly due to its antioxidant properties. The levels of oxidative stress marker such as 8-hydroxy-2-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) were also found to be downregulated after astaxanthin treatment [Dong et al., 2013].

BRAZILIAN GREEN PROPOLIS

Propolis is a resinous compound prepared by honeybees from bark of trees. Shimazawa et al. (2005) have elucidated the neuroprotective role with both in vivo and in vitro studies against retinal damage. They found that neurotoxicity induced by hydrogen peroxide and staurosporin was counteracted by propolis and RGC-5 cells viability was maintained in the culture. Similarly, the NMDA treated mice showed reduced retinal damage when propolis was administered intra-peritoneally to the mice. Later, it was determined that the protective effect was due to antioxidant properties of propolis which reduced the oxidative stress [Inokuchi et al., 2006]. Choroidal neovascularization is a characteristic of various eye related diseases such as age-related macular degeneration, diabetic retinopathy, the retinopathy of prematurity. VEGF is a well known angiogenic factor which activates various signal cascade pathways [Cort et al., 2010]. The study has revealed that propolis exerts its protective effect by acting on angiogenic processes and preventing angiogenesis. The angiostatic property of the propolis is believed to be due to its caffeoylquinic acid derivatives [Chikaraishi et al., 2010]. Thus, these findings suggest that propolis has a potential to be protective and therapeutic agent against diseases caused by angiogenesis.

OTHER HERBAL REMEDIES

A Chinese herb Astragalus membranaceus (AME) extract, when used in animal glaucoma model, lowered the intraocular pressure significantly. AME altered the MDA and GPx levels signifying antioxidant property of extract (Horng, 2011). Another study on herb *Foeniculum vulgare,* known to have antioxidant properties, [Baliga et al., 2003] has been evaluated for treatment of glaucoma. The aqueous extract of plant administered to rabbit model of chronic glaucoma comparatively reduced IOP in treated group as compared to controls [Agarwal et al., 2008]. Similarly, *Eisenia bicyclis* is a brown algae found in Korea and Japan. In vitro and in vivo studies carried out by Kim et al. (2002) showed that ethanol extract of *Eisenia Bicyclis*, seaweed, is protective for RGC cell death caused by ischemia. Some other herbal extracts used in in-vivo animals studies are listed in Table II.

Most herbal extracts prevent the progression of apoptotic signal and promote cell survival by activating the cell survival signal cascade (Fig. 3).

MECHANISM OF ACTION

Herbal extracts preserve the retina from damage by either preventing the apoptosis of the cell or by increasing the survival signals.

TABLE II. Herbal Extracts Used in Different Animal Models

S. no.	Model	Model animal	Extract used	Reference
1	NMDA	Rat	*Hong hua*	[Romano et al., 1993]
2	Transient global ischemia	Rat	*Bacopa monniera*	[Saraf et al., 2010]
3	Transient global ischemia	Mice	*Bacopa monniera and Valeriana wallichii*	[Rehni et al., 2007]
4	IOP elevation	Rat	*Erigeron breviscapus* extract	[Lu et al., 2011]
5	IOP elevation	Rat	Curcumin	[Wang et al., 2011]
6	MCAO	Rat	*Majun Baladar*	[Yousuf et al., 2007]
7	MCAO	Rat	*Embelia ribes*	[Nazam-Ansari et al., 2008]
8	Optic nerve transaction	Hamster	*Panax quinquefolius L.* extract (PQE), *Ginkgo biloba* extract (GBE) & *Hypericum perforatum* extract (HPE),	[Cheung et al., 2004]
9	Optic nerve crush	Rat	*Paeonia* extract paeoniflorin	[Li et al., 2007]

Imbalance in ROS and antioxidant enzymes leading to oxidative stress is a common phenomenon in various optic neuropathies. Almost every herbal extract possesses an antioxidant property which is exploited in exerting neuroprotection [Ritch, 2007]. Herbal extracts inhibit the activity of oxidases such as lipoxygenases (Bhanot and Shri, 2010) in cells. On the other hand, levels of antioxidant enzymes, catalase, glutathionase, superoxide dismutase are increased [Yao et al., 2010] which help in ROS scavenging. The resulting effect includes decreased oxidative stress and restoration of homeostasis. Carotenoids present in the extracts help in absorption of short wavelength light and prevent lipid peroxidation of cell membrane (Demmig-Adams and Adams, 2013). Accumulation of transition metals in ocular tissue is known to result in retinal degeneration by catalyzing the oxidative stress mechanisms. Chelating properties of some herbal extracts have been shown to protect the retina against such damage. Chelation bound accumulated free metal renders it unavailable for toxic effects (El-Beltagi and Badawi, 2013). Besides protecting retinal cells from oxidative

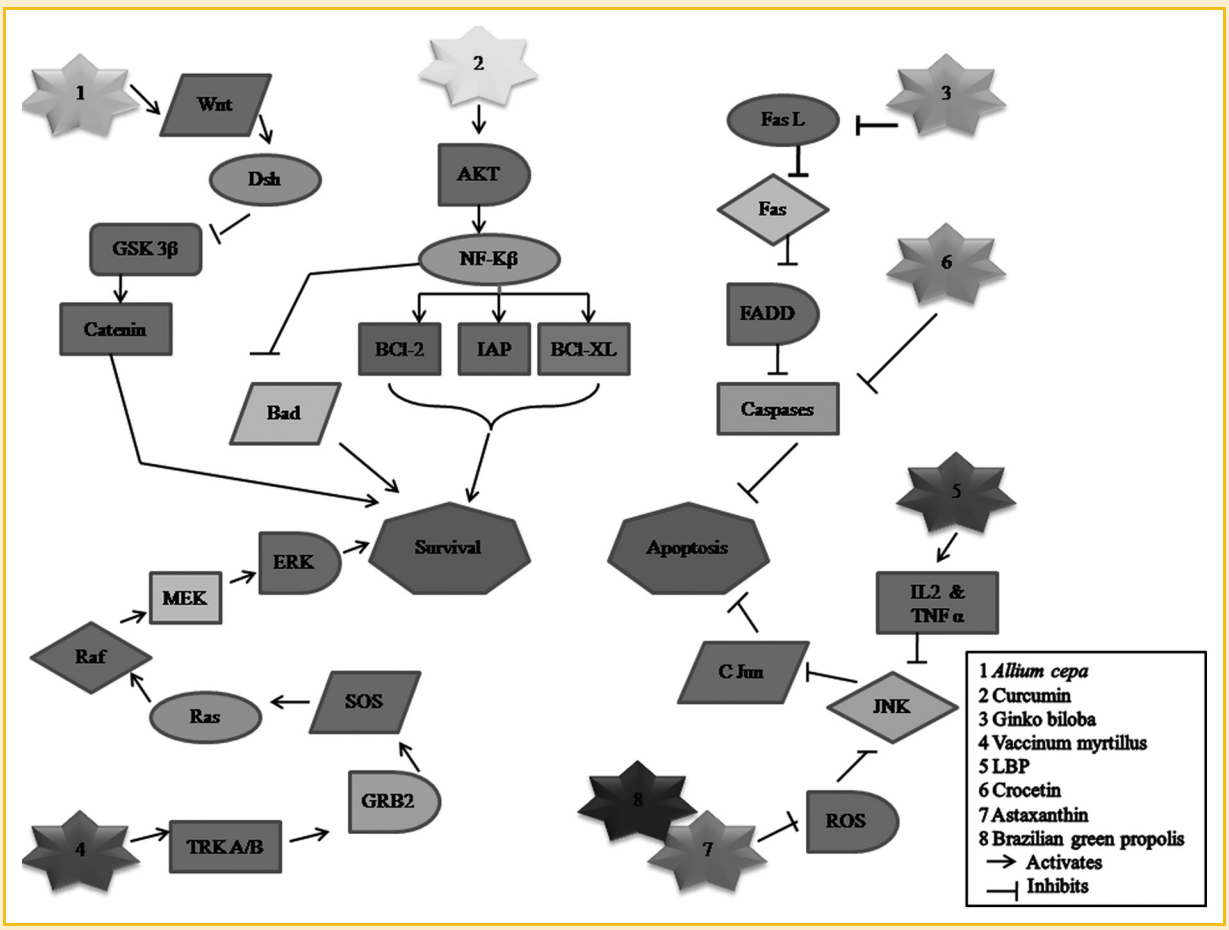

Fig. 3. Cascade of signal molecules activated/inhibited by different herbal extracts to promote cell survival.

stress, flavanoides in herbal extracts also enhance the survival of cells by elevating the expression of neurotrophic factors which are essential for normal cell functioning such as GDNF, VEGF, etc. [Zheng et al., 2000]. Immunostimulatory effects of some herbal extracts have been documented which include increased level of interleukin 2 and TNF-α resulting in retinal microglia activation (Chang and So, 2008). Herbal extracts also increase the cell survival in retina by interacting with anti-apoptotic gene *Bad* and *Bcl-XL* and decreasing caspase activity [Mi et al., 2013], thereby preventing apoptosis. Thus, herbal extracts have various mechanisms to preserve retina from neurodegeneration. These herbs and the related mechanism of action must be explored extensively for their use as therapeutic agent (Fig. 4).

CLINICAL RELEVANCE OF THE HERBAL EXTRACT

Herbal extracts are being used from time immemorial for the treatment of various diseases, however, scientific validation of these therapies still need to be evaluated. Current literature lacks research based on natural extracts [West et al., 2006]. Clinical trials of herbal extracts are very limited and thus there is a need of more and more preclinical studies on such extracts so that they can be promoted to successful clinical trials.

A herbal formulation named as Opthocare containing mixture of different herbal extracts was tested in a clinical trial. The patients who were recruited were having a wide range of ophthalmic disorders such as dry eye, pterygium, conjunctivitis, etc. The eye drop contained mixture of herbs known for their anti-inflammatory activity. In most of the patients there was an improvement with eye drop treatment [Biswas et al., 2001]. Anthocyanins purified from bilberry and blackcurrant when provided in form of capsules to participants in a clinical trial showed marked reduction in levels of NF-k-B inflammatory markers [Karlsen et al., 2007]. The marijuana effect on eye was studied and it was found to exert an effect on aqueous humor dynamics [Zhang et al., 2005].

So far, *Ginko biloba* is the herb of interest for clinicians and there have been some human studies to evaluate protective effect of this herbal extract in addressing eye related problems. Chung et al. (1999) carried out clinical trial of GBE for evaluating its efficacy against glaucoma. Patients with glaucoma were divided into two groups and treated with either GBE or placebo as a control three times daily for two days and blood flow was measured before and after treatment. The extract was shown to increase the ocular blood flow in ophthalmic artery significantly as compared to placebo.

Efficacy of GBE and bilberry, for improving visual field, was evaluated in 332 subjects with normotensive glaucoma (NTG). Both extracts were found to increase visual acuity in the patients (Shim et al., 2012). Clinical studies on patients of NTG have showed improved vision field. 40 mg of GBE for 4 weeks improved the damage in visual field of the patients with NTG [Quaranta et al., 2003]. Further clinical trials on NTG patients using *Ginko biloba* extract have also reported increased blood flow in the

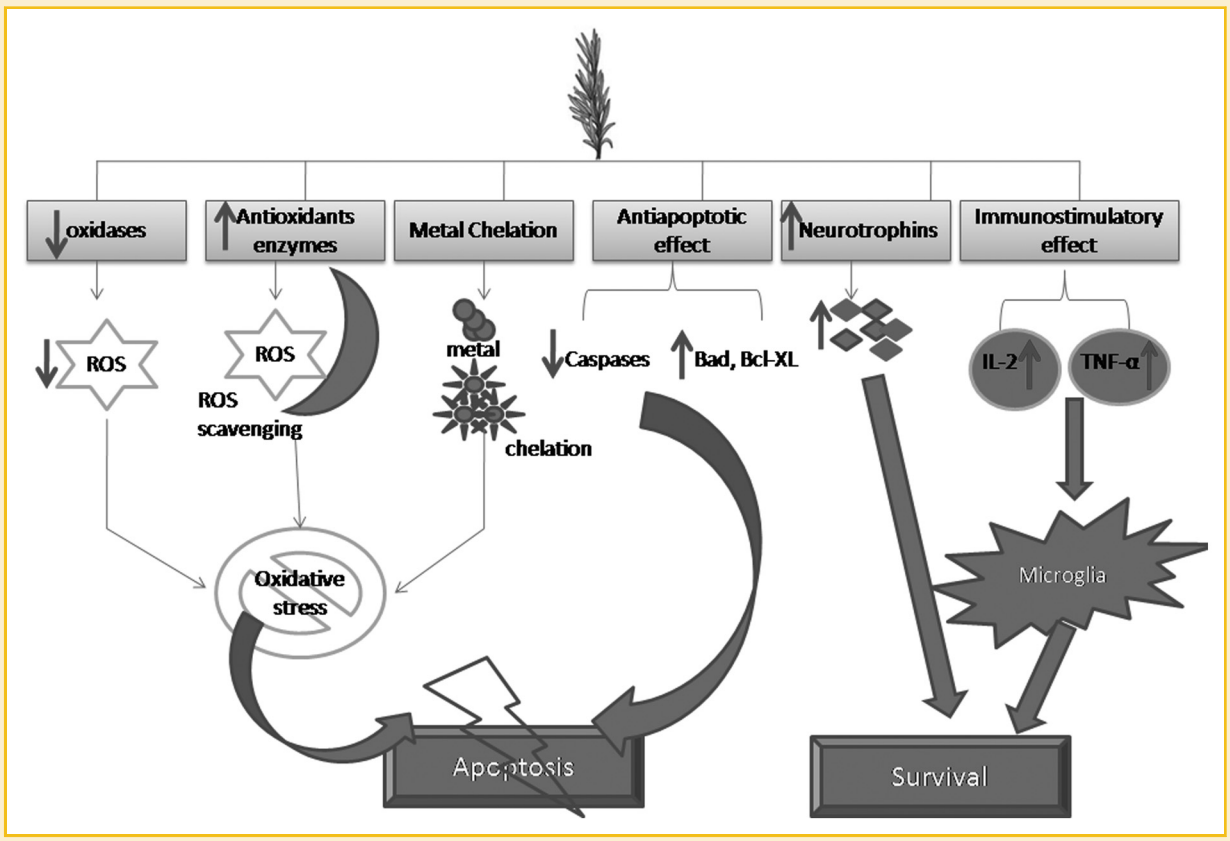

Fig. 4. Physiological and immunomodulatory pathways through which herbal extracts exerts their neuroprotection.

peripapillary area [Park et al., 2011]. Randomized control trial on 44 patients with Type 2 diabetes involving screening of GBE as a neuroprotective for eye was performed and it was revealed that the dry extract improves the microcirculation of diabetic patients. This effect was more pronounced in elderly patients [Spadiene et al., 2013].

CONCLUSION

The characteristic features of optic neuropathies include oxidative stress and cell damage. Thus, therapeutic approaches to eye diseases include not only lowering of pressure to normal level or maintaining the level of antioxidant mechanisms but also in salvaging the retinal cells. Various naturally occurring herbs possess antioxidative properties which have not been adequately explored using appropriate models. These properties of easily available herbal extracts can be tested to provide insight into treatments and mechanisms which could benefit a range of ocular diseases. Preclinical studies have revealed the effectiveness of herbal extracts for ocular diseases; however, clinical studies in the field are still lacking. The few clinical trials mentioned above have revealed the success of herbal interventions in treating the optic neuropathies. However, more preclinical and clinical studies must be carried out to collect robust data supporting the efficacy of herbal extracts in treating eye diseases especially when the synthetic drugs are not yielding any successful results. In addition, no serious side effects have been reported from the previous studies in the field. Therefore, preclinical and clinical data from further studies will help in promoting the use of herbal therapies in ocular diseases. Easy accessibility of the herbal sources may constitute a rather inexpensive strategy to combat eye disorders.

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Characterization of Lin-ve CD34 and CD117 Cell Population Reveals an Increased Expression in Bone Marrow Derived Stem Cells

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Abstract: The purpose of the study was to evaluate the expression of CD45, CD34, Sca-1 and CD117 in mouse bone marrow, Lin-ve and Lin+ve population. Bone marrow cells were isolated from C57/BL6J mouse and mononuclear population was separated from rest of the cell population. With the help of Magnetic associated cell sorter (MACS), Lin-ve and Lin+ve cells were separated from the bone marrow. The expression of CD45, CD34, Sca1 and CD117 was evaluated in bone marrow, Lin-ve and Lin+ve population by flow cytometry. We found a significant increase in the expression of CD34 and CD117 in Lin-ve as compared to the bone marrow and Lin+ve population. These findings suggest that Lin-ve population has higher expression of stem cell progenitor markers and could be useful for tissue repair and regeneration.

Keywords: Bone marrow, CD34, CD45, CD117, EPCs, Sca1, Stem cell.

INTRODUCTION

Stem cell can be isolated from various sources- bone marrow, umbilical cord blood, Wharton jelly, peripheral blood, amniotic fluid. On the basis of surface markers, stem cells population can be identified and selected such as Nestin, Sca1, CD34, CD133, CD117, and Thy1. Hematopoietic stem cells are defined by following characteristics – ability to self-renew, ability to form all blood-cell types [1-4]. The lineage-negative/CD117 (c-kit)$^+$/Sca-1$^+$ cells define the primitive hematopoietic stem cell population in bone-marrow [5]. To enrich adult murine hematopoietic stem cells (HSCs), the most common marker used is Sca1 and CD34. Bone marrow consists of heterogeneous population and known to contain hematopoietic stem cell population which is further divided into Lin-ve and Lin+ve subpopulations. This division is based on their potential to differentiate into blood elements. For the transplantation of hematopoietic stem cells, it is important to quantify the expression of CD34+ cells. The **human CD34** is highly glycosylated 105 to 120-kD hematopoietic, transmembrane cell surface antigen. The overall structure of the human and murine CD34 gene is similar [6]. It is highly expressed on stem cells, progenitor cells and vascular endothelial progenitor cells (EPCs) [7-10]. It was shown that EPCs have angiogenic potential but the cell surface markers of EPCs are not well defined. A study by Yang J *et al.* group suggested that in mouse bone marrow, CD34+ markers represent the functional endothelial progenitor cell population that can be helpful in EPCs therapy. Through various adhesion molecules, CD34+cells have a better homing capacity to ischemic tissue. In myocardium infarction (MI) mouse model, CD34+ cells were found to be incorporated into functional vessels resulting in decreased MI size and enhanced angiogenesis [11]. **CD117 (c-Kit),** a transmem-brane tyrosine-kinase receptor which is encoded by the *Kit* gene. It is expressed on CD34-postive cells [12]. The c-Kit ligand (also known as steel factor, stem cell factor) encoded by the *Kit1* gene, is a co-mitogen for hematopoietic stem cells, myeloerythroid progenitors and a mast-cell differentiation factor. In the adult bone marrow, CD117 is expressed on hematopoietic progenitor cells, including CD90 (Thy-1) low, TER-119-, CD45R/B220-, CD11b (Mac-1)-, Ly-6G (Gr-1)-, CD4-, CD8-, and Sca-1 (Ly-6A/E)+ multipotent hemotopoietic stem cells, progenitors committed to myeliod and/or erythroid lineages, and precursors of B and T lymphocytes [13]. Due to widespread expression of CD117 in hematopoietic, c-Kit and its ligand regulates several hematopoietic lineages. Intrathymic expression of c-Kit and c-Kit ligand suggest that CD117 is also involved in the regulation the development of T lymphocytes. **Sca-1 (Stem Cell Antigen)** is 18-kDa mouse glycosyl phosphati-dylinositol-anchored cell surface protein (GPI-AP), member of Ly6 gene family [14]. Sca-1-positive HSCs can be expressed by a mixture of stem, progenitor, and differentiated cell types of adult bone marrow, fetal liver and peripheral blood and spleen in the adult animal. Sca-1 is used in combination with negative selection against mature markers for enrichment of stem and progenitor cells. **CD45** is a transmembrane, tyrosine phosphatase protein and a marker of all hematopoietic stem cells. It play important role in receptor mediated cell signaling in B and T cells. There are several distinct isoforms of CD45, weighing 180- 240kD, that can be expressed in different cell lineages due to alternate splicing of its mRNA [15]. It is responsible for the interaction of several molecules that help in the mobilization of HSC from bone marrow into the blood.

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Table 1. List of antibodies used in flow-cytometric Characterization of Cells.

S. No.	Antibodies Used	Fluorochrome	Make	Stock Concentration of Antibody	Working Concentration of Antibody	Dilution of Antibody Used
1	CD45, Mouse	FITC	Miltenyi biotec	NA	NA	500X
2	CD45, Mouse	PE	Miltenyi biotec	NA	NA	500X
3	Rat anti mouse CD34 Clone: RAM34	Alexafluor647	BD Pharmingen	0.2 mg/ml	0.05µg/10^5 cells	200X
4	Rat Anti- mouse Ly-6A/E Clone E13-161.7	FITC	BD Pharmingen	0.5 mg/ml	0.1µg/10^5 cells	500X
5	Rat Anti- mouse CD117 (2B8)	FITC	BD Pharmingen	0.5 mg/ml	0.1µg/10^5 cells	500X

The role of flow-cytometry has grown in the recent times. Flow cytometry has provided us a tool to analyze different cell populations and their sub-populations and their identification and purification [16]. Thus, in this study, we isolated mouse bone marrow (BM) cells, BM derived Lin-ve and Lin+ve cells. The aim of the study was to evaluate the expression of CD45, CD34, Sca1 and CD117 in mouse bone marrow, Lin-ve and Lin+ve population.

MATERIALS & METHODS

Bone marrow (BM) Isolation: All experiments were conducted with the approval of Institute Animal Ethical Committee. For bone marrow isolation C57/BL6J, 6-8 week old, weighing 20-25gm mice were used. Mice were euthanized with a high dose of xylazine and ketamine. Sterilization was done by soaking the sacrificed animal in 70% alcohol. Femur, tibia, humerus and radioulna were separated from the body with scissors and muscular tissue was removed. Marrow was flushed from the bones with the help of 1mL syringe filled with cold 1X PBS (Miltenyi biotec, Germany). Flushing was done 2-4 times from both end of the all the bones. Bone marrow (BM) cells were then incubated at 37 ºC water bath for 15 minutes, and were vortexed gently after every 5 minutes. Cells were centrifuged at 2000 rpm at 4 ºC for 10 minutes. Supernatant was discarded and to the pellet, 5ml of 1X lysis buffer (BD) was added, and was mixed immediately. Cells were incubated on ice for 15- 20 minutes. To dilute the further effect of lysis buffer, cold 1X PBS was added and then was centrifuged at 2000 rpm at 4ºC for 10 minutes. Pellet was suspended in 25 ml of cold 1X PBS. BM cells were filtered through 70 micron nylon filter (Miltenyi biotec, Germany) to remove cell aggregates and tissue debris. Cells were washed with cold 1X PBS by centrifuging at 2000 rpm at 4 ºC for 10minutes. Finally the cells were suspended in 1ml of 1X cold PBS.

BM cell counting: BM cells were counted using automated cell counter (Sceptor Hand-held Automated Cell-counter, Millipore). To 99µl of 1X PBS, 1µl of BM cell suspension was added. It was mixed properly and kept for 5 minutes. 10µl of cells were loaded on the 60µm sensor.

CELL COUNT FORMULA

N X Dilution Factor per ml

Where N = Number of cells from cell-counter

Lineage Cell Depletion from Mouse Bone Marrow Cells by MACS Separation

Lineage negative cells were isolated using lineage cell depletion kit (Miltenyi biotec, Germany) that allows depletion of cells that express lineage antigens (negative selection). Lineage cell depletion kit depletes mature hematopoietic cells such as T cells, B cells, monocytes, macrophages, granulocytes and erythrocytes. Lineage positive cells were magnetically labelled using a cocktail of biotin conjugated monoclonal antibodies and anti-biotin conjugated monoclonal antibodies conjugated to microbeads. Biotin conjugated monoclonal antibodies cocktail contains CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4 and Ter-119 antibodies.

Magnetic labelling: BM cells were centrifuged and then the pellet was resuspended in 40µl MACS buffer per 10^7 cells. 10µl of biotin- antibody cocktail was added per 10^7 cells. Cells were incubated for 10 minutes at 4ºC. 30µl of MACS buffer and 20µl of anti- biotin microbeads were added per 10^7 cells. Cells were incubated for 15 minutes at 4ºC. Cells were washed with 1-2 mL of MACS buffer per 10^7 cells and then centrifuged at 2000 rpm at 4ºC for 10 minutes. These were suspended in 500µl of MACS buffer per 10^8 cells.

Magnetic separation: Large sized (LS) MACS column was placed in the Midi MACS magnetic separator (Miltenyi biotec, Germany). Column was prepared by rinsing it with 3mL of MACS buffer containing BSA. Cell suspension was loaded onto the column slowly along the sides of column. When cell suspension has completely entered the column, 1mL of MACS buffer was added. Lineage–ve cells were eluted with 3mL of MACS buffer. Column was removed from magnetic field and Lineage +ve cells were eluted with the help of plunger. Lin-ve and Lin+ve cells were centrifuged at 2000 rpm at 4°C for 10 minutes. Finally Lin-ve cells were resuspended in 200µl of MACS buffer and Lin+ve cells were resuspended in 500µl of MACS buffer.

Table 2. Detailed depiction of expression of CD45, CD34, CD117 and Sca-1 in cell populations in different samples.

Sample	BMCs				Lin $^{-ve}$				Lin^{+ve}			
	CD 45	CD 45/ CD 34	CD 45/ Sca-1	CD 45/ CD 117	CD 45	CD 45/ CD 34	CD 45/ Sca-1	CD 45/ CD 117	CD 45	CD 45/ CD 34	CD 45/ Sca-1	CD 45/ CD 117
Sample 1	92.70%	2.90%	18.40%	14.50%	87.90%	18.70%	24.40%	69.90%	94.60%	0.70%	9.50%	14.20%
Sample 2	91.30%	2.10%	4.60%	9%	91.30%	19.80%	7.90%	63.10%	91.20%	5%	9.40%	7.70%
Sample 3	89.40%	16.80%	7.50%	12.10%	90.90%	58.80%	14.40%	57%	84.30%	21.80%	6.80%	11.60%
Sample 4	88.60%	2%	4.20%	9.05%	93%	25.10%	12.75%	56.65%	79%	7.30%	6.05%	12.95%
Sample 5	90.40%	10.10%	5.95%	8.15%	89.80%	30.30%	6.60%	62.35%	81.45%	9.70%	5.55%	6.75%
Sample 6	86.90%	1.80%	9.20%	7%	87.40%	0.80%	10.30%	49.50%	87.70%	1.50%	6.70%	5.60%
Average	89.88%	5.95%	8.31%	9.97%	90.05%	25.58%	12.73%	59.75%	86.38%	7.67%	7.33%	9.80%
Std. Dev.	0.0205	0.0619	0.0528	0.0279	0.0213	0.1908	0.0641	0.06975	0.0593	0.0772	0.0170	0.0357

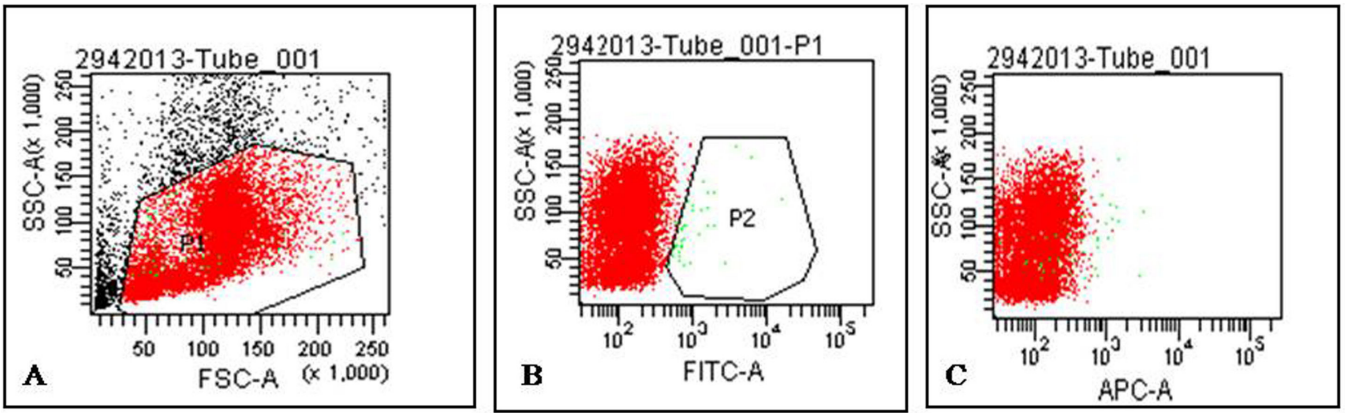

Fig. (1). The dot-plots of unstained bone-marrow derived cells. (**A**) Forward-scatter vs side-scatter. (**B**) and (**C**) No FITC-positive and Alexa fluor-positive cells in respective channels.

Lin-ve and Lin+ve cells were counted with the help of automated cell counter (Millipore).

FACS Characterization: Expression of CD45, CD34, Sca1 and CD117 was analyzed in BM, Lin-ve and Lin+ve cell population with flow cytometry. In 10^5 cells (BM, Lin-ve, Lin +ve cells), 10µl of FcR blocking reagent (mouse) (Miltenyibiotec) and 40 µl of buffer was added. Cells were incubated on ice for 10 minutes. Fluorescent conjugated antibodies (Table **1**) were added and then again cells were incubated on ice for 1hour. Antibodies used were FITC-CD45, Mouse (Miltenyibiotec), PE-CD45, Mouse (Miltenyi biotec), Alexafluor647 rat anti mouse CD34 Clone: RAM34 (BD Pharmingen), FITC rat Anti-mouse Ly-6A/E Clone E13-161.7 (BD Pharmingen), FITC rat Anti- mouse CD117 (2B8) (BD Pharmingen). Cells were washed twice with 1X PBS by centrifuging at 2000rpm, 4ºC for 10 minutes. Finally cells were resuspended in 200µl of 1XPBS and cells were fixed with 100µl of 1X fixation buffer (BD Pharmingen). Fixed cells were acquired by Flow cytometer (FACS Calibur).

Statistical Analysis: The expression levels were presented as mean ± SD of six samples as depicted in Table **2**. One-way ANOVA was used to compare the expression levels of different markers in the three cell populations. P-values ≤ 0.05 were considered as statistically significant.

RESULTS

Gating of mononuclear cell population: The bone-marrow derived stem cells were gated on the basis of their size and granularity in forward scatter versus side scatter dot-plots. These gated cells were further used to check the expression of different markers in the three populations (Fig. **1**).

Expression of CD45 in bone marrow, Lin-ve and Live+ population: In bone marrow cells, the CD45 expression was 89.88% ± 0.02% and in the lineage negative cell population, the expression of CD45 was found to be 90.05% ± 0.02%, which was not statistically significant (Fig. **2**).

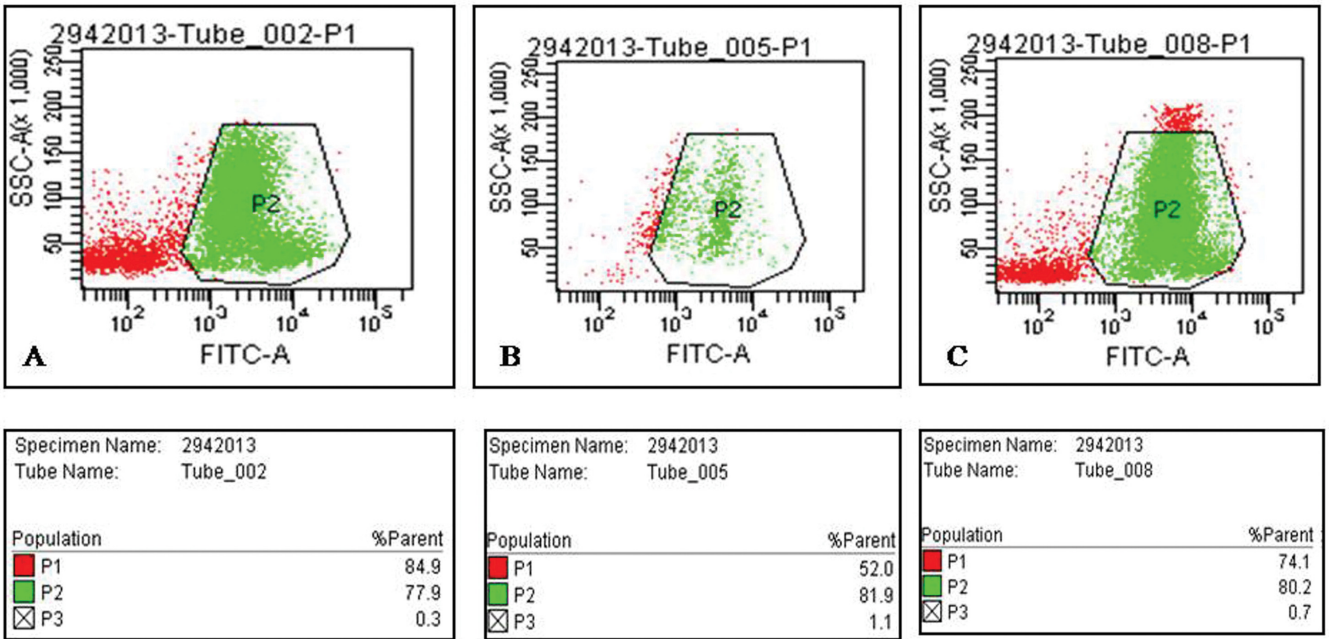

Fig. (2). The dot-plots of FITC stained CD45-positive cells. (**A**) CD45 expression in bone-marrow cell population (**B**) CD45 expression in lineage-negative population (**C**) CD45 expression in lineage-positive population.

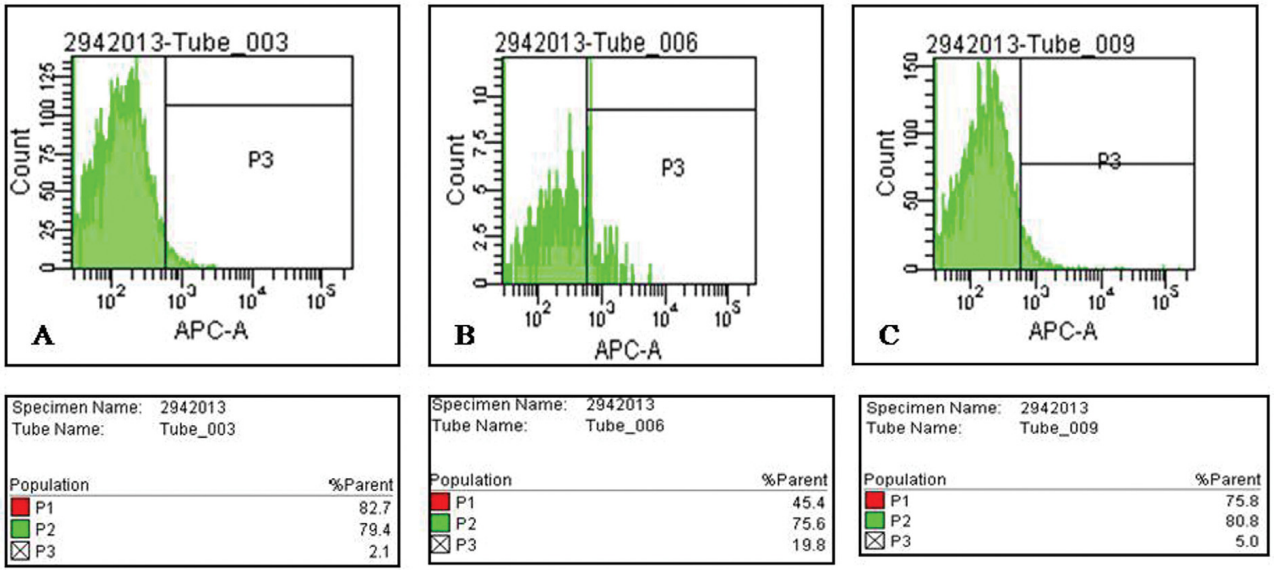

Fig. (3). The histograms of Alexafluor labeled CD34-positive cells . (**A**) CD34 expression in bone-marrow cell population (**B**) CD34 expression in lineage-negative population (**C**) CD34 expression in lineage-positive population.

Expression of CD34 in bone marrow, Lin-ve and Live+ population: In bone marrow cells, the CD34 expression was 5.95% ± 0.06% but when lineage negative cell population was gated for the same, the expression of CD34 was increased significantly to 25.58% ± 0.19% (Fig. **3**).

Expression of Sca-1 in bone marrow, Lin-ve and Live+ population: Also the expression of Sca-1 on lineage negative was 12.73±0.064%, which is more than on bone marrow cells (8.31% ± 0.528%) and on lineage positive cells (7.33% ± 0.017%) (Fig. **4**).

Expression of CD117 in bone marrow, Lin-ve and Live+ population: The expression of CD117 in bone marrow cells isolated from adult C57BL/6 was determined. We found significantly increased expression of CD117 in lineage negative cell population (59.75% ± 0.069%) as compared to bone marrow cells (9.97±0.027%) and lineage positive cells (9.80% ± 0.035%) (Fig. **5**).

Expression of Hematopoietic stem cell marker: Fig. (**6**) shows cumulative expression of different stem cell markers in the three cell populations. Significant increase was seen in the case of CD34 and CD117 from bone marrow to lineage negative cell population.

DISCUSSION

Stem-cell therapy holds promise as the therapeutic approach for different disorders. Before pre-clinical or clinical transplantation is attempted, proper characterization

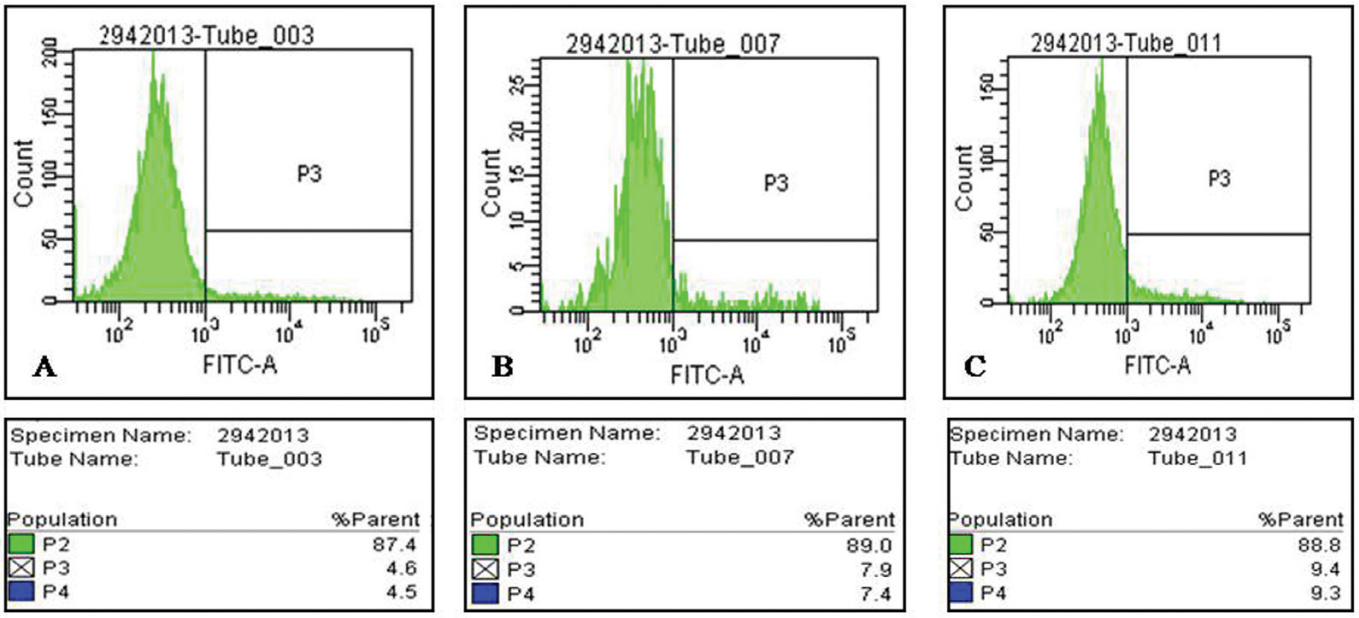

Fig. (4). The histograms of FITC labeled Sca-1-positive cells . (**A**) Sca-1 expression in bone-marrow cell population. (**B**) Sca-1 expression in lineage-negative population. (**C**) Sca-1 expression in lineage-positive population.

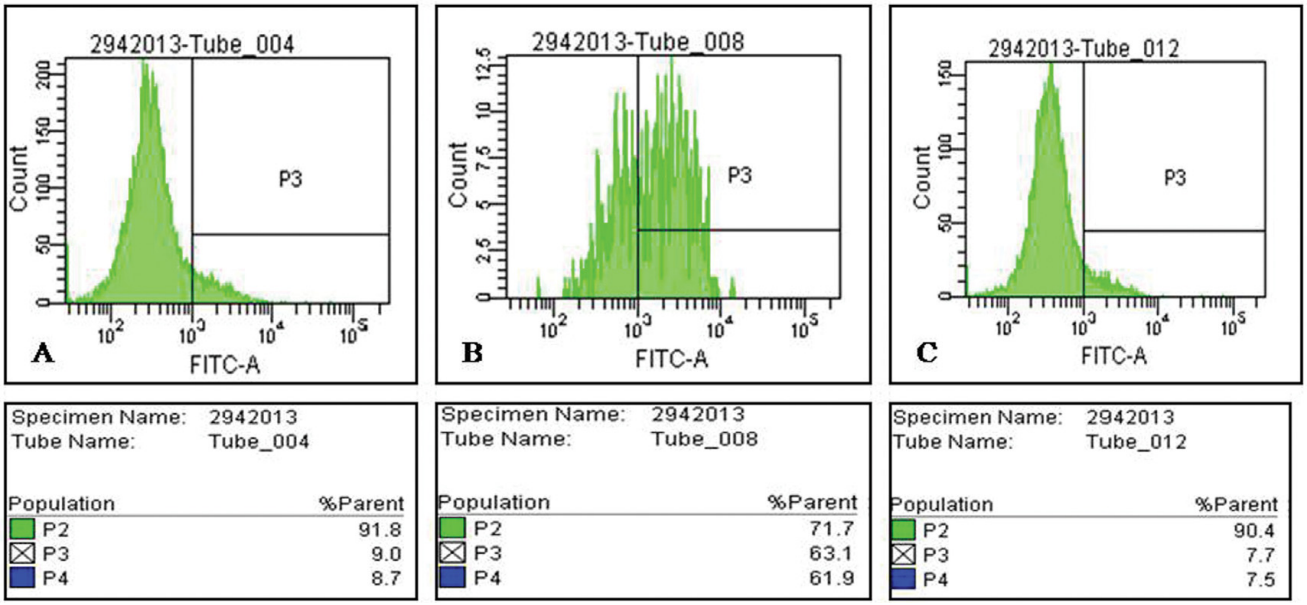

Fig. (5). The histograms of FITC labeled CD117-positive cells . (**A**) CD117 expression in bone-marrow cell population (**B**) CD117 expression in lineage-negative population (**C**) CD117 expression in lineage-positive population.

of different populations and their markers are required. The characterization of bone-marrow derived cells allows understanding the population of cells that constitute the stem cells. Various surface receptors have been discovered and found to represent these cells, making these markers to be important for their characterization and enrichment [17]. In this study, isolation and characterization of lineage-negative population from mouse bone marrow cells result in the increase of expression of important stem cell markers. Since the lineage-negative fraction is predominantly characterized being not committed to any lineage, it forms an attractive source for examining its potential in transplantation biology. It has been shown by research groups to possess the potential to repair and renew damaged cells. The Lin-ve HSCs have been shown to possess the capability to repopulate the bone marrow cells in lethally irradiated hosts [2, 4] suggesting that its prospects in therapeutic regeneration need comprehensive evaluation. In a study by Nilsson *et al.* Lin-ve cells from the male donor engrafted in the non-ablated female recipient showed proliferation and survival for upto six months [18]. In 1988, Spangrude *et al.* succeeded in enriching multipotential progenitors in the lineage marker that includes TER119, Mac1, Gr1, CD45R/B220, CD3, CD4, CD8- Thy-1^{low} Sca-1+ fraction of mouse bone marrow [19]. 50% of lethally irradiated mice were rescued following intravenous injection of Lin- Thy-1^{low} Sca-1+ cells [20]. In another related study Ogawa *et al.* has reported that the cells which are expressing c-kit (receptor for stem cell factor), have hematopoietic

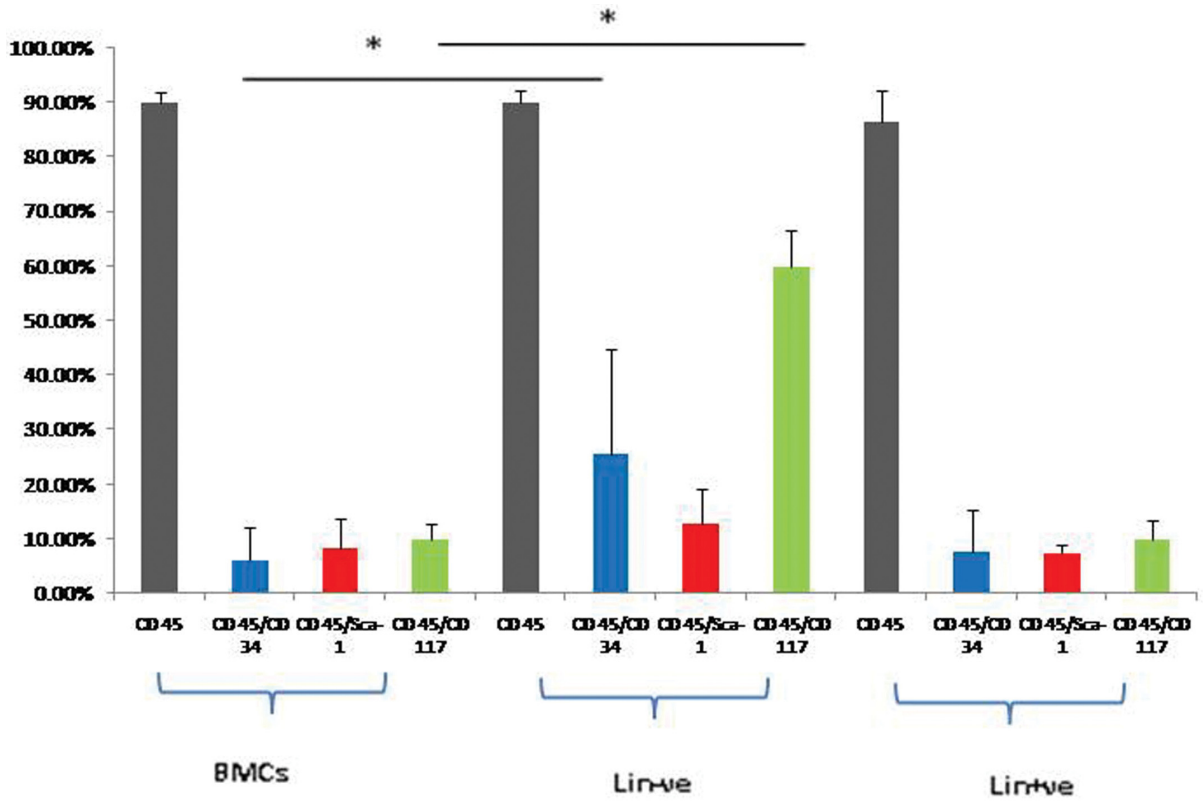

Fig. (6). Comparative expression of CD45, CD34, CD117 and Sca-1 in three different cell populations.

progenitor activity [21]. Since then, Lin- Sca-1+ c-kit+ (LSK) have been generally used as a canonical marker set for HSC enrichment. There are many studies that demonstrate the positive effects of bone marrow derived lineage negative population upon transplantation. One study by Otani *et al.*, 2004 has demonstrated that Lin– HSCs exerts vasculotrophic and neurotrophic effects thus preserve retinal vasculature and providing neuronal retinal rescue when injected intravitreally in mice with hereditary retinal disease [22]. In a similar study, Singh *et al.* described that the bone marrow derived lineage negative stem cell incorporated in laser injured mouse retina and survived up to 21 days after transplantation [23]. In addition to such studies, the Lineage-negative population has also shown positive effects in other diseases, such as chronic renal failure. For example, Alexandre *et al.* in 2009 showed long-term protective effects of Lin-ve transplantation in rats for upto 120 days. The authors demonstrated decreased levels of VEGF, MCP-1, pro-inflammatory cytokines and p21 arresting the disease progression [24]. In cerebral ischemia-reperfusion injury model, a decrease in pro-inflammatory cytokine levels was reported after Lin-ve cell transplantation. Similar reports were emerged from Schwarting and co-workers using the MCAO mouse model. After about 72 hours of intravenous transplantation, significant decrease in infarct volume and apoptosis was observed [25]. Interestingly, none of the studies described above undertook comprehensive analysis of these fractions used for transplantation thus impacting clinical translation. In contrast, majority of studies have successfully exploited the application of CD34+ cells derived from bone-marrow in clinical trials showing well-tolerated and long-term effects in acute ischemia-reperfusion mouse model established by elevated IOP [26]. Jamous and colleagues tested the differentiation capacity of Sca-1+ cells isolated from mouse bone-marrow derived Lin-ve population and showed that these cells differentiate into neuronal lineage *in-vitro* [27]. Bone marrow cells or even a subpopulation of BM cells, CD117+ cells, as progenitors of HSCs, could enhance the corneal healing process [28]. Given the increased testing of these cells, better understanding of hematopoietic stem cell population is required which is based on their characterization. Such investigations will help in identifying the key molecules critical in regeneration evident from their cell surface phenotype and thus enable development of therapies for various degenerative disorders.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Vascular Endothelial Growth Factor (*VEGF*) Induced Proliferation of Human Fetal Derived Ciliary Epithelium Stem Cells is Mediated by *Jagged - N Cadherin* Pathway

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Abstract The pigmented ciliary epithelium (PCE) of mammalian eye harbors resident population of stem cells that lie in apposition with endothelial cells which release vascular endothelial growth factor (VEGF) that may influence the fate and function of these stem cells in ways that remain unclear. We examined the role of VEGF in proliferation of PCE stem cells and expression of *Notch, Jagged, N-Cadherin and β-Catenin* which are known to maintain proliferation state of neural stem cells. We cultured human PCE cells obtained from 12-20 weeks old fetal eyes. The neurospheres were analyzed for the proliferation capacity of PCE stem cells in presence of VEGF on 3,6 and 9 day. Real time PCR was used to quantitate the mRNA expression of above mentioned genes on PCE derived neurospheres on 3,6 and 9 day. We found increased number of neurospheres when PCE stem cells were stimulated with *VEGF* alongwith epidermal growth factor (*EGF)* and basic fibroblast growth factor (*bFGF)* than *EGF* and *bFGF*. BrdU immunostaining was done to analyze the proliferation of CE cells and presence of neural and retinal progenitor markers such as Nestin and Pax6 were also investigated. An increased *Notch* and *Jagged* mRNA was observed on 6th day in *VEGF, EGF* and *bFGF* treated PCE cells as compared to 0,3 and 9 day. A similar pattern was noticed with *N-cadherin* and *β-catenin* mRNA levels. These findings may clarify the role of *VEGF* on PCE stem cell proliferation with possible involvement of *Notch*, *Jagged*, *N-cadherin* and *β-Catenin*. The data may suggest importance of harvesting 6th day neurospheres for transplantation purposes in preclinical investigations pertaining to retinal degenerative diseases, however, additional studies are needed to substantiate the findings.

Keywords: Basic fibroblast growth factor, β catenin, ciliary epithelium, epidermal growth factor, jagged, N cadherin, notch, vascular endothelial growth factor.

INTRODUCTION

Age related macular degeneration (AMD), diabetic retinopathy and glaucoma [1-3], are common causes of vision loss where a progressive loss of one or other cell layer of retina has been reported. Photoreceptors and retinal pigmented epithelium (RPE) are affected in AMD and diabetic retinopathy [4, 5] whereas in glaucoma, ganglion cells are lost [6-8]. Despite several strategies including gene therapy and anti-angiogenic therapy which have been used to ameliorate progression of this disease [9, 10] none has yielded promising results. Cell based therapies including stem cell therapy, however, has led to hope in treating some of these retinal diseases even though complete retinal regeneration has not been achieved.

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Resident stem cells within the pigmented ciliary epithelium provide a potential source of retinal stem cells which may be useful in retinal regeneration after transplantation [11]. To attain this goal, a better understanding of many factors such as the microenvironment, vascular mediators and the effects that exogenous and paracrine growth factors exert on these cells is needed. A key signaling pathway which maintains stem cells within the ciliary epithelium is the *Notch* pathway [12-15]. Generation of ciliary epithelium (CE) neurospheres has previously been reported to be markedly influenced by *Notch* signaling in adult CE [15]. The *Notch* receptor along with its ligand *Jagged* initiates signaling events that activate *HES* and *HES* related (*HEY*) genes [16]. Emerging evidence suggests that *Notch* signaling also mediates cell-cell interaction during vertebrate development. On the other hand, *Wnt* proteins participate in the regulation of retinal progenitor cells during development as well as muller stem cell maintenance and their proliferation [17,18]**.** Expression of *Wnt* signaling molecules was observed in CE stem cells and alteration of these molecules affected the proliferation of CE stem cells in culture [15]. Proliferative action of *Wnt*

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proteins is predominantly mediated by the canonical *Wnt/β-catenin* pathway. Inhibition of *Wnt/β-catenin* pathway is known to decrease the proliferation of retinal precursor cells [19].

Along with *Notch* and *Wnt* signaling pathways, cell to cell adhesion and interaction also plays an important role for proliferation. *N-cadherin* is one such cell adhesion molecule which plays critical role in cell proliferation and neurosphere formation from neural precursor cells and also regulates cell-to-cell adhesion in development of CNS via *β-catenin* [20,21].

Anatomically and functionally, the PCE is in close association with vasculature and endothelial cells. We, therefore, hypothesized that vascular endothelial growth factor (*VEGF*), which is secreted by endothelial cells (and non endothelial cells) may play an important role in the maintenance of CE stem cells mediated by *Notch*/jagged, *β-catenin/wnt* and/or *N-cadherin* signaling pathways. Therefore, we analysed the effect of VEGF on proliferation of PCE stem cells and its effect on expression of *Notch*, jagged, *β-catenin*, and *N-cadherin*.

MATERIALS AND METHODS

Subjects

For isolation of pigmented ciliary epithelial stem cells, eyes were obtained from aborted human fetus (12-20 weeks) after obtaining prescribed informed consent. Ethical approval of the study was obtained by institute human ethical committee and procedures were accomplished according to Indian Council of Medical Research-Department of Biotechnology (ICMR-DBT) guidelines.

Ciliary Epithelial Stem Cells Isolation and Culture

Immediately after enucleation, eyes were transported in a sterile ice cold Hank's Balanced Salt Soultion (HBSS) and dissected under sterile conditions using dissection microscope. PCE cells were isolated using previously published surgical methods. Since no protein markers specific for PCE exists in the literature, identification and isolation of PCE was entirely based on its anatomical location [22]. Briefly, eyes were placed in 30mm petridishes containing ice cold HBSS and cornea and lens were removed. A strip of ocular tissue containing the CE was obtained by cutting the anterior edge of the parsplana. Ciliary rings were collected and washed with HBSS. Precautions were taken not to contaminate the tissue with non pigmented epithelium, RPE, iris and retina. For dissociation of CE tissue by trypsinization, ciliary rings were transferred to 15ml tube containing 4ml - 5ml of 0.25% trypsin with EDTA and incubated at 37°C for 20-30 min, with intermittent mixing. Trypsin was immediately neutralized with equal quantities of DMEM/F12 (Gibco, USA). Cell suspensions were filtered through 0.70µm cell strainer (BD Biosciences, USA) to remove unwanted debris and centrifuged at 800xg for 10min. The cells were washed thrice or more with DMEM/F12 at 800xg for 10min until the pigment was washed off. Finally, the cell pellet was suspended in retinal culture medium (RCM) containing DMEM/F12, N2 supplement (Gibco, USA), 2mM L-glutamine (Gibco, USA), 100U penicillin–streptomycin (Gibco, USA) and fungizone (Gibco, USA). Pigmented cells were counted with automatic cell counter (Millipore, USA). Since suspension culture was required, dissociated pigmented ciliary epithelial cells were plated in an uncoated 96-well culture plate. These cells were grown in the presence of proliferation conditions in retinal culture medium containing three different combinations of growth factors *rhEGF* (20ng/ml; R&D systems, USA) and *rhFGF* basic (20ng/ml; R&D systems, USA)[E+F], rh*EGF*(20ng/ml), *rhFGF* basic (20ng/ml) and *rhVEGF*165 (50ng/ml; R&D systems, USA) [E+F+V] and *rhVEGF*165 (50ng/ml) alone [*VEGF*] in CO_2 incubator at 37^0C. The proliferating cells i.e., neurospheres were collected at different time intervals, i.e 0, 3, 6 and 9 days of culture, for subsequent RNA isolation and self renewal analysis (Fig. **1**).

Counts and Size Analysis of PCE Derived Neurosphere

Neurosphere counting and size analysis was done to assess the effect of *VEGF* on the proliferative capacity of pigmented ciliary epithelium. Neurospheres were counted by using a grid that covers one entire well of a 96-well plate. The counting was done by three independent observers in a blinded fashion by using Nikon inverted microscope. The size of the neurospheres produced in culture was measured using in built cell^A software of Olympus inverted microscope. Since the size variations among the neurospheres for a given condition was not significantly different, ten neurospehres for each condition (*EGF+bFGF*, *EGF+bFGF+VEGF* and *VEGF*) were picked to measure the average size of neurospheres.

Total RNA Isolation and Reverse Transcription

Total RNA was extracted from cultured cells in the presence of different factors such as *EGF+bFGF*, *EGF+bFGF+VEGF* and *VEGF* alone at different time points i.e., 0, 3, 6 and 9 days using RNAeasy columns (Qiagen, Valencia, CA) according to manufacturer's recommendations. DNA contamination was eliminated by using DNA digestion kit (Ambion,TX,USA). RNA concentration was measured in UV–visible spectrophotometer (DU7400, Beckman, Fullerton, CA) by taking absorbance at 260nm (A_{260}). About 100.00ng – 200.00ng total RNA was used to synthesize cDNA as per cDNA synthesis kit protocol (Fermentas, MD, USA).

Quantitative Real Time PCR

Real Time PCR was used to quantitate expression of *Notch*, *Jagged*, *β* catenin and *N cadherin* at mRNA levels using primer sequences (Table **1**) available at primer bank (http://pga.mgh.harvard.edu/cgibin/primer bank) in CE neurospheres, and was performed in the 48 wells model Step One™ (Applied Biosystems Inc., Foster city, CA).

Real time PCR was carried out for 10.0 µl containing SYBR green master mix (5.0ul), 0.2µl ROX dye as passive reference, 200nM (0.20µl) of each sense and antisense primers, 5.0 ng cDNA template and molecular biology grade water was added to make the volume 10.0 µl. All reactions were carried out using SYBR green based kit (Fermentas, USA) according to manufacturer's recommendations. Each sample was run in duplicate. The relative expression or fold change of different factors was analyzed using the comparative

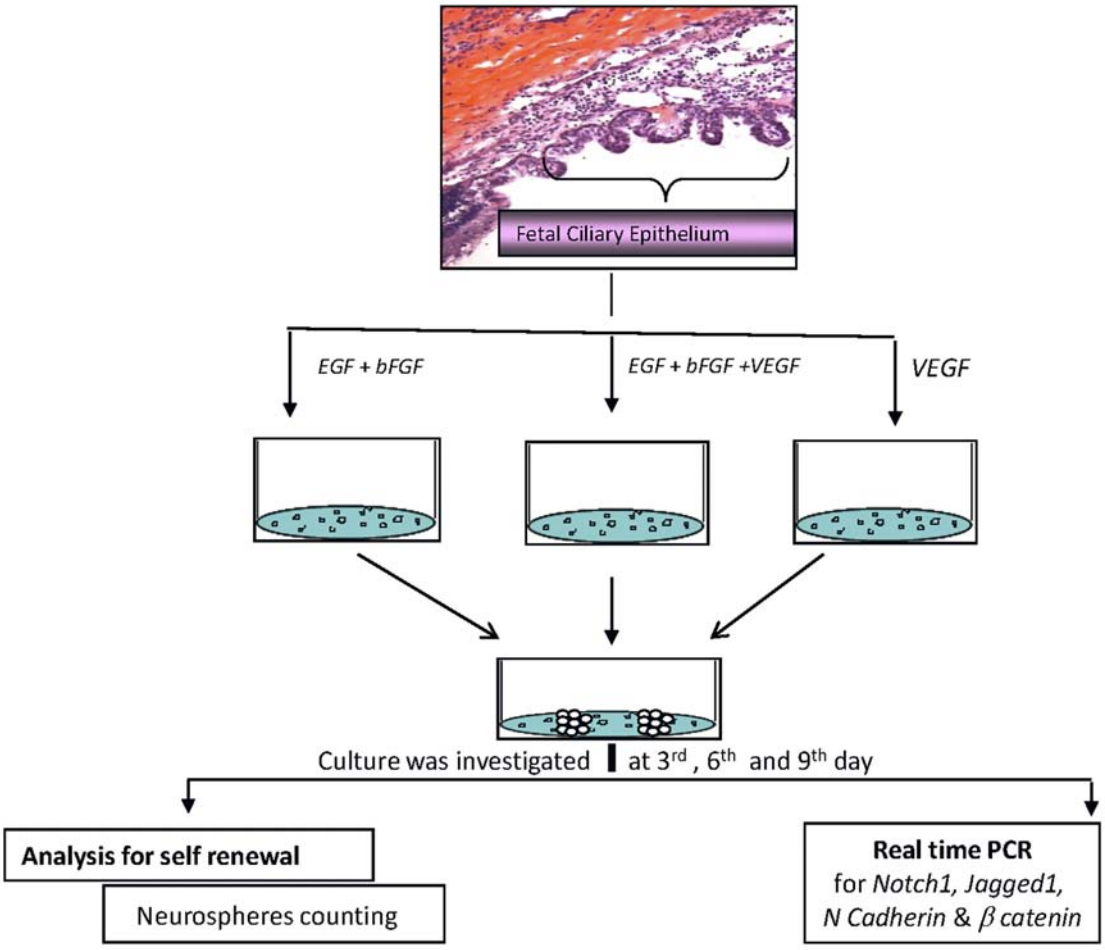

Fig. (1). Schematic representation of ciliary epithelium stem cell culture. Pigmented CE cells were isolated and cultured in three different combinations of growth factors for 3-9 days. 0 day CE cells and neurospheres were then anlaysed at different time points (3,6 and 9 day) for each condition for the self newal capacity and also for the expression of genes *Notch*, *Jagged*, *N cadherin* and *β catenin* by real time PCR. CE, ciliary epithelium.

Table 1. Real Time Quantitative PCR Primers

Gene	Sequence (5'→ 3')
Notch	F: GCTGGACTGGTGAGGACTG R: AGCCCTCGTTACAGGGGTT
Jagged	F: TCAGTTCGAGTTGGAGATCCT R: CCTTGAGGCACACTTTGAAGTA
N cadherin	F: CAGGCACCGTATTTGTGATTGA R: AAAATGGGTGGATTGTCGTTGA
β Catenin	F: TACCTCCCAAGTCCTGTATGAG R: TGAGCAGCATCAAACTGTGTAG
GAPDH	F: CAAGGTCATCCATGACAACTTTG R: GTCCACCACCCTGTTGCTGTAG

Ct (threshold cycle) method. Fluorescence data were acquired at the annealing step. Melt curve was also performed to rule out the presence of non specific amplification and primer dimer. Software StepOne™ v 2.0 (Applied Biosystems Inc., Foster city, CA) was used to perform amplification and melt curve and to acquire Ct values.

Immunocytochemistry

Cultured CE neurospheres were fixed with 4% paraformaldehyde (PFA) before immunostaining. These neurospheres were blocked with blocking solution containing 1X PBS with serum of host of secondary

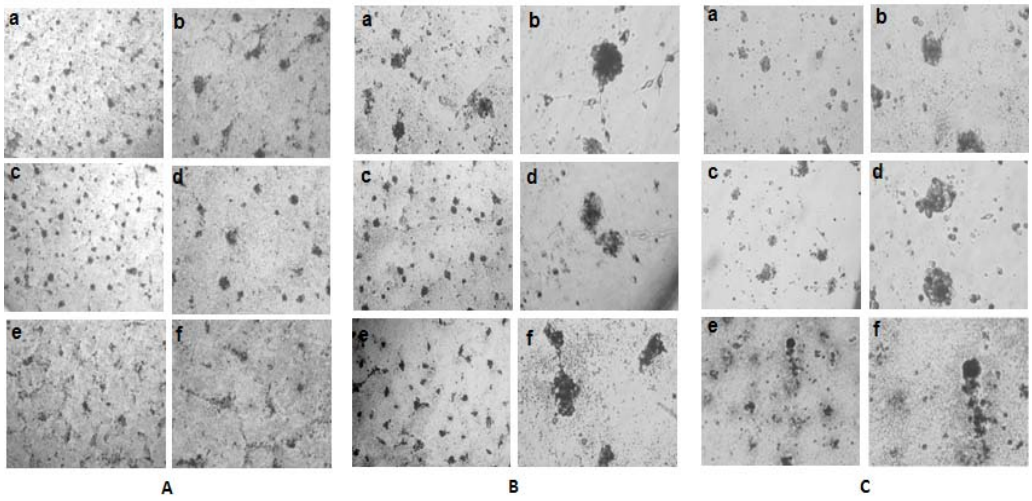

Fig. (2). Bright field images of CE cells on 3rd day (**A**). (a,b) in the presence of E+F; (c,d) in the presence of E+F+V; (e,f) in the presence of VEGF. (a,c,e) is at 10X and (b,d,f) is at 40X. Bright field images of CE cells on 6th day (**B**). (a,b) in the presence of E+F; (c,d) in the presence of E+F+V; (e,f) in the presence of VEGF. (a) is at 20X (c,e) is at 10X and (b,d,f) is at 40X. Bright field images of CE cells on 9th day (**C**). (a,b) in the presence of E+F; (c,d) in the presence of E+F+V; (e,f) in the presence of *VEGF*. (**a**,**c**,**e**) is at 10X and (b,d,f) is at 40X. CE, ciliary epithelium, *EGF* – Epidermal growth factor, *bFGF* – basic Fibroblast growth factor and *VEGF* – Vascular endothelial growth factor.

Table 2. Number of Ciliary Epithelium Derived Neurospheres/Well

	E+F	E+F+V	VEGF	*p* value
3rd day	194±9.4	383±18.8	203±13.8	0.005* 0.006#
6th day	118±13.2	230±10.4	157±13.0	0.005* 0.006#
9th day	118±16.4	119±16.8	54±18.0	0.038† 0.037#

Number of neurospheres/well at various time points (day 3, 6 & 9 of culture) under different combinations of growth factors (E+F, E+F+V & VEGF). Number of neurospheres/well is indicated as mean ± standard error (SE). One way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test was used to analyze the level of significance among the given conditions. Each dataset has been collected from three repeated experiments (n=3) and counting of neurospheres has been done in three adjacent wells for each condition in each experiment by independent masked observer. *, # and † represents significant difference E+F and E+F+V, E+F+V and VEGF, and E+F and VEGF respectively at given time point ($p<0.05$). E, EGF (epidermal growth factor); F, bFGF (basic fibroblast growth factor); V, VEGF (vascular endothelial growth factor).

antibody and 0.4% Triton X100 at room temperature for 30min. These cells were incubated with 10 Ab for over night at 4°C. Different antibodies, source and dilutions included were: anti Brdu at 1:5 (Upstate); anti Nestin (BD bioscience) at 1:100 and anti Pax6 (BD bioscience) at 1:100. 20 Ab incubations were done for one and half hour at room temperature. Secondary antibodies used were, FITC conjugated Goat anti mouse IgG at 1:200 (Jackson) and goat anti rabbit IgG at 1:200 (Jackson).

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc analysis was applied for multiple statistical comparisons of normally distributed data. Normal distribution of data was elucidated using quantile-quantile (Q-Q) plot. Data is shown as mean±standard error of mean (SEM). *p*-value was considered significant at ≤0.05. Real time PCR data is represented as fold change or relative expression of respective mRNA transcript expression of particular molecule. Statistical analyses was performed by Statistical Package and Service Solutions (SPSS) 16 software. No value was excluded from the analysis. Analysis was done by an independent masked observer and validated by another masked researcher.

Results

Proliferation capacity and gene expression profiling of CE stem cells in the presence and absence of *VEGF* was studied to evaluate the effect of *VEGF* on CE stem cells. For this, CE stem cells were grown under proliferation conditions using three different combinations of growth factors. These included *EGF+bFGF* (E+F); *EGF+bFGF+VEGF* (E+F+V) and *VEGF* alone. Gene expression of *Notch*, *Jagged*, *N-cadherin* and *β catenin* was studied at different time intervals i.e., 0, 3, 6 and 9 day of CE cell culture.

VEGF Induced Proliferation of CE Stem Cells

Human fetal pigmented ciliary epithelial cells begin to form neurospheres from day 3 of culture and attain a maximum size at day 6 in the presence of 20 ng/ml *EGF* and 20ng/ml *bFGF*. E+F+V group yielded significantly more number of neurospheres when compared to its control E+F and there was a substantial reduction in neurosphere number in the *VEGF* group when compared to E+F+V group on 3rd day of the culture (Fig. **2A**; Table **2**).

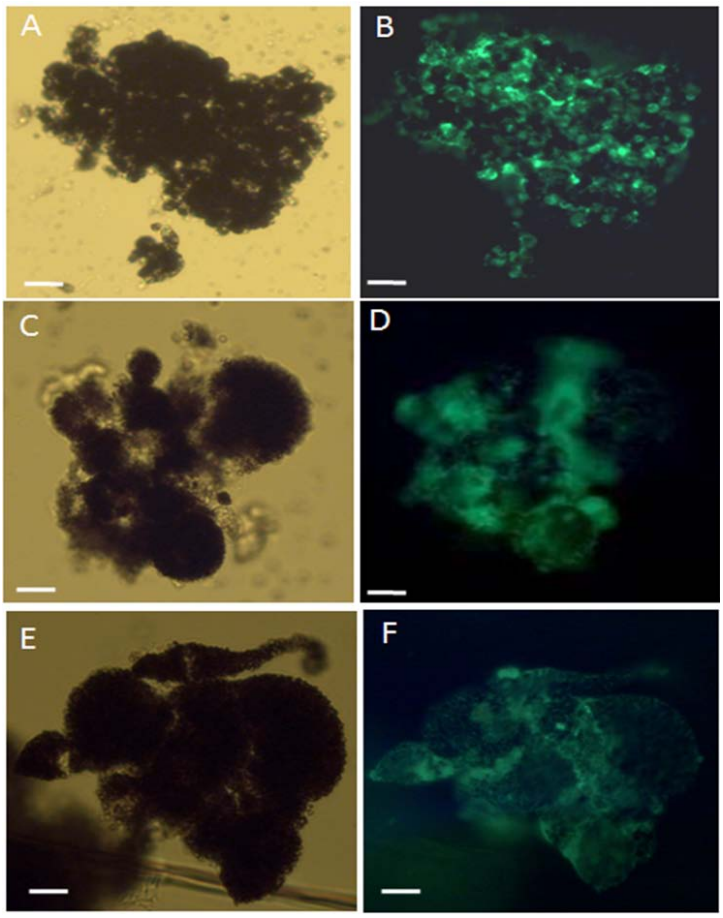

Fig. (3). Human fetal CE derived neurospheres are proliferating and expressing neural/retinal progenitor markers. (**A**,**C** & **E**) Bright field image of CE derived neurospheres on 9th day of culture in the presence of E+F+V. (**B**) Proliferation of CE neurospheres was confirmed by BrdU immunostaining (green immunoflourescence). Neurospheres were pulsed with BrdU 12hrs before harvesting and stained with anti BrdU antibody. (**D**) 9th day CE derived neurospheres showed positive staining for neural progenitor marker Nestin (green). (**F**) Presence of retinal progenitor marker in CE neurospheres were confirmed by immunostaining of Pax6 (Green). CE, Ciliary Epithelium; E+F+V, EGF (Epidermal Growth Factor) + bFGF (basic Fibroblast Growth Factor) + VEGF (Vascular Endothelial Growth Factor); BrdU, Bromodeoxy Uridine. (scale bars in A ,B,C,D,E & F is 3.00µm).

Table 3. Size of Ciliary Epithelium (CE) Derived Neurospheres in Micrometer (µm)

	E+F	E+F+V	VEGF	*p* Value
3rd day	24.3±1.1	23.3±2.0	22.7±3.3	ns
6th day	36.0±3.3	34.4±4.4	31.7±2.9	ns
9th day	36.0±1.2	44.3±5.2	30.8±3.7	ns

Size of neurospheres at various time points (day 3, 6 & 9 of culture) under different combinations of growth factors (E+F, E+F+V & VEGF). Size of neurospheres is indicated as mean ± standard error (SE). One way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test was used to analyze the level of significance among the given conditions on the same day ($p>0.05$). Each dataset has been collected from three repeated experiments (n=3) and size of neurospheres has been measured in three adjacent wells for each condition in each experiment by independent masked observer. E, EGF (epidermal growth factor); F, bFGF (basic fibroblast growth factor); V, VEGF (vascular endothelial growth factor); ns, non significant.

Significantly higher number of neurospheres were observed in a condition where CE cells were exposed to E+F+V as compared to E+F and *VEGF* ($p=0.005$ and $p=0.006$ respectively) on 6th day of CE culture (Fig. **2B**; Table **2**). There was no difference in number of neurospheres between E+F and *VEGF* alone group ($p>0.05$) . On contrary, there was no significant difference in number of neurospheres between E+F and E+F+V conditions on 9th day of CE culture (Fig. **2C**; Table **2**). However, there was a substantial drop in number of neurospheres in *VEGF* alone condition when compared to E+F and E+F+V condition ($p=0.036$ and $p=0.037$ respectively) on 9th day of CE culture. The proliferation of CE cells were also analyzed by BrdU immunostaining on 9th day neurosphere in the presence of E+F+V and further validated by presence of neural and retinal progenitor markers such as Nestin and Pax6 respectively (Fig. **3**).

Size of the neurospheres did not differ significantly among the given conditions (E+F, E+F+V and VEGF) when analysed at different time points i.e. on 3, 6 and 9th day (Table **3**; $p>0.05$).

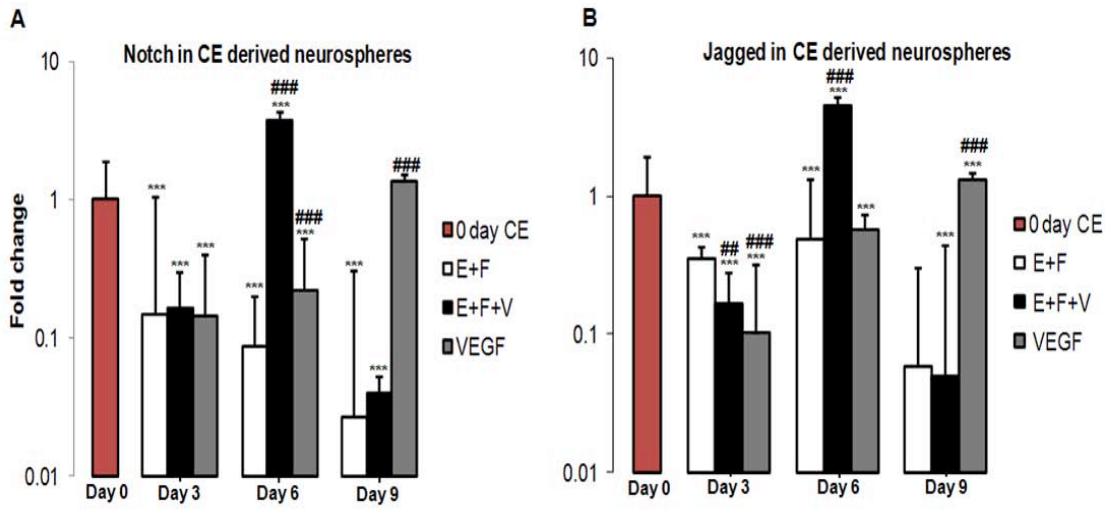

Fig. (4). Relative mRNA expression of *Notch* (**A**) and *Jagged* (**B**) in CE derived neurospheres in different conditions. Fold change along Y axis is presented in log scale. Values were plotted as mean±SE. Data was analyzed by parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. ∗indicate significant difference of given conditions in comparison to 0 day and # represents significant difference within the group as compared to E+F condition ($p<0.05$). mRNA expression of *Notch* and *Jagged* was normalized against endogenous control GAPDH. CE, ciliary epithelium; E, EGF (epidermal growth factor); F, bFGF (basic fibroblast growth factor); **,##$p<0.005$; ***,###$p<0.0005$.

Over the course of time, number of neurospheres decreased from day 3 to day 9 and there was increase in size from day 3 to day 6 irrespective of the conditions used for culture. However, no difference was observed in the neurosphere size between 6th day and 9th day. The present study is limited to 9th day of culture since no significant difference in neurospheres size was observed from 6th day to 9th day of culture, however, future extended studies are recommended to see behavior of neurospheres in culture.

Quantitative Analysis of Effect of VEGF on Gene Expression of Notch1 on CE Stem Cells by qRT PCR at Different Time Points of the Culture:

There was a substantial reduction in *Notch1* mRNA expression in CE derived neurospheres when treated with E+F on 3,6 and 9 days as compared to 0 day CE cells (6.7 fold, 11 fold and 37 fold reduction respectively). Although *Notch1* expression was comparable in 3rd and 6th day neurospheres, expression was significantly reduced by 5.5 fold and 3.2 fold in 9th day neurospheres when compared with 3rd and 6th day neurospheres (Fig. **4A**).

Relative mRNA expression of *Notch* in E+F+*VEGF* treated CE cells on 0, 3, 6 and 9 day revealed that, there was substantial reduction in *Notch1* mRNA expression in CE derived neurospheres when treated with E+F+*VEGF* on 3rd and 9th day neurospheres when compared to 0 day CE cells (6 fold and 24.5 fold respectively). On contrary, 3.7 fold upregulation of *Notch1* mRNA was seen in 6th day neurospheres when compared with 0 day CE cells. When comparison was made between 3rd day and 9th day E+F+V treated neurospheres a significant 4 fold decrease was reported in 9th day neurospheres. *Notch1* expression was increased by 22 fold and 93 fold in 6th day E+F+V treated neurospheres as compared to 3rd day and 9th day respectively (Fig. **4A**).

Analysis of relative mRNA expression of *Notch1* in *VEGF* treated CE cells on 0, 3, 6 and 9 day showed that there was no difference between 0 day cells and 9th day neurospheres. However, a significant 6.5 fold and 4.5 fold reduction was observed on 3rd and 6th day neurospheres when compared to 0 day CE cells. No significant change in *Notch1* expression was seen between 6th and 9th day, and 6th and 3rd day neurospheres. There was 9.3 fold increase in *Notch1* expression in 9th day neurospheres as compared to 3rd day neurospheres (Fig. **4A**).

Enhanced Jagged1 mRNA Expression in CE Cells on 6 Day of Culture

Relative mRNA expression of *Jagged1* in E+F treated CE cells on 0, 3,6 and 9 day showed that there was a substantial reduction in *Jagged1* mRNA expression in CE derived neurospheres in 3 and 9 day neurospheres as compared to 0 day CE cells (2.8 fold and 17 fold respectively). *Jagged* expression was 25.8 fold, 73.1 fold and 445.0 fold higher in 6th day neurospheres as comapared to 0, 3 and 9 day neurospheres (Fig. **4B**).

In the case of E+F+VEGF treated CE cells, there was a significant decrease in *Jagged1* mRNA expression in 3rd day and 9th day CE derived neurospheres as compared to 0 day CE cells (6 fold and 20 fold respectively). On contrary, 36.2 fold upregulation of *Jagged1* mRNA expression was observed in 6th day neurospheres when compared with 0 day CE cells. Although SD was high, we found significant decrease in 9th day E+F+V treated neurospheres when compared to 3rd day E+F+V treated neurospheres. *Jagged1* expression was increased by 218.3 fold and 725 fold in 6th day E+F+V treated neurospheres as compared to 3rd day and 9th day neurospheres respectively (Fig. **4B**).

Quantitative analysis of mRNA expression of *Jagged1* in VEGF treated CE cells on 0, 3rd, 6th and 9th day showed that there was no difference in *Jagged1* expression between 0 day CE cells and 9th day neurospheres. A significant 9.6 fold reduction on 3rd day and 25.4 fold increase on 6th day was observed when compared with 0 day CE cells. *Jagged1*

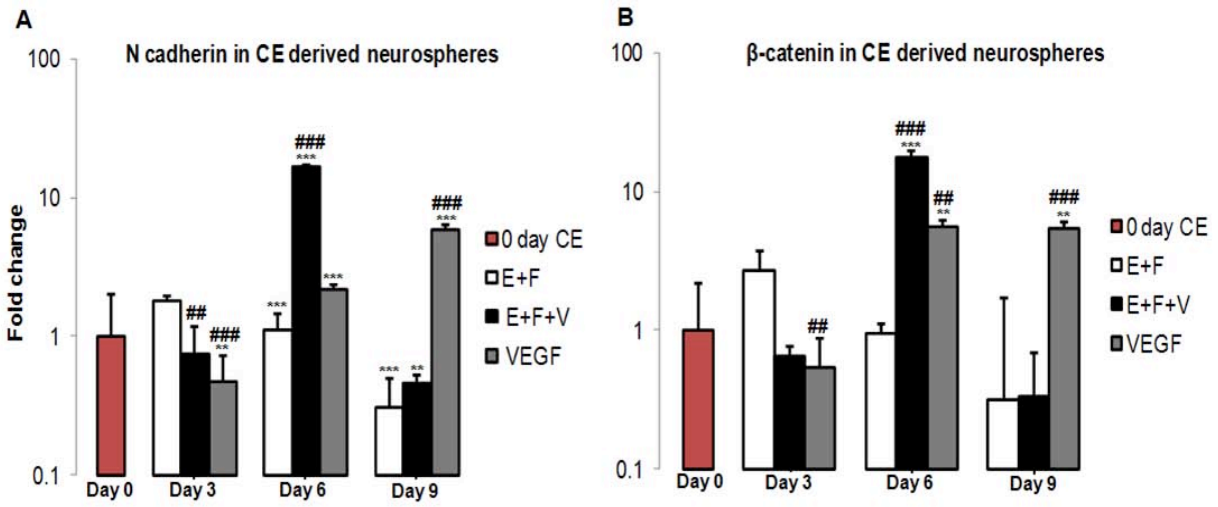

Fig. (5). Fold change in mRNA expression of *N-cadherin* (**A**) and *β-catenin* (**B**) in CE derived neurospheres. Values were plotted as Mean ± SE. Fold change along Y-axis is presented in log scale. Data was analyzed by parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. ∗indicate significant difference of given conditions in comparison to 0 day and # represents significant difference within the group as compared to E+F condition ($p<0.05$). mRNA expression of *N-cadherin* and *β-catenin* was normalized against endogenous control GAPDH. CE, ciliary epithelium; E, EGF (epidermal growth factor); F, bFGF (basic fibroblast growth factor); **,##$p<0.005$; ***,###$p<0.0005$.

mRNA was ~ 25 fold higher in 6th day neurospheres compared to 9th day *VEGF* alone treated neurospheres. There was 12 fold and 244 fold increment in *Jagged1* expression in 9th day and 6th day *VEGF* treated neurospheres as compared to 3rd day *VEGF* treated neurospheres (Fig. **4B**).

The Effect of VEGF on N Cadherin mRNA Expression in CE Cells on 0, 3, 6 and 9 Day of Culture

Effect of *VEGF* on *N cadherin* mRNA shows similar pattern as that of *Jagged1*. No substantial change was observed in *N cahderin* mRNA expression in E+F treated CE derived neurospheres on 3rd day as compared to 0 day CE cells. There was significant 32.6 fold increase in the *N cadherin* expression on 6 day E+F treated CE derived neurospheres as compared to 0 day CE cells and significantly lower expression of *N cadherin* was observed in 9th day E+F treated neurospheres as compared to 0 day CE cells. The mRNA expression was significantly reduced by 5.7 fold and 7.06 fold in 9th day E+F treated neurospheres when compared with 3rd and 6th day E+F treated neurospheres respectively (Fig. **5A**).

Relative mRNA expression of *N cadherin* in E+F+V treated CE cells on 0, 3, 6 and 9 day showed that there was a significant reduction in *N cadherin* expression in CE derived neurospheres when treated with E+F+V on day 9 as compared to 0 day CE cells (2.1 fold). On contrary, 43.7 fold upregulated mRNA was seen in 6th day E+F+V treated neurospheres when compared to 0 day CE cells. There was no difference between day 3 E+F+V treated neurospheres and 0 day CE cells. *N cadherin* mRNA expression remained unchanged, when comparison was made between 3rd and 9th day for E+F+V treated neurospheres. A significant 58.3 fold and 94 fold increase in expression was reported in 6th day E+F+V treated neurospheres as compared to 3rd and 9th day E+F+V treated neurospheres respectively (Fig. **5A**).

Analysis of relative mRNA expression of N-cadherin in *VEGF* treated CE cells on 0, 3, 6 and 9 day showed that although there was a 2 fold downregulation of N-cadherin expression in 3 day *VEGF* treated neurospheres, a significant 41.3 fold and 5.8 fold upregulation was observed on 6th and 9th day *VEGF* treated neurospheres when compared to 0 day CE cells. 7.03 fold increase in expression was observed in 6th day *VEGF* treated neurospheres as compared to 9th day *VEGF* treated neurospheres and 86.5 fold increase on 6th day *VEGF* treated neurospheres as compared to 3rd day neurospheres. There was significant 12.25 fold increase in *N-cadherin* expression on 9th day *VEGF* treated neurospheres as compared to 3 day *VEGF* treated neurospheres (Fig. **5A**).

β Catenin mRNA Expression is Upregulated when CE Cells are Treated with VEGF at 6 Days of Culture

When relative mRNA expression of *β catenin* was compared between 0 day CE cells and E+F treated 3, 6 and 9 day neurospheres, no substantial change was observed among the groups. Expression of *β-Catenin* on 3rd day and 9th day did not significantly differ from 6th day neurospheres when treated with E+F. The expression was significantly reduced by 8.3 fold in 9th day neurospheres when compared with 3rd day E+F treated neurospheres (Fig. **5B**).

There was a significant increase in *β-Catenin* expression in CE derived neurospheres when treated with E+F+*VEGF* on day 6 as compared to 0 day CE cells, 3rd day and 9th day E+F+V treated neurospheres (17.5 fold, 27.3 fold and 53 fold respectively). There was no difference between day 0 CE cells, 3rd day and 9th day E+F+V treated neurospheres (Fig. **5B**).

Although there was significant 5.5 fold upregulation of *β-Catenin* expression in 6th and 9th day VEGF alone treated neurospheres, a non significant 2 fold downregulation was observed on 3rd day VEGF alone treated neurospheres compared with 0 day CE cells. No significant change in *β-Catenin* expression was seen between 6th and 9th day *VEGF* treated neurospheres. When induced with *VEGF* alone, 10

fold rise in *β-Catenin* expression in 6th and 9th day as compared to 3rd day neurospheres was observed (Fig. **5B**).

DISCUSSION

We demonstrate the effect of *VEGF* on expression of *Jagged, Notch, N-cadherin* and *β-Catenin* of CE stem cells from human fetal eyes**.** Increased number of neurospheres in the presence of *VEGF* along with *bFGF* and *EGF* indicates that *VEGF* promotes proliferation of CE stem cells in tendem with other growth factors. However, *VEGF* alone did not increase the number of neurospheres. These results are in agreement with the previously published report of the effect of VEGF on neural stem cell proliferation [23]. BrdU positive cells in our neurospheres suggests that neurospheres are formed by clonal multiplication, however existing literature suggests that neurospheres can be formed by clonal multiplication as well as aggregation of stem cells depending on initial seeding density of the cells [24-28]. Further, these neurospheres are primarily composed of neural and retinal stem cells as shown by Nestin and Pax6 immunostaining. Our finding of temporal decrease in neurosphere number and their increase in size from day 3 to day 9 suggests a possible inverse association between number and size of neurospheres and indicates that the aggregation of individual cells may be one of the potential contributor to neurosphere formation apart from proliferation of CE stem cells. However, future temporal cell death analysis is needed to account for reduced neurospheres with time.

The increased expression of *Notch1* receptor in the presence of *VEGF*, *EGF* and *bFGF* indicates increased proliferation of CE stem cells. It is, therefore, possible that enhanced mRNA expression of *Notch1* on 6th day in *EGF*+*bFGF*+*VEGF* (E+F+V) group may result in increased number of neurospheres with the same conditions. Though, there was a trend towards increased *Notch1* expression in *VEGF* alone on 6th day, it was not as pronounced as in the case of (E+F+V) group. It was earlier reported that, *bFGF* enhances the expression of *VEGFR2* (*Flk1*) via phosphorylation of ERK1/2 pathway [29] and *Flk1* is the major receptor for *VEGF* localized to a variety of cells including retinal progenitors and ciliary epithelial cells [30]. It is possible that increased Flk1 on CE cells by *bFGF* stimulation is essential for *VEGF* mediated upregulation of *Notch1* as evidenced in E+F+V group. The same effect was lacking in *VEGF* alone group due to absence of necessary induction by *bFGF*. Existing reports show that apart from *VEGF*, *bFGF* can also enhance *Notch1* expression [31]. To rule out the possibility that increased expression of *Notch1* is predominantly mediated by *VEGF* instead of *bFGF*, a control with *EGF* and *bFGF* was used. The expression of *Jagged1*, the canonical ligand of *Notch1* receptor, followed a similar trend. It can be argued that *Notch1* expression was increased in response to elevated *Jagged.* Existing literature also suggests the aggravation of *Notch1* signaling in response to increased *Jagged1* [32]. The increase in *Jagged1* in presence of E+F+*VEGF* on 6th day is partly inconsistent with the report of Kiec-Wilk *et al*, where authors observed that *bFGF* inhibits *Jagged1*. However the same group also reported an upregulated *Jagged1* in the presence of *VEGF* [33].

It has been seen that lack of proper diffusion of nutrients in the core of neurospheres and hypoxia may induce differentiation of neural stem cells in a time dependant manner [34]. Expectedly, the decreased expression of *Notch1*/*Jagged1* was observed on 9th day in both E+F+V group and control group (E+F). We are unable to rule out that 9th day neurospheres contained differentiated cells with proliferating foci localized in the periphery. It therefore, seems reasonable to suggest that *Notch1* expression may be restricted to peripheral cells because these cells suitably respond to *bFGF* and *VEGF*, and may account for the observed results. The reduced *Notch1* levels are further confirmed by the finding that the size of neurospheres remained constant with time (across 6th and 9th day). Although the size of the neurospheres was comparable between 6th and 9th day, *Notch1* expression was pronounced at day 6 and may account for higher state of proliferation.

Notch1 expression in *VEGF* treated neurospheres was significantly higher as compared to E+F+*VEGF* on 9th day. It has earlier been reported that *bFGF* helps in differentiation of different types of stem cells including neural stem cells via p38 MAPK pathway [35]. Conversely, increased expression of Jagged1/*Notch1* was observed on inhibition of p38 MAPK pathway which in turn maintains the cells in proliferative stage. As neurospheres are heterogeneous in nature, having both immature and mature cells, it is anticipated that E+F+*VEGF* neurospheres have higher proportion of mature cells partly contributed by presence of *bFGF* in a temporal manner as compared to *VEGF* alone neurospheres which are composed of relatively less mature and highly proliferating cells. Additionally, activation of *Notch* signaling is known to reduce levels of *VEGF*R2 on cultured human embryonic cells [36]. It is likely that increased expression of *Notch1* on 6th day causes reduction in *VEGF*R2, expressed by CE stem cells. Reduced *VEGF*R2 will further hinder *VEGF* mediated increase in *Notch1* expression. Based on these reports, feedback inhibition of *Notch1* on 9th day is indicated.

Like *Notch1* and *Jagged1* the increased levels of *N-cadherin* in the presence of *EGF* and *bFGF* on 6th day suggests the pivotal role of cell adhesion in cell proliferation. These findings are in consensus with existing report where authors showed that *bFGF* induces mRNA expression of *N cadherin* via protein kinase C and *Src* kinase pathways as inhibitors of protein kinase C demolish the observed increase in N cadherin [37]. There was significant upregulation of *N Cadherin* in presence of E+F+*VEGF* as compared to E+F on 6th day. We believe that *VEGF* complements *bFGF* induced proliferation by enhancing *N cadherin* expression of CE cells. This is evidenced by increased number of neurospheres (Fig. **2**; Table **2**). The downstream cascade by which *VEGF* upregulates *N cadherin* is not clear and needs more investigations, however, *VEGF* is known to decrease protein expression of VE cadherin *via* Src dependant phosphorylation of guanine nucleotide exchange factor [38]. As *N cadherin* is known to upregulate *β-catenin* via *Wnt* pathway [39] and participates in the proliferation of CE stem cells we also analysed the *β-catenin* levels of these CE cells. The *β-catenin* mRNA levels showed similar expression presumably induced by *N cadherin* on 6th day.

Recently, it was reported that *in vivo* knockdown of *N-cadherin* results in reduced *β–catenin* signaling arresting cell proliferation and promoting neuronal differentiation [20].

The phenomenon of contact inhibition after prolonged stimulation of neurospheres in culture (9^{th} day) is known to initiate differentiation of proliferating cells. It is, hence, speculated that the decreased levels of *N-cadherin* and *β-catenin* in E+F+*VEGF* group on the 9^{th} day were a result of contact inhibition. A similar trend of *N cadherin* and *β-catenin* expression levels was seen in E+F group. On the contrary, Gao *et al* reported increased mRNA and protein levels of *N-cadherin* and *β-catenin* during neuronal differentiation of embryonic carcinoma PC12 cells suggesting that *N-cadherin* may play a role in differentiation mediated via *Wnt* and *β-catenin* signaling [40]. A significant upregulation of *β –catenin* on 9^{th} day in presence of *VEGF* is in agreement with existing literature. It has earlier been observed that *VEGF* increases the levels of *β–catenin* mediated by protein kinase C (PKC) and helps in maintaining endothelial cells in proliferative stage [41].

Altered expression of candidate genes at different time points may indicate time dependent change in the proportion of proliferating and differentiating PCE stem cells inside the neurospheres. Our findings emphasize the importance of real time ananlysis, as the biological outcome is primarily mediated by signaling pathways and is chiefly governed by gene expression changes can be totally different, depending on the cellular context and stage of differentiation [42, 43].

Since it is known that VEGF is secreted by both CE stem cells as well endothelial cells of microvessels in the vicinity of CE i*n vivo* and expression of VEGF receptor (VEGFR) 1 & 2 has already been observed on CE cells [44], it will therefore be interesting to investigate if and how VEGF might act as an paracrine and/or autocrine fashion in these stem cells to induce proliferation.

At the moment, it is difficult to conclude whether there is a causal relationship between altered expression of *Jagged1, Notch1*, *N-cadherin* and *β–catenin* in response to VEGF and proliferation of CE cells or it is coincidental. Nevertheless, the data may suggest the possible involvement of these factors in proliferation of CE stem cells in a time dependant manner and lays the foundation for further comprehensive analysis of other determinants such as Sonic Hedgehog and SCF/c-kit pathways alongwith VEGF-VEGFR1 & 2. It will be interesting to screen downstream signalling molecules of these pathways including Notch Intracellular Domain (NICD) and HES (1 or/and 5) for Notch pathway; Frizzled1 (FZD1) for Wnt pathway; PTCH1 and GLI1 for Hedgehog pathway; SCF, c-kit, AKT and JNK/ERK for SCF/c-kit pathway. Moreover, inhibition using antagonists such as Dkk, an inhibitor of Wnt pathway; GSI, an inhibitor of Notch pathway; ZM323881, PP2, Herbimycin A, inhibitors of VEGF pathway and PD173074, an inhibitor of FGF2 receptor, and loss of function experiments for VEGF can establish direct association of VEGF with these signalling pathways. The harvesting time of stem cells should be taken into consideration before attempting clinical translation for possible therapeutic intervention.

ETHICAL APPROVAL

Ethical approval was obtained by institute ethical committee, PGIMER, Chandigarh, India – 160012.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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AUTHOR'S CONTRIBUTIONS

CA, Acquisition of data and writing of manuscript; SP, Grant Co PI; JK, Sample recruitment; AH, Sample recruitment; AA, Interpretation and analysis of data, grant PI and editing of manuscript.

ABBREVIATIONS

CE	=	Ciliary epithelium;
PCE	=	Pigmented Ciliary Epithelium;
VEGF	=	Vascular Endothelial Growth Factor;
EGF	=	Epidermal Growth Factor;
bFGF	=	Basic Fibroblast Growth Factor.

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PROSPECTS
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Neural Stem Cells—Trends and Advances

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ABSTRACT

For many years, accepted dogma held that brain is a static organ with no possibility of regeneration of cells in injured or diseased human brain. However, recent preclinical reports have shown regenerative potential of neural stem cells using various injury models. This has resulted in renewed hope for those suffering from spinal cord injury and neural damage. As the potential of stem cell therapy gained impact, these claims, in particular, led to widespread enthusiasm that acute and chronic injury of the nervous system would soon be a problem of the past. The devastation caused by injury or diseases of the brain and spinal cord led to wide premature acceptance that "neural stem cells (NSCs)" derived from embryonic, fetal or adult sources would soon be effective in reversing neural and spinal trauma. However, neural therapy with stem cells has not been realized to its fullest extent. Although, discrete population of regenerative stem cells seems to be present in specific areas of human brain, the function of these cells is unclear. However, similar cells in animals seem to play important role in postnatal growth as well as recovery of neural tissue from injury, anoxia, or disease. J. Cell. Biochem. 114: 764–772, 2013.

KEY WORDS: NEURAL STEM CELLS; MESENCHYMAL STEM CELLS; NEUROGENESIS; REGENERATION; BIOTHERAPEUTICS

The signals generated and transmitted in response to stimulus are necessary for communication between complex networks of neurons, which once disrupted, cannot be restored. In vitro, differentiation of neuronal–like cells from putative stem cells often is verified by morphology, wherein the cultured cells emit projections that resemble those of neurons derived from animals. However, it is now clear that many antigens considered specific for neural tissue could be expressed by many other cells, especially endothelial cells and monocytes [Vescovi et al., 1993]. Furthermore, nestin, the neuroepithelial stem cells marker seems to be expressed in most of the mitotically active cells. Suggestions regarding the existence of dividing cells in the postnatal central nervous system (CNS) were raised in 1901 by Hamilton. Ramon and Cajal [1913] suggested that neurons are generated exclusively during prenatal phase of development. Kaplan and Hinds [1977], and Kaplan and Bell [1984] proved that new born neurons in hippocampus survived for long periods of time, appeared to receive synaptic inputs and also extend projections to their target area. While, Reynolds and Weiss [1992] in 1992 isolated adult NSCs from adult CNS of rodents, Kukekov et al. [1999] isolated NSCs from human embryo. Belluzzi et al. [2003] showed that the new born neurons in adult mammalian CNS are indeed functional and synaptically integrated.

In 1983, Nottebohm et al. demonstrated the genesis of neurons in the telencephalon of adult male songbirds. In addition, it was found that acquisition of new neurons is hormonally controlled and therefore seasonally regulated, corresponding to the mating season of singing songbirds [Nottebohm, 1981]. The events may thus be independent but stimulated by common factors that arise during seasonal alterations of the environment.

Studies by the group of Stevens and Gage in 2002 indicated that adult NSCs indeed form functional neurons and do not simply express protein markers specific to differentiation. By recording electrical signals of the cultured cells, they showed that these fluorescently labeled precursors formed dendrites and synapses in the rat brain, challenging the dogma that neurons are not replaced in brain [Song et al., 2002]. The adult brain maintains discrete parts of neurogenesis, new neurons migrate from these parts and become integrated into the functional circuitry of the brain. These multipotent stem cells are present in various regions of the brain including the cortex [Marmur et al., 1998], the subventricular zone (SVZ) [Levison and Goldman, 1997] and the ventricular zone [Cai et al., 2002]. NSCs produce neuroblasts that migrate from the SVZ along a separate pathway, the rostral migratory stream; the mature neurons involved in the sense of smell are formed in to the olfactory bulb [Lennington et al., 2003].

Conflicts of interest: None.

Denis English and Neel K. Sharma contributed equally to this work.

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NEUROGENESIS AND TRANSDIFFERENTIATION IN BRAIN

Positional effects of NSCs can be appreciated by the observation that SVZ neuronal precursors have a bias to form olfactory bulb derivatives [Gritti et al., 2002] spinal cord neuron restricted precursors give rise to cholinergic neurons [Kalyani et al., 1998] and hippocampal neuronal progenitor cells develop CA1 and CA3 neurons [Regnell et al., 2012]. It is important to understand the timing of developmental restrictions that occur while identifying the optimal source of stem cells for transplantation. Such phenotypic restrictions occur early in development and cells can be isolated based on characteristics unique to that cell population which may vary according to applications for different neurological disorders.

In some transplant experiments, it has been shown that CNS stem cells will not populate the peripheral nervous system (PNS) and vice versa [Moreno and Fraser, 2002]. NSCs are part of CNS that have the capacity to self-renew and give rise to astrocytes, neurons, and oligodendrocytes. Oligodendrocyte precursor cells and Schwann cells of the CNS have the capability to remyelinate the injured CNS axon. In contrast, remyelination of injured axon in multiple sclerosis (MS) is limited. In addition to this, boundary cap cell which is the type of PNS stem cell population can differentiate into CNS as well as PNS lineage [Reynolds and Weiss, 1992; Fawcett and Asher, 1999; Zujovic et al., 2011]. Neural crest stem cells (NCSCs) are likely regionally specified and the differentiated progeny of NCSCs and olfactory stem cells appears to be capable of enhancing CNS repair and regeneration [Bunge, 2002]. Indeed dramatic results have been reported with OEC (olfactory ensheathing glia) transplants in spinal cord injury models and it is likely that these cells can be harvested from the adult neuroepithelium and amplified in vitro. Undifferentiated embryonic stem (ES) cells may have limited use for therapy due to their propensity to form teratomas while the ES cells derived differentiating neural progenitors and also the matured ones can be valuable for therapy provided they are properly depleted of undifferentiated cells. Depending on the role of transplanted cells, one would choose a cell type based on their properties and ease of availability and isolation. While attempting to replace neurons, as in the case of ALS, one would choose a neuronal precursor, a multipotent stem cell, or a glial cell. Using the human ESC-based ALS model, the Eggan's group has revealed that inhibition of signaling through the classic prostaglandin D2 receptor suppresses the toxic effect of SOD1 glia on motor neurons, hence providing a target for developing new methods of cure for ALS [Giorgio et al., 2008].

It is generally believed that the adult bone marrow cells can bring about the required changes in the tissue adjacent to the site of implantation repopulating the cell lineages during lifetime. A plethora of recent results from many groups suggests that adult stem cells may have a broader differentiation capacity than expected and that their fate may not be as tissue specific as once thought. It has been shown that adult NSCs can differentiate into a broad range of cells of different sources when introduced into myogenic cells and blastocyst. Moreover, skeletal muscle, brain and hepatic cells can give rise to bone marrow stem cells, whereas blood cells can generate from muscle precursors [Vescovi et al., 2002]. However, many argue that original source of tissue used in these studies was contaminated by blood and the hematopoietic stem cells in that blood gave rise to the blood cells formed. There are some reports which indicate that the structures do not develop from a single primordial stem cell but arise from the coordinated and dynamic interactions of many stem cells in what amounts to a ''stem system,'' similar to primitive buds that give rise to limbs amputated from primitive animals. Others believe that in response to distal injury, cells of the sub lamina undergo reverse differentiation and then differentiate to form viable tissues. Lately, induced pluripotent stem cells (iPSCs), are a type of artificially derived pluripotent stem cell from a non-pluripotent cell (from adult somatic cell), by providing the inducing medium to ''forced'' expression of specific genes, have been using for the same.

SOURCES OF NSCS

Large-scale resources of NSCs are crucial for both basic research and for the development of novel approaches for treating neurological disorders. NSCs primarily arise from embryonic ectoderm that forms neuroepithelial cells. The neuroepithelial cells generate radial glia that produces fetal and adult NSCs within CNS [Weiner, 2008]. The alternative sources of neural stem cells are shown in Table I [Nakatsu et al., 2005; Ryan et al., 2007; Robertson et al., 2008; Amit et al., 2010; Polo et al., 2010; Julius et al., 2011; Uri and Benvenisty, 2011]. Cells including multiple subtypes of CNS and PNS neurons, as well as oligodendrocytes, Schwann cells, and astrocytes, are modeled in these large-scale sources. Although most cell lines were initially from rodents, their human counterparts are being characterized and discovered. The prominent regions in the mammalian brain that have a reservoir of stem cells include the ependymal lining, SVZ and the olfactory bulb [Gritti et al., 2002]. The ciliary margin and the limbal regions of the retina have also been shown to be rich in NSCs which have been expanded in culture and shown to grow into neurospheres, many of which have been implanted in animal models to differentiate into retinal neurons. Until recently, it was widely believed that the marrow did not contain any non-hematopoitic cells. Recently, these cells have been recognized as the colony forming fibroblasts and mesenchymal cells. The latter type of cells have also been shown to be expressed in Umbilical Cord, cord blood as well as other tissues and have been found to be useful in neural regeneration [Song and Ramos, 2008]. Mesenchymal stem cells (MSCs) are multipotent stem cells possessing the intrinsic ability to differentiate into different types of cells that include osteocytes, adipocytes, and chondrocytes. MSCs have been postulated to generate cells of the mesoderm, endoderm and ectoderm, including neurons in culture depending of the inducing agents used. Interestingly, progeny of human MSCs infused after ectodermal differentiation has been identified in brains of mice and other animals. Identification of these cells as human is evident by a marker, only the infused cells possess due to their tagging with fluorescent reporter genes such as green fluorescent protein. Furthermore, the validation of these human cells as neural cells relies on immune localization of specific antigens such as nestin, S-100β, Sox2, Map2, GFAP, etc. In vitro MSCs have been

TABLE I. Comparison Between Different Types of NSCs Cells

Types of stem cells	Sources	Advantages	Disadvantages	Refs.
Embryonic NSCs	Embryonic CNS	Non-tumorigenic, committed neural lineage, and regionally specific	Difficulty in long-term preservation and ethical considerations	Julius et al. [2011]
NSCs derived from ES cells	Blastocyst inner cell mass	These cells have the pluripotent, unlimited differentiation, and stable karyotype	Tumorigenicity, ethical considerations and need to commit as neural specification	Amit et al. [2010], Uri and Benvenisty [2011]
Non-NSCs	Umbilical cord, blood, skin, bone marrow, etc.	No ethical consideration, abundant available supply and generate autologous cells	Require neural specification and restricted potential to differentiate in different cells	Stewart and Przyborski [2002]
Adult NSCs	Subventricular zone of Hippocampus	Committed neural lineage	Restricted potential to differentiate, limited availability and difficulty in long-term preservation	Robertson et al. [2008]
Induced pluripotent stem cells (iPSCs)	Adult somatic cells	Genetically matched cell lines, No ethical consideration, easier to create	Patient specific cell lines	Polo et al. [2010]
Mesenchymal stem cells (MSCs)	Wharton's jelly, bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth and umbilical cord	No ethical issues, Immuno-privileged and therefore most beneficial for allogenic transplantation, reduced risks of rejection and complications of transplantation, isolated and easily expandable	Require neural specification, and highly heterogeneous nature isolates	Ryan et al. [2007]
Oncogene immortalized cell lines	PC-3, HeLa and Jurkat cell lines, cancer patients	Organ-specific genes which work as the sensitive or resistant factors	Contain numerous genetic mutations and exhibit an unstable genotype	Nakatsu et al. [2005]

shown to express properties of neuroectodermal cells by researchers and in vivo after transplantation into spinal cord and the brain [Mazzini et al., 2010]. NSC transplantation may be hampered by the limited number of donors available and by the toxicity of immunosuppressive regimens that may be needed after allogeneic transplantation. These limitations may be avoided if NSCs can be generated from clinically accessible sources, such as bone marrow (BM) and peripheral blood samples that are suitable for autologous transplantation. Fu et al. [2008] have reported that NSCs can be generated from human BM-derived mesenchymal stem cells (MSCs). When cultured in NSC culture conditions, 8% of MSCs were able to generate neurospheres. These MSC-derived neurospheres expressed characteristic NSC antigens, such as nestin and musashi-1, and were capable of self-renewal and multilineage differentiation into neurons, astrocytes, and oligodendrocytes. More recently, dental pulp was shown to possess a pool of stem cells that were expanded in culture which upon differentiation acquired a neural fate [Huang et al., 2008]. These had earlier been used for regeneration of dental and craniofacial cells. Spinal cord-derived NSCs have also been isolated and characterized and search for new sources continue to add to an increasing knowledge base in this area.

DETECTION AND MANIPULATION OF NSCS

Mice and humans studies have exposed a important developmental occurrence of aneuploid NSCs, whereas other chromosomal defects, such as inter-chromosomal translocations and partial chromosomal deletions/insertions, are extremely rare.

"Cre-Lox" systems in mice combined with other genetic markers empower researchers to track differentiation markers that are expressed in the growing brain. Neurosphere formation, which is characterized by aggregates of similar looking cells in culture, is another established system of screening the NSCs by virtue of their immunoreactivty with markers specific to NSCs although absence of differentiation markers doesn't necessarily indicate absence of differentiation or vice versa [Yang et al., 2005]. However, because this assay may choose and enlarge a heterogeneous stem/progenitor cell population, rigorous clonal, and serial subcloning analyses are required to detect and document stem cell activity and to unequivocally identify bona fide stem cells (see Fig. 1). Oncogene immortalized cell lines are the beginner's tool to dissect the proliferation and differentiation cues in culture. In fact, recent development of a magnetic bead based assay (Milteny Biotech, Inc.) allows sorting of putative NSCs using a cocktail of antibodies specific for markers expected to be selectively expressed by these cells. Similarly, the study of migration of these cells requires accurate mapping of the traffic of NSCs in the mammalian brain. The migration of stem cells to an injured or infarcted region of the brain requires mobilization, and it is imperative to mark these donor cells in order to differentiate them from resident stem cells. Various methods of labeling exist that include the use of genetically tagged NSCs or Y chromosome labeling that employs donor NSCs from male cells transplanted into female recipients. Lately, several dyes are being used for such types of labeling. Prominent among these is the CFDA-SE label. CFDA is an ester which diffuses into the cell where it

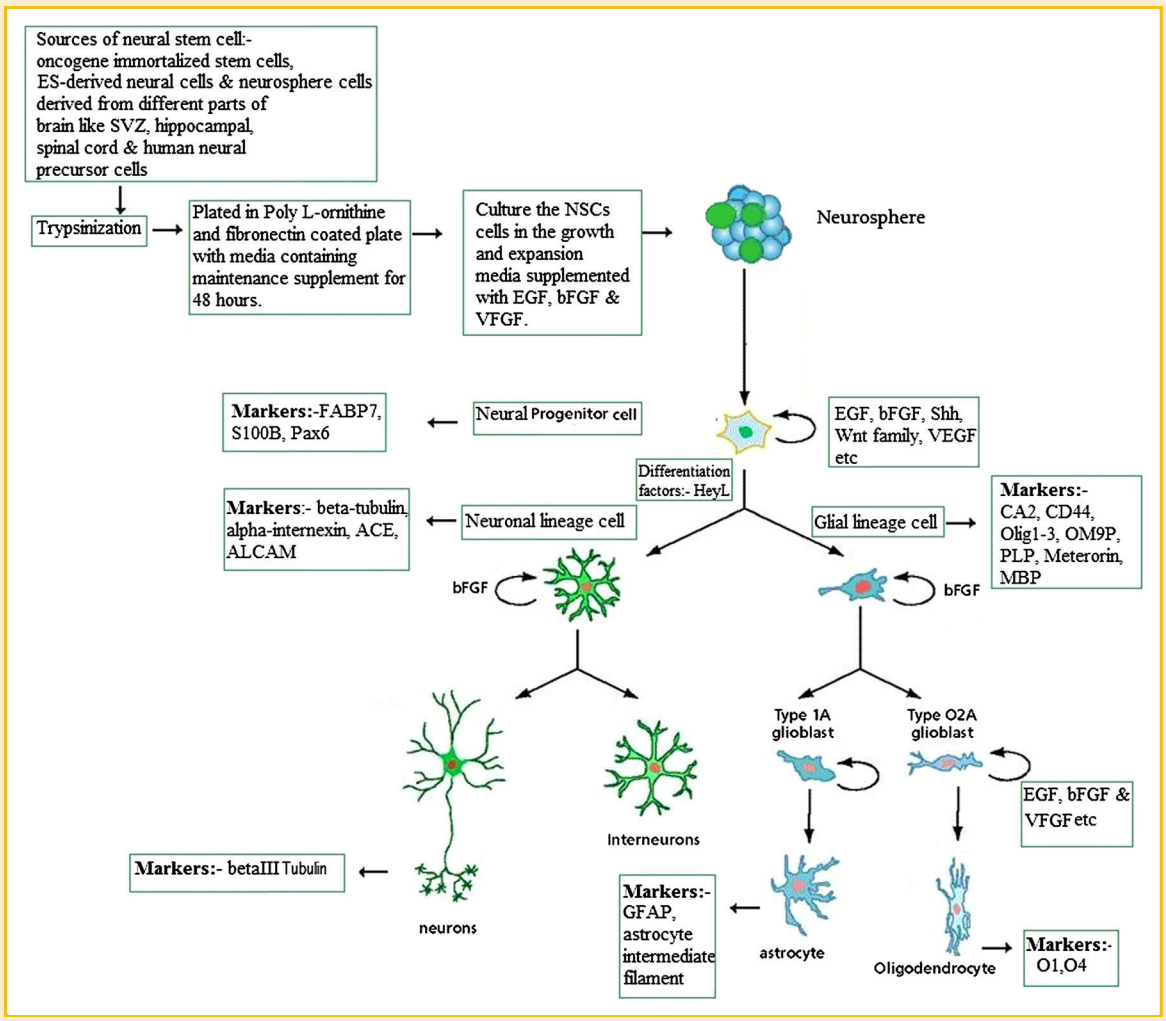

Fig. 1. Schematic representation of manipulation of neural stem cells. SVZ, subventricular zone; NSCs, neural stem cells; EGF, endothelial growth factor; bFGF, basic fibroblast growth factor; VFGF, viral fibroblast growth factor; Shh, sonic hedgehog; VEGF, vascular endothelial growth factor; FABP, fatty acid binding protein 7; S100B, S100 calcium binding protein B; PAX6, paired box protein Pax-6; ACE, angiotensin I-converting enzyme; ALCAM, activated leukocyte cell adhesion molecule; A2B5, type 2 astrocyte precursor marker; Olig1, oligoden drocyte transcription factor-1; Olig2, oligoden drocyte transcription factor-2; Olig3, oligoden drocyte transcription factor-3; PLP, proteolipid protein; MBP, myelin basic protein; O2A, oligodendrocyte-type-2 astrocyte; GFAP, glial fibrillary acidic protein; O1, oligodendrocyte marker O1; O4, oligodendrocyte marker O4; CA2, carbonic anhydrase 2.

is acted upon by endogenous esterases which liberate secondary products that interact with amines and fluoresce at the similar wavelength as GFP and imparts green fluorescence to the transplanted cell. There has been an equally rapid development in the field of cell marker analysis which characterize particular stem cells and enables sorting of these cells from various sources, based on the expression of neural antigens such as nestin and musashi. Two photon microscopy also has been used and represents a powerful tool that has greatly empowered analysis by allowing in vivo imaging of cells [Wang et al., 2006]. Many argue that the neuronal regeneration has no meaning unless there is functional revival of the affected region of brain. Functional MRI and electrophysiological measurements are powerful tools which allow localization of functional neurons in vivo and in vitro, respectively. Investigators have shown that human NSCs express both outward and inward K(+) currents with no evidence of Na(+) currents [Cho et al., 2002] and are useful in evaluating the potential for clinical translation. Similarly, electroretinograms enable assessment of regenerative capacity of stem cells in the eye and are very effective in strengthening the detection of regenerating neurons induced pharmacologically.

REGULATION OF NEURAL STEM CELLS: INTRINSIC CUES VERSUS GROWTH FACTORS

The reductionist view is the heart of breakthroughs in stem cell biology as it allows the clear definition of stem cell characteristics using genetic and molecular tools. The NSCs acquire a neural fate in response to environmental signals, leading to migration or differentiation into defined phenotypes. Apart from genetic signals that shape the pluripotency of these cells, cytokines and growth

factors, particularly bFGF, EGF, and VEGF play prominent role in proliferation and differentiation of the neural proginator cells. Lately, several researchers have provided credible evidence that angiogenesis and neurogenesis engage in a cross talk [Carmeliet and Tessier-Lavigne, 2005; Sharma et al., 2009]. Nakatomi et al. [2002] have shown that the infusion of EGF and FGF-2 into the lateral ventricle of the rat model of ischemia, in which CA1 neurons are selectively lost, leads to recovery of memory and learning functions with concomitant regeneration of pyramidal neurons due to neurogenesis. This has facilitated the discovery of factors that would enable desirable neurogenesis (or angiogenesis). For example, Gage's group showed that enriched environment and exercise improves neurogenesis and learning lending credence to the hope that environment factors can greatly influence the rate of neurogenesis [Van Praag et al., 2005].

Are we entering an era where cellular therapy may finally be able to reverse some of the disorders of brain. NSCs are becoming attractive tools for advancement of cellular therapy in neurodegenerative disorders. NSCs derived from fetuses have also been successfully used for symptomatic treatment after long-term follow-up in PD (Parkinson's disease) patients and functional improvement in patients who received fetal striatal grafts for treatment of Huntington's disease was remarkable [Claire et al., 2008]. PD is the neurodegenative disorder of CNS resulting from the death of substantia nigra (SN) which is located in midbrain. The consequence of SN cell death results in dopamine deficits with accompanying symptoms like movement rigidity, dementia, sleep disorders, and psychological problems, etc. So far, the most effective transplantation strategies concern the paracrine systems in which the affected cells exert modulatory actions on target circuits such as in the case of PD. More requirements have to be met in cases such as focal ischemia where it may be possible to rebuild the anatomical matrix. However, a long distance connection may be difficult to recreate. As a consequence, the behavioral benefits may be attributed to the tropic effects of transplants as the rewiring of the disrupted circuits may be necessary but not sufficient. Even the long-term follow-up of PD patients has shown that the limited functional recovery of such patients comes long after anatomical repair [Gogel et al., 2010]. Cell transplantation should be more accessible where degeneration affects a restricted area, such as in the case of PD, where transplants distributed across large areas require multiple transplantation approaches and have to rely on targeted migration of transplanted cells.

INJURY INDUCES STEM CELL RECRUITMENT

One important property that NSCs possess is migration. They were once thought to be more suited to deliver substances to specific sites in the brain than for regeneration. They appear to home to ischemic and neoplastic areas of brain and at least three physiological processes such as angiogenesis, reactive astrocytosis and inflammation invite their presence. Chemokines such as VEGFR1/R2,VEGF, Ccl2, and cKIT have been reported to be involved in NSC tropism [Chen et al., 2011] and studies have shown that VEGF mediated homing of cells may play a prominent role in the same [Chyi et al., 2010]. There is growing evidence to suggest that there is intimate relationship between CNS morphogenesis and endothelial cells; the basal lamina produced by endothelial cells contains many components that are supposed to be important for the maintenance of a neurogenic niche. Even SDF1 is expressed by both endothelial cells and astrocytes in stroke lesions and could be important for NSCs mobilization. Animal studies discussed below show how the lesion or damaged brain or retina mobilizes stem cells to damaged areas.

MECHANISMS INVOLVED IN NSC BASED REGENERATION

Neural stem cells (NSCs) are heterogeneous population of cells which are mitotically active. These cells have self-renewing and multipotent capacity which shows the differential pattern of gene expression at different times at various damaged regions of Brain [Gage, 2000; Temple, 2001; Ivanova et al., 2002]. Understanding the mechanisms of NSCs are important because these may be critical for driving clinical applications. NSC based investigations for different CNS disorders, for example, PD [Tonnesen et al., 2011], Huntington's disease [Connor, 2011], MS [Carbajal et al., 2010], retinal ganglion cell degeneration [Bull and Marti, 2011] and spinal cord injury (SCI) [Abematsu et al., 2010] have been studied in various animal models. The molecular mechanism of NSCs during recovery of injury induced inflammation, like rolling, adhesion, and extravasations into damaged CNS regions are sequentially mediated by constitutive expression of cell adhesion molecules (e.g., CD44) [Pluchino et al., 2003; Haas et al., 2005; Wang et al., 2010], integrins (such as α4, FAK, β1) [Campos et al., 2004; Leone et al., 2005; Pluchino et al., 2005; Campo et al., 2006; Wang et al., 2010; Battiste et al., 2011], chemokines receptors (Such as CCR3, CCR1, CCR2, CCR4, and CCR5) [Imitola et al., 2004; Ji et al., 2004; Wang et al., 2010; Andres et al., 2011a; Choi and An, 2011] on the surface of NSCs. These factors work as chemoattractive gradient, which leads to specific homing of NSCs in inflammatory regions of the brain. The recruited NSCs could exert bimodal effect depending on the CNS resident cells (such as microglia and astrocytes) which are reactive to pathological insults. First, neuroprotective effect offered by NSCs is accompanied by increased expression of neurotrophins such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), nerve growth factors (NGF), and glial-derived neurotrophic factor (GDNF) which has been demonstrated in experimentally induced neurodegenerative CNS disorder in rodents [Teng et al., 2002; Lu et al., 2003; Chu et al., 2004; McBride et al., 2004; Ryu et al., 2004; Richardson et al., 2005; Lee et al., 2007; Tamaki et al., 2009; Jaderstad et al., 2010]. Secondly, the recruited NSCs might promote immunomodulation by releasing chemokines or cytokines [Pluchino et al., 2003, 2009] and express relevant receptors (such as chemokines receptors and cell adhesion molecules), which are able to change the inflammatory responses. NSCs mediated mechanism is accelerated by pro-inflammatory cytokines (such as INFγ, IL-1β, Thelper 1-like, and TNFα). These recruited NSCs can significantly and specifically reduce the effector functions of inflammatory T-cells as well as macrophages [Einstein et al., 2003].

NSC transplantation also promises new hope in stroke by enhancing the axonal transport and structural plasticity in cerebral ischaemia [Andres et al., 2011b] and infiltration of mononuclear cells has been found to be decreased at the lesion site of ischaemic areas in the CNS where recruited NSCs accumulate in stroke animal model [Park et al., 2002; Kelly et al., 2004].

NEURAL STEM CELLS AND ANIMAL MODELS

Retina is the extension of the central nervous system which provides a convenient tool to examine the complicated nervous system. It can be manipulated with relative ease, making it feasible to test the regenerative potential of different types of stem cells, pharmacological compounds, and neurotropic factors. Human embryonic stem cell-derived retinal pigment epithelium (RPE) has been reported to rescue the visual function in an animal model of retinal disease [Lund et al., 2006]. When fetal neurons were assessed, they appeared to survive transplantation surgery better than adult neurons [MacLaren and Taylor, 1997], highlighting the value of fetal derived NSCs. Improvement in visual performance was twice that of untreated controls (spatial acuity was approximately 70% that of normal non-dystrophic rats) without evidence of untoward pathology [Lund et al., 2006]. Therefore, stem cells applications in the eye have become a center of hope for therapeutic use in regeneration and repair of damaged retina and possibly other neural tissue. The search for additional foci of NSCs led to their localization at the junction of retina and ciliary bodies, which is the remnant of ciliary marginal zone (CMZ) [Mayer et al., 2003]. CMZ is proliferative region at the periphery of the retina where the retinal stem cells are located. Thereafter, it was shown that NSCs could be isolated from mouse, rat, rabbit and human pigmented ciliary epithelium [Tropepe et al., 2000; Tsonis and Rio-Tsonis, 2004]. Under in vitro conditions, these cells can differentiate into retinal neurons such as photoreceptors, bipolar cells and muller glial cells. It has been shown that extrinsic factors strongly influence the progeny of retinal cells [Ezzeddine et al., 1997].

Further experiments in this direction have tested the in vivo potential of the retinal stem cells and their progeny, human retinal sphere cells, in eyes of postnatal (day 1) NOD-SCID mice and in embryonic chicks [Brenda et al., 2004]. RSCs progeny were able to survive, integrate, migrate, and differentiate into the neural retina, especially photoreceptors. The integration and differentiation of stem cells derived from human ciliary epithelium suggests that these cells finally may be precious in treating human retinal diseases [Brenda et al., 2004]. Chacko et al. have also isolated stem cells/progenitors from peripheral nerve type 1 (PN1, which is expressed in high levels throughout the PNS) rat retina and adult ciliary and limbal epithelium and used them for transplantation experiments in 10-day-old rat eyes. These cells survived and differentiated into photoreceptor-like cells expressing opsin but did not integrate into the existing retina. Postnatal PN1 retinal progenitors when transplanted into host retinas where mechanical damage was induced proved that retinal damage was essential for retinal integration [Chacko et al., 2004].

Though retinal stem cells exist in the mammalian eye throughout life, these cells proliferate embyonically and help to build the retina only in the initial phase, but in postnatal mammals they do not proliferate to regenerate the retina in response to injury [Ezzeddine et al., 1997]. However, van der kooy and coworkers [Brenda et al., 2006] reported that there was 3–8-fold increase in stem cell population in the region of ciliary margin in chx10 orj/orj and Mitf mi/mi mutant mice [Coles et al., 2006]. This indicates that loss of the neural retina or RPE progenitor populations results in increase in the resident stem cell population in pigmented ciliary epithelium. Such findings are important in the context of localizing stem cell progenitors.

To evaluate the morphological integration and host photoreceptor rescue as well as the impact on visual behavior, progenitor cells from neural retina of postnatal Day 1 EGFP transgenic mice were transplanted into the C57BL/6 rho $-/-$ mice at 4 weeks of age ($n = 12$) or C3H rd mice at 4 weeks of age [Klassen et al., 2004]. Brain- and retina-derived stem cells transplanted into adult retina have shown slight evidence of being able to differentiate into new photoreceptors and integrate into the outer nuclear layer. Sun et al. [2010] hypothesized that committed precursor or progenitor cells at later ontogenetic phases might have a greater probability of success upon transplantation. They showed that donor cells from the developing retina can integrate into the adult or degenerating retina at a time coincident with the peak of rod genesis. These transplanted cells differentiate, integrate into rod photoreceptors, form synaptic connections, and improve the visual function. Furthermore, they used genetically tagged post-mitotic rod precursors expressing the transcription factor Nrl (neural retina leucine zipper) to show that successfully integrated rod photoreceptors can not be derived from proliferating progenitor, these are derived from immature post-mitotic rod precursors. These results define the ontogenetic phase of donor cells for successful rod photoreceptor transplantation [MacLaren et al., 2006].

The works discussed here has demonstrated that progenitor transplantation can achieve limited photoreceptor replacement in the mammalian retina in rodents; however, replication of these findings on a clinically relevant scale requires large animal models. Large animal models like caprine model, horse, pig, etc. have been successfully used for such proginator transplantation experiments [Wanga et al., 2007; Revishchin et al., 2008]. In order to investigate this, some groups have propagated such cells from the brain, retina, and corneo-scleral limbus and have genetically modified human NSCs so that GDNF could be expressed upon transplantation into the spinal cord of SOD 1 mutant rat model. It was found that there was significant migration of cells into degenerate areas with remarkable early and end stages of the disease within chimeric regions. Combined with this study another group showed that neural stem cell fractions could bring benefits through neurogenesis and release of growth factors in a population double positive for Lewis X and the chemokine receptor CXCR4. The Seventy-day-old transgenic SOD1-G93A mice were transplanted with Le + CX + NSCs (20,000 cells) or only with vehicle (saline solution). There was generation of cholinergic motor neuron-like cells upon differentiation. The transplanted mice survived longer than controls at 23 days [Corti et al., 2007]. A cursory analysis of these reports reveals that fewer

studies have analyzed the dose response or comparative efficacy of these cells when implanted through different routes of administration. Therefore such approaches may aptly supplement the pace with which the field is growing.

CONCLUSIONS

There is plenty of data to suggest that much of what is considered as promise for regeneration may not be limited to neurons but also include myelin-forming cells; however, whether the intact nervous system can be successfully reconstituted remains hotly debated. It is difficult to reconcile the two disparate thoughts and only further advancement of our knowledge in clinical translation studies combined with use of primate models of research will uncover the promise held by demolishing the Cajal's myth that brain cells do not divide. With ever increasing funding in stem cell research and sudden increase in impact factor of stem cell journals, it is the time for disease model specialists to collaborate with those that specialize in in vitro manipulations so that side by side comparisons on potential and efficacy of variety of stem cells from different sources, stages of development, administration routes and doses between species can be appropriately evaluated. This will not only accelerate the pace of clinical translation and consequent stem cell entrepreneurship for societal benefit but also improve the unmet requirements of current healthcare delivery systems.

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Ciliary Epithelium: An Underevaluated Target for Therapeutic Regeneration

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ABSTRACT: The purpose of stem cells in various organs of vertebrates is to replenish dying cells or to replace damaged tissues. However, a few organs have reasonable, while others have very limited regenerative, capacity. Until the last two decades, the organs such as brain, heart, and kidneys were known to lack regenerative capacity for lack of resident stem cell population. However, with advancement of techniques and an increase in scientific communication, new discoveries have brought novel concepts and data to discover and manipulate these valuable resources. Much focus has been devoted to understanding the regulation and maintenance of these stem cells. We discuss the preclinical data emerging from retino-vascular interactions useful in the exploitation of ciliary epithelium–derived stem cells for therapeutic regeneration.

KEY WORDS: stem cells, ciliary epithelium, retina, vascular niche

I. HISTORICAL ASPECTS OF RETINA REGENERATION

French naturalist Charles Bonnet first conducted the studies in retinal regeneration in lower vertebrates, particularly amphibians, as early as 1781.[1] His experiments revealed that the eyes of amphibians (newts) were able to regenerate the lost portion, when a portion of the retina was removed.[2] However, the source of the observed regeneration remained unclear. It was generally believed that the regenerative cells may vary among the species. Several breakthroughs were made during 1950s showing that the new cells for regeneration of the retina were derived either from undifferentiated peripheral growth zone or differentiated retinal pigment epithelium.[3–5] The regenerative capability of fish retina was discovered in the late 1960s, long before the identification of rod precursors and inner nuclear layer stem cells.[6] The earliest experiments showed retinal regeneration by surgically removing a portion of the central retina. INL stem cells had not been identified by then; it was postulated that the CGZ was the source of these regenerated cells. Regeneration restores the original architecture and histology of the retina,[7–10] as well as function.[11,12] When rod precursors were identified, they were originally thought to be the source of new neurons and glia during regeneration.[13] Identification of stem cells in the inner nuclear layer immediately raised the possibility that these cells could be the source of retina regeneration. With the advent of new molecular and biological procedures in the last two decades, it became possible for researchers to demonstrate that retinal regeneration is a phenomenon occurring in various species such as fish,[7,14–16] frogs,[16,17] chicks,[18] and mammals.[6,19–21] However, the regeneration capacity is far more in embryonic stages of vertebrates when compared to adults, and it is more effective in lower vertebrates than in higher vertebrates. The ciliary marginal zone (CMZ) and pigmented ciliary epithelium have been the known source of regeneration in larval frogs, embryonic chicks, urodeles, ambhibians, and fish,[18,22,23] and more recently human

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studies have also shown the existence of stem cells in this region.

II. REGENERATION OF RETINA IN ADULT MAMMALIAN EYE

Mammals are known to lack CMZ, which is a potential stem source in lower vertebrates. The only mammal to have a transient CMZ is the postnatal marsupial.[24] A decade ago, for the first time, Ahmad et al. and Tropepe et al. showed the existence of stem cells in the pigmented ciliary epithelium of mammals.[25,26] Since then, much work has been done on ciliary epithelial stem cells, including in rodent, rabbit, porcine, bovine, monkey, and humans.[27–29] These investigations offered hope for transplantation in retinal degenerative diseases. CE stem cells showed an increasing amount of proliferation when CE cells were grown in serum free media (SFM) containing mitogens such as *EGF*, *bFGF*, and heparin. However, a small number of pigmented epithelial cells, i.e., ~10,000 cells per human eye, were able to proliferate in *invitro* conditions.[30] Although PCE cells have good proliferative and self-renewal capacity in the presence of mitogens, the proliferation capacity is limited in PCE cells when compared to SVZ/SGZ–derived stem cells.[31] Cells from different regions such as iris, pars plana, and pars plicata of large mammalian eye have also been tested for their proliferation and differentiation capacity.[28] The PCE cells were found to be multipotent by differentiating into all retinal markers for rod photoreceptors, ganglion cells, bipolar, horizontal, amacrine, and muller glial cells.[32] However, a few groups have reported that the CE-derived neurospheres fail to differentiate into rod photoreceptor.[28,33,34] It has been proposed that the production of rod photoreceptors and other retinal cell types from CE stem cells might occur through a process of transdifferentiation. Genomic reprogramming would be indicative of a permanent change requiring internal cellular processes for any expansion of cell population. For instance, overexpression of *Crx* or *Otx2* by retroviral infection of ciliary epithelial cells yielded high percentage of cells expressing rhodopsin.[35–37] Recently, Inoue et al. showed that combined transduction of *Otx2* and *Crx* while blocking *Chx10* in human RSCs resulted in enrichment of photoreceptor progeny.[38] Exogenous expression of *Crx* in mouse and postmortem human ciliary epithelial stem cells promoted the differentiation of these cells into functional rod photoreceptors.[39,40] RPE is one of the key components to maintain the normal retina, failure of which may lead to age-related macular degeneration. Since CE stem cells can differentiate into RPE cells, CE transplantation as a resource for damaged RPE and needs more investigation before clinical trials are designed.[41]

Like CNS stem cells, CE stem cells also respond effectively to retinal injury. Recently, it has been shown that rod and cone photoreceptor lineage cells were identified within the ciliary epithelium of pars plana in response to retinal degeneration in adult mice.[42] In a wild-type normal mouse at P24, a few recoverin-positive cells were seen in the pars plana region. However, in an adult wild-type mouse with retinal degeneration induced by N methyl N nitrosourea, there was a huge increase in the number of proliferating cells as well as recoverin-positive cells.[42,43] In a RGC (retinal ganglion cell) injury model by optic nerve axotomy in mice, nestin-positive cells in CB showed proliferation with axotomy before the onset of RGC death. This proliferation continues and peaks along with RGC death.[44] Two different studies by Wohl et al. showed that there was a tremendous increase in proliferating cells in an optic nerve lesion model of mice.[45,46] In *Chx10*(*orJ/orJ*) and *Mltf*(*ml/ml*) mutant mice, there was reduction in retinal progenitor population, but ciliary epithelial stem cell population was increased in these mutants, indicating that ciliary epithelial stem cells are responsive to a fewer number of retinal progenitor cells in genetically modified mutants.[47] Recently, this kind of activation of stem cells present in the ciliary epithelium was also reported in RCS (Royal College of Surgens) rats during the development of retinitis pigmentosa.[48] Combined together, these studies signify that CE stem cells are reactive in response to injury in the retina.

Because CE stem cells have a capacity to mi-

grate, and cellular plasticity to integrate and differentiate in the host retina, efforts have been made to replace the lost photoreceptors in retinal degeneration using the stem cell transplantation approach. Chacko et al. showed that adult CE stem cells, when transplanted into diseased retinas, adopted retinal phenotypes.[49] The study highlighted the necessity of some factors that are released by injury leading to recruitment and differentiation of the transplanted cells in the host retina. Van der kooy and coworkers have also attempted transplantation of human RSCs into neonatal animal eyes, resulting in cell migration, integration, and differentiation toward retinal cell fates.[47] Therefore, these cells seem to hold promise for retinal regeneration.

III. DEVELOPMENT OF RETINA AND CILIARY EPITHELIUM

Development of ciliary body has an important role in the tissue interactions that occur during the development of the eye, since the growth of many ocular tissues and the geometric relationship between all parts of the eye are dependent on the intraocular pressure generated by embryonic ciliary epithelium. Development of the ciliary body has not been adequately discussed in the existing literature even though eye has been a favorite source of material for developmental biologists. It is believed that the location, morphology, and function of ciliary body is determined by environmental cues during development. Any errors in the formation of ciliary body or in its interaction with other structures in the eye may lead to severe ocular diseases such as myopia and microphthalmia.

A. Formation of the Optic Cup

Vertebrate ocular development involves a series of inductive interactions between neural ectoderm, surface ectoderm, and periocular mesenchyme. From embryonic day 8 (E8) to 8.5 (E8.5) in mouse, the neuroepithelium of the diencephalons thickens and evaginates toward the surface ectoderm, forming the optic vesicles with the optic stalks connected to the forebrain.[50,51] The optic vesicles grow laterally and eventually make contact with the surface ectoderm, inducing a thickening, called the lens placode, in this tissue.[50,52] At E10, the optic vesicle invaginates to form the bilayered optic cup. The outer layer of the optic cup, which is destined to become the retinal pigment epithelium (RPE), does not grow at this stage. The inner layer undergoes rapid proliferation to form a multicellular layer that will give rise to the neural retina. The invagination of the optic cup flips the retinal neuroepithelium to an opposite orientation of that seen in the CNS, with the apical surface of the epithelium oriented toward the prospective RPE. The space between the lens vesicle and the future neural retina gets filled with vitreous humor at later stages. The optic stalk, which connects the optic cup to brain, remains open before E11. Later on, a groove forms at the inferior aspect of the optic cup and optic stalk, known as the embryonic fissure. Through the embryonic fissure, the hyaloid artery enters the optic cup and branches anteriorly to connect with anterior ciliary vessels. The interface between the optic stalk and the retina develops and gives rise to the optic disc, through which axons from developing retinal ganglion cells grow toward the presumptive optic nerve and exit the optic cup.

During the development, the peripheral part of optic cup differentiates into ciliary body and iris. Understanding the molecular mechanisms of development of ciliary body is crucial because it is involved in a critical role of vision. The first identified signaling pathway that was involved in the ciliary body formation was BMP signaling.[53] Along with existing reports of the role of Wnt signaling pathway in ciliary epithelial cell fate determination,[54,55] it was recently demonstrated that secreted frizzled related proteins (Sfrps) play an important role in the development of the optic cup periphery.[56]

IV. ANATOMICAL RELATIONS OF VASCULATURE WITH RETINA AND CILIARY EPITHELIUM

There is a coordinated interaction between the neural and vascular systems in the developing ver-

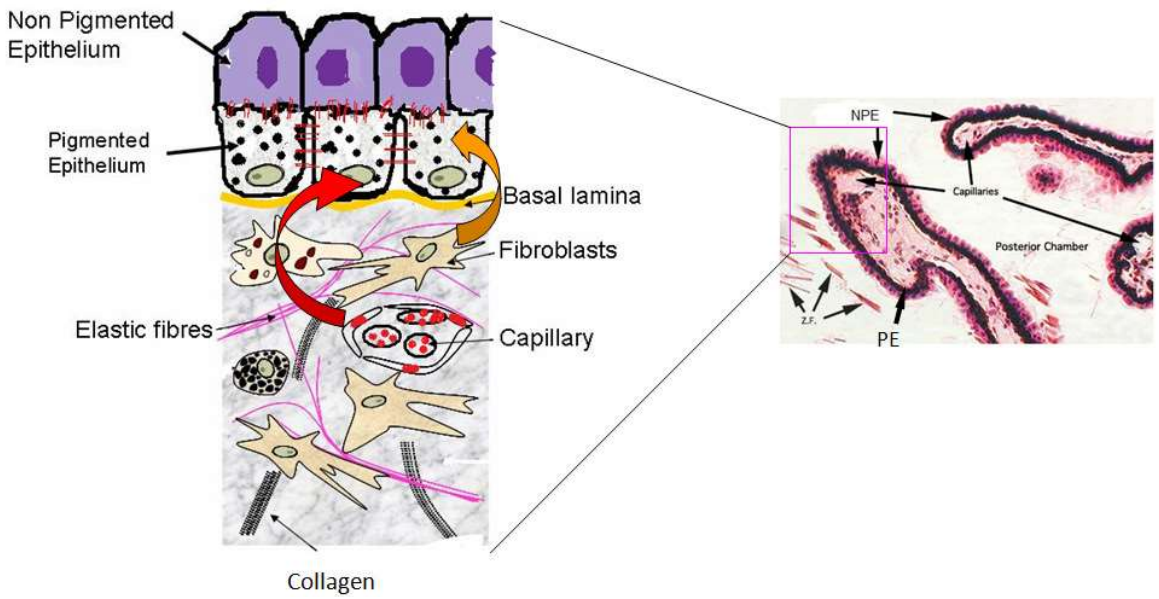

FIGURE 1. Schematic representation showing the close association of pigmented CE with its microenvironment, including vasculature, fibroblasts, and basal lamina. The factors secreted by endothelial cells of blood vessels and capillaries, such as VEGF, may have an effect on proliferation and migration of stem cells residing in pigmented (CE, ciliary epithelium; VEGF, vascular endothelial growth factor).

tebrate retina. Under normal physiological conditions in the developing retina, vasculature follows the existing astrocytic meshwork and shows similar pattern as that of the astrocytes growth, and also shows the radial orientation of ganglion cell axons.[57] The region that lacks astrocytes in the central retina or fovea is also completely devoid of retinal vessels. This cellular pattern of retinal blood vessels and astrocytes, ganglion cell axons, and other neuronal elements clearly indicates the close association of neural and vascular structures during retinal development. There is interdependence between ganglion cells, astrocyte, and vascular endothelial cells in retinal vascular development. Astrocytes follow the radial plan of ganglion cell axon bundles where platelet-derived growth factor-A (PDGF-A) secreted by ganglion cells stimulates the proliferation of astrocytes, which in turn induces VEGF-dependent vascular growth. In the adult vertebrate eye, photoreceptors are the most highly metabolically active cells, and there is continuous demand for oxygen supply.[58] Under pathological conditions such as proliferative diabetic retinopathy (PDR) and age-related macular degeneration (AMD), hypoxia-induced neovascularization regresses after the onset of photoreceptor degeneration, in which oxygen demand will be less, and more oxygen is available to the remaining retina.[59] Muller glia is one of the sources of retinal stem cells that form on the large retinal blood vessels at the inner surface of the retina.

In order to ensure the consistent perfusion of the anterior segement and to facilitate the homeostatic control of aqueous humor production, the ciliary body (CB) of eye receives the highest density of blood in the eye. Ciliary epithelium of CB is encapsulated with a capillary network embedded in connective tissue (Fig. 1). As in the case of development of astrocytes and blood vessels, there is a pattern between ciliary capillaries and the organization of ciliary processes, and both vasculature and ciliary processes are associated during development.[60]

V. ROLE OF VASCULATURE AND ENDOTHELIAL CELLS ON CNS STEM CELLS AND IN OTHER ORGANS

In the central nervous system (CNS), NSCs are localized in highly vascularized regions instead of being distributed throughout the brain.[61–63] In the dorsal SVZ, NPC chains are built in the orientation of blood vessels, and vessels guide the migration of these chains toward the olfactory bulb.[64] This provides an intimate interaction of vasculature with neural stem cells in the subventricular zone (SVZ) and subgranular zone (SGZ) to create a specialized vascular microenvironment or vascular niche in which neural progenitor cells (NPCs) proliferate, self-renew, and differentiate. The vascular niche is not only involved in the maintenance of NSCs and NPCs through direct contact with endothelial and mural cells of blood vessels, but also helps in the constant supply of soluble factors, signaling molecules, and nutrients.[65,66] It has been shown that in case of poststroke traumatic brain injury or Alzheimer's disease, the damaged areas are regenerated through vascular secreted factors such as the stromal cell–derived factor (SDF-1) and angiopoeitin (Ang-1).[67]

Among the various vascular components such as endothelial cells, pericytes, and the outer adventious layer of the vascular niche, endothelial cells play an important role in the maintenance of NSC self-renewal.[63,68–70] It has been shown that disruption of endothelial cell interaction with NPC by radiation in SGZ resulted in the loss of neurogenic potential.[71] Similarly, in *in vitro* studies, a coculture of bovine pulmonary artery endothelial (BPAE) cells and cerebral endothelial cells from SVZ of the stroke boundary with NPCs showed an enhanced self-renewal and proliferation capacity of NPCs.[68,72] Furthermore, a coculture of SVZ explants with endothelial cells showed increased neurite outgrowth, enhanced neuronal migration, and maturation, thus indicating the role of endothelial cells on the maintenance of neural stem cells. In *in vivo* studies, cotransplantation of endothelial cells with NPCs increased the survival and proliferation of ischemia-induced NPCs compared to NPCs alone.[73] Endothelial cell regulation of adult NSCs and NPCs is believed to be mediated either by the secreted factors and cytokines released by the endothelial cells or by direct contact. Some of the known soluble factors secreted by endothelial cells that modulate the neurogenesis include vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF).

Among these factors, VEGF is one of the factors that has been extensively studied in neural progenitor cell proliferation and migration. VEGF-induced proliferation of neural precursors was demonstrated in both *in vivo* and *in vitro* conditions.[74,75] Fabel et al. found that VEGF is necessary for physical exercise–induced hippocampal neurogenesis.[76] Gene transfer of VEGF in adult rat hippocampus resulted in a twofold increase in neurogenesis, which further improved cognitive ability.[77] Similarly, transplantation of NPCs overexpressing VEGF into the area of a cerebral hemorrhage increased the survival of transplanted NPCs.[78] Infusion of VEGF into the lateral ventricle of cerebral ischemic brain resulted in increased neurogenesis in SVZ.[74] The role of VEGF has also been evaluated in regulation of NSC migration.[79–84] *In vitro* studies have also demonstrated the importance of the role of VEGF in the migration of neural stem cells.[79,82] It was evident from the studies of Wittko et al. that endogenous VEGF is required for the proper migration of neuronal progenitors along with the rostral migratory stream in adult mice.[84] It has been shown that injury to the CNS results in enhanced VEGF expression, which futher induces NSC migration to the area of damage.[80,81,83]

VI. ROLE OF VEGF AND ENDOTHELIAL CELLS ON RETINAL AND CE STEM CELLS

Yang and Cepko demonstrated the pattern of VEGF and its receptor Flk-1 (VEGFR2) expression at various stages of developing mouse retina. At P0, VEGF was mainly localized in the differentiated ganglion and amacrine cells, and in

the developing inner plexiform layer. In the later stages, more VEGF mRNA was localized to the inner nuclear layer. At P12, higher levels of VEGF mRNA were expressed in the inner nuclear layer, especially in the Muller glia. It was also shown that VEGF is mostly concentrated in differentiated cells adjacent to the proliferative zone, thus indicating the neurotropic and neurogenic role of VEGF.[85] The mitogenic role of VEGF was demonstrated on retinal progenitor cells by Yourey et al.[86] They also showed that VEGF treatment induces the number of photoreceptors, amacrine cells, and levels of rhodopsin. In order to investigate the importance of the role of Flk-1 and its ligand VEGF in early stages of retinal neurogenesis, studies have been conducted in avascular chick retina.[87] These experiments revealed that VEGF signals mediated through the Flk-1 receptor directly modulate the behavior of uncommitted retinal progenitor cells. Although much attention was focused on VEGF in retinal diseases involving vasculopathies, the role of VEGF in retinal degeneration was not explored extensively. Koji et al. studied the effect of VEGF on retinal progenitor cells (RPCs) in *rd1* mice, and showed that VEGF stimulates the proliferation of RPCs isolated from *rd1* mice in *in vitro* conditions. Intravitreal injection of VEGF also enhanced the proliferation of RPCs in the peripheral retina, indicating the potential role of VEGF in regeneration of degenerating retina.[88] A few studies have been carried out to understand the interaction of endothelial cells with retinal cells. When retinal cells were cocultured with endothelial cells, mRNA expression of VEGF and its receptors was increased in retinal cells when compared to retinal cell culture alone, indicating the cross talk between endothelial and retinal cells.[89]

It was shown that retinal stem cells present in the CMZ of zebra fish have as close contact with the apical surface and basal lamina of large blood vessels as that of neuroepithelial cells and SSEA-1, where an adult neural stem cell marker was found to be concentrated around the circumferential blood vessels located between the neural retina and ciliary epithelium overlying the CMZ.[90] Although the expression of VEGF and its receptors VEGFR1 and VEGFR2 have been demonstrated on CE cells,[91] the role of VEGF has not been studied on CE-derived stem cells.

VII. CONCLUSIONS

During both development and pathology, the retina and ciliary epithelium maintain close association with vasculature. Extensive work has shown the role of vasculature, endothelial cells, and VEGF in various retinal diseases such as diabetic retinopathy, age-related macular degeneration, and glaucoma. However, the interaction of stem cells present in CE and retina with endothelial cells in relation to VEGF in the maintenance of these stem cells has not been adequately investigated. Understanding the effects resulting from the supplementation of VEGF with and without CE stem cells in various preclinical models of retinal degeneration can uncover the therapeutic benefit from VEGF-induced CE stem cell proliferation and differentiation. Through combining existing *in vitro* data about CE stem cells with *in vivo* studies that address the role of various doses, routes, and types of CE stem cells in transplantation models, the chasm between basic research and translational studies can indeed be bridged.

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Preclinical models to investigate retinal ischemia: advances and drawbacks

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Retinal ischemia is a major cause of blindness worldwide. It is associated with various disorders such as diabetic retinopathy, glaucoma, optic neuropathies, stroke, and other retinopathies. Retinal ischemia is a clinical condition that occurs due to lack of appropriate supply of blood to the retina. As the retina has a higher metabolic demand, any hindrance in the blood supply to it can lead to decreased supply of oxygen, thus causing retinal ischemia. The pathology of retinal ischemia is still not clearly known. To get a better insight into the pathophysiology of retinal ischemia, the role of animal models is indispensable. The standard treatment care for retinal ischemia has limited potential. Transplantation of stem cells provide neuroprotection and to replenish damaged cells is an emerging therapeutic approach to treat retinal ischemia. In this review we provide an overview of major animal models of retinal ischemia along with the current and preclinical treatments in use.

Keywords: animal model, retinal ischemia, stem cells, therapeutics

INTRODUCTION

The term "ischemia" implies reduction in the blood supply to a tissue resulting in insufficiency to meet the metabolic requirements. Thus, retinal ischemia is the clinical condition when the blood supply to the retina is obstructed. The retina is an extension of central nervous system (CNS) and thus shares many embryological, functional, and anatomical characteristics with brain. The response to ischemia in retinal neurons is similar to those in other parts of CNS. Nevertheless, retina is more resistant to ischemic injury than the brain (Tso and Jampol, 1982).

As far as the blood supply to the retina is concerned, the mammalian retina has a dual circulation system. The major portion of blood supply goes to the choroid, i.e., 65–85% of the total supply, whereas the remaining goes to the retina through central retinal artery, which originates from ophthalmic artery (Henkind et al., 1979). **Figure 1** depicts the blood supply to the eye. The extent of retinal ischemia and the region of retina affected depends on the blood supply that is obstructed (Saint-Geniez and D'Amore, 2004). The retina has a high metabolic demand. Any hindrance in the blood supply affects the exchange of substrates and products, leading to many morphological and functional changes in retina. The pathophysiology of retinal ischemia is not completely known but involves imbalance in ion transport, changes in neurotransmitter levels, oxidative stress, and finally cell death. Thus, there is a need to study the pathophysiology involved so that better therapeutic agents can be tested. As retina can be studied non-invasively the investigations into retinal ischemia may lead to a better understanding of cerebral ischemia.

NEED FOR ANIMAL MODELS

Although humans and animals have varying phenotype, they share strikingly common physiological and anatomical details. Therefore, to understand the human physiology and to test new treatments through preclinical investigations, animal experimentation has always been a core of basic and clinical research. The importance of developing animal models for retinal ischemia originates from the limitations found in the use of *in vitro* models. Retinal ischemia at molecular and biochemical levels can be studied in depth using an *in vitro* model of ischemia but the pathophysiological details can only be understood using an appropriate animal model. An ideal animal model for retinal ischemia should have parallelism with humans at anatomical, vascular, and retinal levels besides offering ease in handling and manipulation.

ANIMAL MODELS OF RETINA ISCHEMIA

ELEVATION OF INTRAOCULAR PRESSURE

The model often used to cause retinal ischemia is by increasing intraocular pressure (IOP). This model involves elevation of the IOP above the systemic arterial pressure for a fixed duration of time (Peachey et al., 1993). High IOP results in global ischemia as it hampers the blood supply from both uveal and retinal circulation. In rodents, different groups have shown that IOP-induced retinal ischemia mimics the features observed in human central retinal artery occlusion (CRAO) and primary open angle glaucoma (Smith and Baird, 1952; Buchi et al., 1991).

The animal model of retinal ischemia model was first described by Smith and Baird (1952) and was further validated by Flower and Patz (1971) in cats. In rodents, the elevated IOP-induced retinal ischemia injury was induced in rats by Buchi et al. (1991). In all these studies, the IOP was increased through cannulation of the anterior chamber with a 26-gauge needle connected to an elevated chamber containing normal saline. The IOP was raised to 110 mm Hg. This method has been used in a wide range of experiments to study the alteration in protein expression, ion channel imbalance, excitotoxicity in various animal models (Hirrlinger et al., 2010; Joachim et al., 2011). Ji et al. (2005) used this model

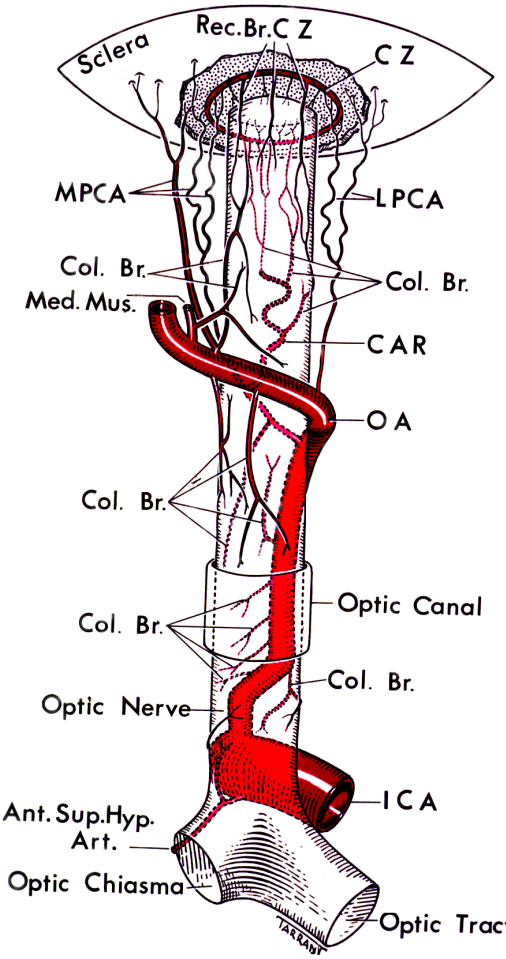

FIGURE 1 | The figure depicting the circle of Zinn-Haller and the blood supply to eye (Hayreh, 1963).

in mice to investigate the mechanism behind the retinal ganglion cell death due to retinal ischemia.

The advantages of using this model are that it is temporary and reversible, easy to create, and reproduce and there is minimal requirement of surgery or special equipments. But there is a limitation to this model that the elevated IOP can itself cause damage and hence, lead to incorrect interpretation of the data (Peachey et al., 1993).

MIDDLE CEREBRAL ARTERY OCCLUSION

It has been reported that the cerebral stroke incidents are invariably accompanied with temporary (amaurosis fugax) or permanent vision loss. A purely vascular model of retinal ischemia is the middle cerebral artery occlusion (MCAO). As the ophthalmic artery which is the source of blood supply to the inner retina originates proximal to the origin of middle cerebral artery (MCA), any hindrance in the blood flow in MCA obstructs the flow to the ipsilateral retina. This method involves occlusion of blood supply by the use of a filament inserted through external carotid artery (ECA) and internal carotid artery (ICA) and advanced into the MCA.

This procedure was first time demonstrated by Block et al. (1997) in rats that led to MCAO induced retinal ischemia. Later the same model was used to depict damage to retina through MCAO in mice (Steele et al., 2008). This model is a non-invasive and does not disrupt the blood–retina barrier or cause any mechanical damage to the retina (Kaja et al., 2003). The MCAO model has other advantages including reproducibility making it permissive for reperfusion related investigations.

CHRONIC CAROTID OCCLUSION

Retinal ischemia can also be caused by carotid artery disease in humans. This model was first induced by Block et al. (1992) in rats in order to show that the bilateral common carotid artery occlusion in rats causes functional damage to the retina. The electroretinogram studies have also shown a decrease in amplitude of b-wave 7 days after the bilateral carotid artery occlusion or two-vessel occlusion (2VO). The b-wave represents the bipolar and Muller cell activity (Barnett and Osborne, 1995). Not only functional, 2VO model also causes structural damage to the retina. In another study in rats, Lavinsky et al. (2006) showed that in the animals that showed functional damage, the retinal thickness was found to be decreased and the layers that are most affected included inner and outer plexiform layers.

The 2VO model for retinal ischemia leads to permanent occlusion without any reperfusion. Due to collateral blood circulation partial blood supply to retina is retained through Circle of Willis leading to variable retinal damage.

PHOTOTHROMBOSIS OF RETINAL VESSELS

The photothrombosis or photocoagulation induced retinal ischemia model is a simple and non-invasive method. It is comparatively a new method to perform vessel occlusion. This method was first described in adult rat retina by Mosinger and Olney (1989), where the authors injected Rose Bengal, a photosensitive dye intravenously and then exposed the retina to light of a fixed wavelength (550 nm) leading to photothrombosis. Miller et al. (1994) used this same method in a non-human primate model, cynomolgus monkey (*Macaca fascicularis*), where a laser at 577 nm was used for causing occlusion. Buchi et al. (1994) used this model to study morphological and histological changes in retina. Schimdt-Kastner also used Photothrombosis to induce ischemia in rat retina and showed the ganglion cell death (Schmidt-Kastner and Eysel, 1994). Another photosensitive dye, apart from Rose Bengal, that can be used in this model, is chloro-aluminum sulfonated phthalocyanine (Kliman et al., 1994).

Like any other method, this too has its own advantages and disadvantages. Photothrombosis leads to variability in retinal damage and hence irregular damage due to variation in light exposure. It leads to permanent ischemia and can therefore not be used to study damage due to reperfusion. This method results in damage due to free-radicals.

OCCLUSION OF CENTRAL RETINAL ARTERY

The central retinal artery is the first intraorbital branch of ophthalmic artery. The CRAO causes complete inner retinal ischemia.

In humans, the clinical features were first described by von Graefe (1859). The CRAO model has been used to induce retinal ischemia in different species. Hayreh induced transient CRAO in rhesus monkeys by clamping the central retinal artery for different time durations. This study also showed that the damage induced depends on the tolerance time for the particular species (Hayreh et al., 1980). Zhang et al. (2005) created this model in rats by intravenous injection of Rose Bengal and treating the animals with green laser. This model is used to study the pathways involved in transient retinal ischemia by mimicking the clinical features of CRAO in humans. In another study in CRAO model in rhesus monkeys, amino acid profiling was done to evaluate the role of glutamate excitotoxicity in ischemia (Kwon et al., 2005). The mouse model showing similar changes as human CRAO has been generated, where central retinal artery was occluded by laser photoactivation of Rose Bengal. The occlusion of 6–24 h showed molecular and histological changes (Goldenberg-Cohen et al., 2008). CRAO can also be achieved by another method which leads to ischemia – reperfusion model. In this model a suture is placed behind the eye globe and then obstructing the blood flow by pressing the tube through which both ends of suture are passed (Prasad et al., 2010).

ENDOTHELIN ADMINISTRATION

Endothelin-1 or ET-1 is a 21 amino acid long peptide with vasoconstrictor activity. It was first purified and characterized in 1988 from the conditioned medium of cultures of porcine aortic endothelial cells (Yanagisawa et al., 1988). In humans, cardiovascular disease, renal, and ocular disorders have been associated with endothelin-1. The vasoconstrictor activity of endothelin has been demonstrated in retinal arteries in rats. In a study by Bursell et al. (1995) an approximate 17% reduction in diameter after 10^{-7} M concentration of ET-1 was shown. Sakaue et al. also studied the effect of endothelin on the retinal vessels after intravitreal administration of ET-1 in rabbit eyes. The authors showed a dose-dependent response, where the concentration of 10^{-6} M caused vasoconstriction and lower concentrations caused vasodilation first with vasoconstrictions later, leading to hypoxia and retinal ischemia (Sakaue et al., 1992; Sato et al., 1993; Takei et al., 1993). In another study, ET-1 administration was used to obstruct the central retinal artery causing ganglion cell loss in retina (Masuzawa et al., 2006).

No problems such as inflammation or infection are associated with this model, but this has a limitation of ET-1 dose. Also it may show some undesirable effect due to systemic circulation to other tissues.

CURRENT AND POTENTIAL THERAPEUTIC STRATEGIES FOR RETINAL ISCHEMIA

Current treatments for retinal ischemia involve recovery of the blood circulation in retina that will prevent further damage and permanent vision loss. Combination of strategies are being used to treat retinal ischemia including intravitreal or retinal vein administration of anticoagulants such as tissue-plasminogen activator (t-PA), hemodilution, pan-retinal laser photocoagulation, or anti-VEGF antibodies (Lucentis or Avastin). Ischemic conditions in retina have been shown to up-regulate the expression of vascular endothelial growth factor or VEGF, a potent angiogenic factor. This change in VEGF levels leads to retinal neovascularization or growth of abnormal blood vessels. Neutralizing VEGF through monoclonal antibodies has been shown to block neovascularization. This technique was tested in a primate model of retinal ischemia induced by laser. The study showed that VEGF was inhibited *in vivo* when proteins containing human (Flt) or mouse (Flk) VEGF receptors attached to IgG were administered (Aiello et al., 1995). This showed almost complete inhibition of neovascularization. Laser photocoagulation or pan-retinal laser treatment is used to decrease the neovascularization or growth of abnormal blood vessels, thus decreasing the damage to the retina.

All these strategies that are being used have their own limitations. The anticoagulants and vasodilators are effective only in limited cases of retinal ischemia, such as CRAO patients, while the laser treatment is effective only when given early (Rumelt et al., 1999). This unavailability of effective treatment options has led to development of various other neuroprotective agents. Other possible therapeutic agents include NMDA receptor blockers or inhibitors, catalase and thioredoxin (free-radical scavengers), calcium channel blockers, and many others. These novel drugs and chemical compounds have been tested in different animal models of retinal ischemia, showing positive outcome and potential as future therapeutics. Various antagonists and blockers have been demonstrated to reduce the damage caused through ischemia. NMDA receptor antagonists have shown neuroprotective effect in retinal ischemia animal models. Different NMDA receptor blockers that have been tested in preclinical studies are dextromethorphan, MK-801, memantine (Cao et al., 1994; Lam et al., 1997; Osborne et al., 1999). Blockers of calcium channels have also been tested in animal models and have shown to decrease neurotoxicity (Melena et al., 1999). Retinal ischemia damage is exacerbated by free-radicals. Free-radicals are generated by re-oxidation of compounds during reperfusion and leads to oxidative stress (Gilgun-Sherki et al., 2002). Thus, different free-radical scavengers can be used for treatment. Rios et al. compared the free-radical scavengers – SOD or superoxide dismutase and DMTU or dimethylthiourea. In an IOP-induced retinal ischemia model in rats, intravitreal administration of both the compounds has shown to potential in recovery. In this model, DMTU led to 40% functional recovery while SOD treatment showed a 99% recovery when assessed through electroretinogram (Rios et al., 1999).

Apart from these, growth factors such as CNTF, bFGF, NGF, BDNF, PEDF, HGF have been tested in retinal ischemia–reperfusion models for therapeutic potential (Unoki and LaVail, 1994; Ogata et al., 2001; Shibuki et al., 2002). These therapeutic agents have short half-life and thus require repeated administration resulting in side-effects. This limitation can be overcome by the delivery of the gene of neurotrophic factors directly using gene therapy. Viral vectors have been used successfully to transfect cells in eye (Bennett et al., 1994; Flannery et al., 1997; Di Polo et al., 1998). Wu et al. used recombinant Adeno-associated virus (AAV) vector to transfer GDNF intravitreally in rats subjected to elevated IOP. GDNF is a known neurotrophic factor

that reduces ischemic injury (Wu et al., 2004). It has also been shown in different studies that GDNF protects photoreceptors and inhibits apoptosis. Another group of neuroprotectants that have shown positive results in animal models by inhibiting apoptosis are the inhibitors of apoptosis (IAP) which inhibit caspases. Most tested IAP is the XIAP which blocks caspases 3, 7, and 9 (Deveraux et al., 1997; Xu et al., 1999; Eberhardt et al., 2000; Holcik et al., 2001; McKinnon et al., 2002; Petrin et al., 2003). Renwick et al. reported that XIAP overexpression protects the retina from transient ischemia induced by elevation of IOP in rats. In this study the authors intravitreally administered AAV vector expressing XIAP and demonstrated structural and functional protection in retinal ischemia model (Renwick et al., 2006). But it has a limitation of being ineffective in the cases where retinal ischemia occurs suddenly.

ROLE OF STEM CELL THERAPY IN RETINAL ISCHEMIA

The treatment strategies discussed in the previous section have limited potential. Stem cells are an emerging branch used in the treatment of a wide variety of disorders (Lenka and Anand, 2009). The use of stem cells from different sources are being studied and clinical trials are being carried out for disorders such as diabetes, spinal cord injury, fractures, cardiovascular, and neurological disorders. Replenishment of neuronal and retinal cells by stem cell transplantation is therefore a promising approach to treat retinal ischemia.

The stem cells have an ability to self-renew and differentiate into specialized cells. They can act through various mechanisms. Stem cells can induce angiogenesis and thus, increase vascularization. Stem cells can also enhance the endogenous repair mechanisms, reduce inflammation, and release trophic factors. In the case of retinal ischemia, the eye is easily accessible for transplantation of stem cells. Thus, the stem cell therapy has a huge potential to restore visual function in case of retinal ischemia (Cogliati and Swaroop, 2009).

Stem cells from various sources have been used for treatment. Most commonly used are the stem cells from the bone-marrow as they are easier to obtain. Two types of cell population is present in the bone-marrow – hematopoietic progenitor cells and hematopoietic stem cells. The sub-population of hematopoietic stem cells has been shown to differentiate into lineage-negative and lineage-positive sub-types. This division is according to their ability to differentiate. The cells in lineage-negative fraction can lead to vascular endothelial cells or the endothelial precursor cells after the differentiation. These progenitor cells can then mobilize from the bone-marrow and home to target sites of angiogenesis in different injuries. Bone-marrow mesenchymal stem cells (BM-MSCs) differentiate into retinal neural cells *in vivo* and *in vitro*, and when implanted at a site of injury in experimental animal models, they show the ability to migrate to the injury site, initiate tissue repair, and restore function (Goes et al., 2008).

Another source of stem cells for the therapeutic purposes is those obtained from the early embryos. These embryonic stem cells have two characteristic properties, their ability to replicate indefinitely and their pluripotency. Thus the embryonic stem cells can differentiate into various cell types. These cells have also been tested in the cerebral ischemia model, showing structural as well as functional recovery (Wei et al., 2005). Various research groups have shown the potential of these stem cells in neurodegenerative diseases including retinal damage. The use of embryonic stem cells for transplantation has its own limitations. The foremost is the ethical concerns over its use and secondly there is a risk of immune rejection. To overcome these limitations, another stem cell source from non-pluripotent cells was discovered. In 2006, Takahashi and Yamanaka identified four transcription factors – Oct3/4, Sox-2, Klf-4, c-myc. These factors can reprogram the DNA leading to the formation of stem cells from a non-pluripotent cell. These stem cells are known as induced pluripotent stem cells or iPCs (Takahashi and Yamanaka, 2006). iPCs have been used in various animal models and have shown to form different cell types. In humans, iPCs have been isolated from the patients with neurodegenerative disorders such as Parkinson's disease, muscular dystrophy, amyotrophic lateral sclerosis (Dimos et al., 2008; Park et al., 2008). iPCs have been used to generate human neuronal cells, photoreceptors, and retinal pigmented epithelium cells (Takahashi and Yamanaka, 2006; Hirami et al., 2009; Karumbayaram et al., 2009). Another source of stem cells that is being tested as a potential therapy is the umbilical cord blood. It has a large percentage of hematopoietic stem cells, higher than those found in bone-marrow and also these cells show lesser immune rejection.

Earlier the CNS was thought to be a non-renewable tissue in mammals. Neural stem cells are normally found in the developing CNS, but studies have shown that these are also present in adult CNS. These cells are found in restricted region of CNS, such as hippocampus, subventricular zone, spinal cord, and ependyma and

Table 1 | Advantages and limitations of different animal models of retinal ischemia.

Animal model	Advantages	Limitations
Elevation of intraocular pressure	Temporary and reversible; easy to create and reproduce; minimal requirement of surgery or special equipments	High IOP can cause damage
Middle cerebral artery occlusion	Transient (can study reperfusion); reproducible	Requires specialized skills; incomplete ischemia; alter blood flow to the brain
Chronic carotid occlusion	Reproducible; easy	Variable damage; permanent ischemia
Photothrombosis of retinal vessels	Simple	Variable degrees of exposure and variable damage; permanent ischemia; free-radical damage
Occlusion of central retinal artery	Complete retinal ischemia	–
Endothelin administration	No inflammation or infection	Can enter systemic circulation

can form astrocytes, neurons, and oligodendrocytes. The neural stem cells were first isolated from adult rat hippocampus (Palmer et al., 1997). These cells have shown the ability to migrate and differentiate into neuronal cells in retinal injury (Nishida et al., 2000). But more studies need to be done to estimate the efficiency of the neural stem cells.

The stem cell transplantation in retina has some limitations, such as poor cell homing, cell migration, and integration. Thus, many of the transplanted cells do not reach the retina. A study has shown that only 1% of the cells transplanted intraocularly reach the retina (Johnson et al., 2010).

CONCLUSION

Retinal ischemia is the clinical condition caused by insufficient supply of blood to retina and is found in many of the disorders. Retina, like the CNS originates from the ectoderm. Thus, with the use of animal models of retinal ischemia pathophysiology can be studied and therapeutic agents can be tested. Each of the animal models described in this review has its own advantages and limitations. **Table 1** summarizes the advantages and limitations of the retinal ischemia models discussed in this review. Thus, the choice of animal models for pre-clinical testing will depend on the research problem involved.

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Pathophysiology of Stroke and Stroke-Induced Retinal Ischemia: Emerging Role of Stem Cells

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The current review focuses on pathophysiology, animal models and molecular analysis of stroke and retinal ischemia, and the role of stem cells in recovery of these disease conditions. Research findings associated with ischemic stroke and retinal ischemia have been discussed, and efforts towards prevention and limiting the recurrence of ischemic diseases, as well as emerging treatment possibilities with endothelial progenitor cells (EPCs) in ischemic diseases, are presented. Although most neurological diseases are still not completely understood and reliable treatment is lacking, animal models provide a major step in validating novel therapies. Stem cell approaches constitute an emerging form of cell-based therapy to treat ischemic diseases since it is an attractive source for regenerative therapy in the ischemic diseases. In this review, we highlight the advantages and limitations of this approach with a focus on key observations from preclinical animal studies and clinical trials. Further research, especially on treatment with EPCs is warranted.

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Among the ischemic diseases, heart disease and stroke are the leading causes of adult disabilities and death, in the world. On average, every 45-sec someone in the United States has a stroke, totaling 700,000 strokes in 2000 alone. Although 25% of all strokes result in death, 75% constitute survival. Of the individuals who survive, 50–70% regain some degree of functional independence. However, 15–30% are permanently disabled; making stroke a leading cause of serious, long-term disability in the United States (AHA, 2002). In India, the changing pattern of diseases occurring due to efforts in control of communicable diseases have brought in a sharp focus on stroke as one of the major health problems and many centers are being funded to address both preventive and therapeutic aspects of the disease.

Ischemic stroke has an enormous economic burden in all developed countries and recent community surveys from many regions of India depict a prevalence rate presumed to be in the range of 40–270/100,000 in rural population and of 400–800/100,000 in metropolitan cities (Dhamija and Dhamija, 1998; Anand et al., 2001). In recent years, a considerable interest towards stroke research has increased greatly. One of the first effective treatments for stroke is tissue plasminogen activator (t-PA) which is a thrombolytic agent. The brain tissue damage can be prevented, if it has been given in the first few hours after stroke. Hence, it has been accepted rapidly as an acute therapeutic agent and a number of major efforts have been directed toward stroke awareness due to the recognition of the importance of diagnosing a stroke very early after its onset. The significant development of sophisticated neuroimaging techniques (e.g., functional magnetic resonance imaging and magnetoencephalography) also plays a role in the stroke research. These techniques have the capability to describe the biochemical and anatomical events occurring in the damaged brain tissue, as well as the functional changes that take place in the uninjured areas.

Ischemia is one of the key factors determining the pathophysiology of many brain and retinal diseases. Ischemic injury is caused by a series of events such as energy depletion and cell death which are mediated by the intermediate factors such as excess of extracellular excitatory amino acids, free-radical formation, and inflammation (van der Worp and van Gijn, 2007). An interesting observation in recent studies show that patients with diabetic retinopathy, retinal arteriolar emboli, and AMD may have a higher risk of ischemic stroke, suggesting that a retinal examination may be useful for stroke risk stratification and patients with ischemic stroke have a high prevalence of hypertension and diabetes which are risk factors for potentially blinding retinal diseases. Therefore, understanding the detailed molecular mechanism of ischemic conditions may provide clues to understand the pathophysiology of stroke and related ischemic diseases. Many retinal conditions are associated with stroke which reflects the possible concomitant pathophysiological processes affecting both the eye and the brain (Baker et al., 2008). So far, the incremental value of a retinal examination for prediction of future stroke risk remains to be determined. However, acute ischemic stroke patients' prevalence and types of retinal diseases in patients with acute ischemic stroke are unknown.

Current treatments are not sufficient to restore brain and visual function after severe damage of neurons and retinal nerve cells. To treat such conditions, neuroprotection could be a new therapeutic approach addressing the replenishment of neurons and retinal cells by stem cells as a way to treat neurodegenerative and retinal diseases.

Pathophysiology of Ischemic Stroke

The degree of brain injury depends on the severity and duration of the cerebral blood flow interruption. The artery occlusion leads to reduced blood flow most crucially in the region of the brain where that artery supplies blood (infarct core), and in graded fashion centrifugally from the core (ischemic penumbra) normally supplied by the occluded artery, due to residual perfusion from collateral blood vessels. Gradually, the ischemic penumbra is integrated into the core region if the blood flow is not restored within hours and become nonfunctional, however, retaining structural integrity (Durukan and Tatlisumak, 2007).

The pathogenic mechanisms of ischemic cascade include energy failure, elevation of intracellular Ca^{2+} level,

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excitotoxicity, and spreading depression, generation of free radicals, BBB disruption, inflammation, and apoptosis (Fig. 1).

This process produces brain injury via a variety of cellular and molecular mechanisms that impair the energetic required to maintain ionic gradients. At the beginning, the CBF reduction results in the depletion of substrates particularly oxygen and glucose, that causes accumulation of lactate via anaerobic glycolysis. Energy failure results in neuron depolarization, which causes activation of glutamate receptors, which in turn alters ionic gradients of Na^+, Ca^{2+}, Cl^-, and K^+ (Dirnagl et al., 1999). The result of increasing intracellular Ca^{2+} is an upregulation of a variety of enzyme systems such as lipases, proteases, and endonucleases. As a result, oxygen free radicals are generated via a variety of biochemical pathways, and apoptotic cell death occurs. The formation of free radicals induce a variety of inflammatory mediators, such as platelet activating factor, tumor necrosis factor α, and an assortment of interleukins. Moreover, oxygen radicals are produced on enzymatic conversion of arachidonic acid to prostanoids and the degradation of hypoxanthine during reperfusion (Lo et al., 2003). In ischemic conditions, mitochondrial permeability transition pore is formed, that causes explosion of free radicals and the release of proapoptotic molecules (Mergenthaler et al., 2004). Furthermore, the generation of free radicals is also observed during the inflammatory response after ischemia. The consequences of free radicals are lipid peroxidation, membrane damage, dysregulation of cellular processes and mutations of the genome. Oxygen radicals play a major role in signaling pathways which trigger inflammation and apoptosis (Dirnagl et al., 1999).

Brain edema worsens the ischemic process by its volumetric effect causing local compression of microcirculation, rise of intracranial pressure, and dislocation of parts of the brain and the intensity of edema depends on the size of the infarct. In the case of severe stroke it can give rise to fatal transtentorial herniation which is responsible for 30–90% of acute stroke fatalities (Gartshore et al., 1997).

Neutrophils transmigrate from the blood into the brain parenchyma followed by macrophages and monocytes which produce toxic mediators by activated inflammatory cells due to the expression of adhesion molecules at the vascular endothelium. While astrocytes protect neurons by multiple mechanisms, including regulation of ionic homeostasis, control of extracellular glutamate levels, and upregulating glycolytic capacity during ischemic condition (Dienel and Hertz, 2005; Raghubir, 2008), under certain conditions, activated astrocytes contribute to the ischemic cell death (Trendelenburg and Dirnagl, 2005). After ischemic injury, particularly within the ischemic penumbra, apoptosis is triggered by a number of processes, including excitotoxicity, free radical formation, inflammation, mitochondrial and DNA damage, and cytochrome c release from mitochondria (Mergenthaler et al., 2004). As apoptosis is an energy-consuming process, reperfusion could potentiate apoptosis by restoring cellular energy (Schaller and Graf, 2004).

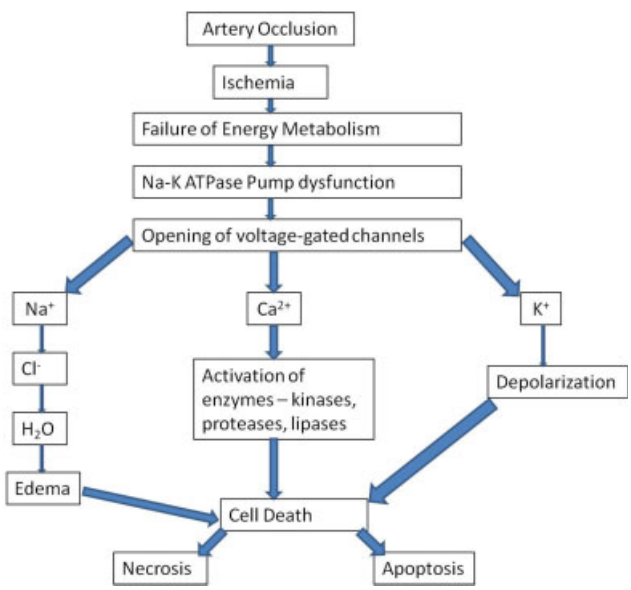

Fig. 1. Flow-chart depicting the pathophysiology involved in stroke. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

Cerebral and Retinal Ischemia

Stroke occurs due to the interruption of blood supply to a part of brain by a thrombus occlusion or embolus or hemorrhage. The symptoms of stroke are blurred vision, vertigo, dizziness, convulsion, and loss of consciousness depending on the area of the nervous system affected. There are wide range of motor and sensorimotor deficits, including tremor, lack of co-ordination, and partial or complete paralysis. Major disability results from loss of ability to communicate, ambulate, coordinate, or reason.

Of all strokes, 80% are categorized as ischemic stroke which results from a thrombotic or embolic occlusion of a major cerebral artery (most often middle cerebral artery, MCA) (Durukan and Tatlisumak, 2007). Current treatment options are limited to supportive care and the management of complications. In spite of significant research in neuroprotection, there is no approved therapy, which can reduce stroke size or neurological disability. The reperfusion following an ischemic event is an important factor which affects the stroke outcome. Early reperfusion after a stroke seems beneficial and capable of reversing the majority of ischemic dysfunctions. However, the development of new therapeutic approaches continues to be a crucial challenge. Various neurotransmitters and neuromodulators are proposed to be involved in the ischemic injury and neuronal death associated with stroke (Koroshetz and Moskowitz, 1996). Though a number of experimental agents reduce infarction size in well-controlled animal stroke models, they have failed to show efficacy in clinical studies, that may be due to several factors such as toxicity, narrow therapeutic time window, difficulty in finding a clinically relevant delivery system to administer compounds intracerebrally over a long period of time and difficulty in transposing standardized experimental settings to human situations. Furthermore, the mechanisms of neuronal death in humans might differ significantly from those studied in experimental systems (in vitro and in vivo), heterogeneous processes might lead to stroke in the human population, and a limited therapeutic window in humans might preclude effective therapeutic opportunities at the time patients become available for treatment (Ginsberg and Busto, 1998). Based on recent advances in our understanding of molecular pathways, many advanced therapeutic approaches are currently developing, that could be considered as potential therapeutic targets.

As the retina is an extension of the diencephalon, the retinal blood vessels share similar anatomic, physiological, and embryological characteristics to the cerebral vessels and possess a blood–retinal barrier which is analogous to the blood–brain barrier (Tso and Jampol, 1982). Retinal ischemia is the characteristic feature of various clinical retinal disorders, including ischemic optic neuropathies, obstructive arterial, and venous retinopathies, carotid occlusive disorders, retinopathy

of prematurity, chronic diabetic retinopathy, and glaucoma (Osborne et al., 2004).

Changes in the retinal vessels probably reflect similar changes in the cerebral vessels (Wong et al., 2003). However, the retinal vasculature is unique in which it can be directly and noninvasively visualized in vivo. Hence, studying retinal signs may provide clues to understanding the pathophysiology of stroke. However, the retinal examination for prediction of future stroke risk remains to be further elucidated.

Animals Models of Stroke

The value and effect of therapeutic approaches in human subjects could be predicted well with appropriate animal models. The goal of cerebral ischemia (focal and global) models is to reduce oxygen and glucose supply to brain tissue (Fig. 2). Most focal cerebral ischemia models involve occlusion of one major cerebral blood vessel such as the MCA in small animals (Hossmann, 1991). The most frequently used focal cerebral ischemia model in rodents is filamentous middle cerebral artery occlusion (fMCAO). This model occludes arterial blood flow intraluminally and allows reperfusion by removing the inserted filament (Steele et al., 2008).

In recent years, animal models of focal cerebral ischemia have gained increasing acceptance as it mimics the human disease (Ginsberg and Busto, 1998). Ischemic stroke is a very heterogeneous disorder; hence it is not possible to mimic all aspects of human stroke in one animal model. Experimental ischemic stroke models help to understand the events occurring in ischemic and reperfused brain. MCAO in the rat has been in use since 1975 (Robinson et al., 1975). The rat and mouse are the most commonly used animals in stroke studies due to many reasons such as their resemblance to humans in cerebrovascular anatomy and physiology (Macrae, 1992). In the initial period, cauterization of the MCA via craniotomy was the most widely used technique of MCAO (Tamura et al., 1981), but it is invasive and does not permit reperfusion. In the case of human cerebral embolism, recirculation occurs frequently after focal ischemia. Koizumi et al. (1986) reported a novel, relatively noninvasive method of achieving reversible MCAO by the use of an intraluminal suture (Koizumi et al., 1986). Longa et al. (1989) reported a variation of this method and stated that their technique reliably produced regional infarcts (Longa et al., 1989). However, brain injury produced by MCAO in rodents varies considerably in its size and distribution; this variability in infarct volume from animal to animal necessitates the use of large numbers of animals to discern statistical significance in drug testing. Among experimental stroke models, the intraluminal suture MCAO in rodents is the most frequently used model, as it is less invasive and easy to perform both permanent and transient ischemia in a controlled manner, and provides reproducible MCA territory infarctions and allows reperfusion by retracting the suture.

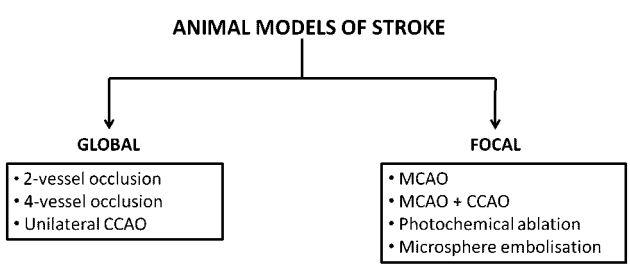

Fig. 2. Flow-chart depicting different types of animal models of stroke.

Animals Models of Retinal Ischemia

The ophthalmic artery originates from the internal carotid artery and it is proximal to the origin of the MCA (Fig. 3). Therefore, fMCAO simultaneously obstructs blood flow in the ipsilateral retina (Steele et al., 2008).

Block et al. demonstrated the first evidence of retinal ischemia by fMCAO. In their study, male Sprague–Dawley rats were subjected to 3 h fMCAO and electroretinogram was recorded. During fMCAO, the amplitude of the a- and b-wave was markedly suppressed (Block et al., 1997). The a-wave is associated with rod photoreceptor activity (Penn and Hagins, 1969) and b-wave reflects the combined activity of depolarizing bipolar cells and potassium currents of Müller cells (Miller and Dowling, 1970).

Among the experimental models of cerebral ischemia, the most common are focal models that either permanently or transiently occlude blood flow of the MCA. It can be achieved either by ligation or coagulation of the MCA resulting in a permanent occlusion (Tamura et al., 1981) or by advancement of an intraluminal suture to the bifurcation between the MCA and the anterior cerebral artery resulting in a transient occlusion depending on the duration of the stay of the suture (Longa et al., 1989). The duration of ischemia can be determined easily which is considered as the advantage of this model. In this method, the common carotid artery, the external carotid

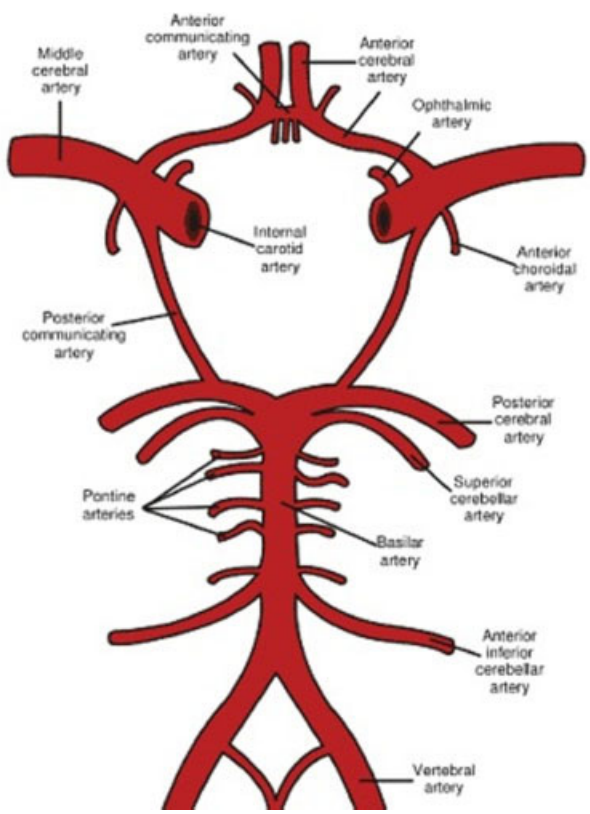

Fig. 3. Circle of Willis exhibiting the blood circulation in brain and eye (With permission from Block et al., 1997). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

artery, and the pterygopalatine artery is occluded by ligation and the branches of the internal carotid artery are occluded by the intraluminal suture. As the ophthalmic artery originates from the internal carotid artery that mainly supplies the inner retina and it is proximal to the origin of MCA, the vascular supply to the retina and the whole eye also gets simultaneously interrupted during occlusion of the MCA which results in retinal ischemia.

The other two methods to induce complete retinal ischemia are ligation of the optic nerve which occludes the central retinal and posterior ciliary arteries or by escalating the intraocular pressure above the arterial opening pressure by infusion of saline into the anterior chamber of the eye (Barnett and Osborne, 1995). Nonetheless, these methods are associated with some unspecific effects which may possibly contribute to retinal changes observed. The optic nerve could be damaged by its ligation and the increased intraocular pressure might affect any of the retinal layers. But in an intraluminal suture method, the vascular supply is interrupted and all the other structures remain intact. Hence, the MCAO model to induce retinal ischemia seems to be an appropriate approach to study pathophysiological processes.

Growing evidence suggests that the bilateral occlusion of the common carotid artery (2VO) compromises the visual system. 2VO affects retinal signal transmission through the elimination of the b-wave of the electroretinogram after 7 days, but no other structures are affected (Barnett and Osborne, 1995). Several studies have reported that chronic 2VO causes atrophy of the optic nerve at 16 weeks postocclusion (Takamatsu et al., 1984).

After reperfusion, the a-wave recovered completely but the amplitude of b-wave recovered approximately 50% of the pre-fMCAO level and remained at this level up to 2 days after reperfusion. The changes in electroretinogram were associated with upregulation of glial fibrillary acidic protein (GFAP) in Muller cells. Kaja et al. (2003) demonstrated histological evidence of retinal ischemia by fMCAO (Kaja et al., 2003). Ovariectomized female Sprague–Dawley rats were subjected to 60 min fMCAO in which they demonstrated apoptosis in approximately 4% of neurons in the ganglion cell layer (GCL) at 1 day after reperfusion. This study also documented a reduction in the Vesl-1L/Homer immunopositive synapses in the inner plexiform layer, indicating early degeneration of retinal ganglion cells. It has been extrapolated that ischemic damage is restricted to GCL.

Thus, fMCAO is a more applicable model for studying these changes and testing the efficacy of therapeutic strategies to prevent or ameliorate any lost functions. Since vision loss resulting from retinal ischemia is often associated with stroke, it is important to show that a neuroprotective reagent or strategy effectively preserves vision by protecting the retina, in addition to the brain damage.

Monocular Amaurosis Fugax

In humans, acute thrombotic/embolic stroke and transient ischemic attack are often associated with temporary diminishment, that is, amaurosis fugax or even permanent loss of vision (Babikian et al., 2001). In the last 7 years of an episode of amaurosis, the risk rate of subsequent hemispheric infarction is increased to approximately 14%. Changes in the retina tend to influence the brain, even in the absence of traditional risk factors (De Schryver et al., 2006). To study neurodegenerative processes and neuroprotective strategies in the central nervous system (CNS), the retina and its projections are an excellent model, since they are easily accessible for experimental procedures and functional evaluation. Animal model of monocular amaurosis fugax is noninvasive with respect to the eye and does not induce blood–eye barrier infarction, mechanical lesions of the retina or optic nerve, contralateral eye lesions, or associated brain lesions in contrast to other currently available models (Osborne et al., 2004). Furthermore, it is reproducible and easily reversible and involves the vascular structure of the entire eye as a result of spontaneous reperfusion which gradually proceeds from the central retina to the periphery, and its duration increases with the duration of ischemia due to microvascular occlusion (Zhang et al., 1999).

Histological and Molecular Analysis of Retinal Ischemia

The intensity of histological damage in ischemia and reperfusion-induced retinal injury is critically dependent on the duration of the period of ischemia. In previous reports, ischemia- and reperfusion-induced cell swelling was well recognized and documented in the inner plexiform layer of the eye (Juarez et al., 1986). The migration of neutrophil leukocyte was also observed after 24 h of reperfusion. In the vertebrate retina, Muller cells are the major type of nonneuronal cells and they are morphologically similar to radial glia and Bergmann glial cells, but unlike these cells, the Muller cells normally do not express the glial cell-specific protein, GFAP. It has been reported that Muller cells accumulate GFAP in response to neuronal injury (Bignami and Dahl, 1979) and degeneration (Drager and Edwards, 1983). Furthermore, GFAP immunoreactivity is enhanced in the Muller cells in response to experimental as well as genetically induced photoreceptor degeneration (Eisenfeld et al., 1984). Recent study has explicitly demonstrated that regenerative processes involving a remodeling of the retinal network may occur following retinal injury in addition to degenerative processes (Isenmann et al., 2003). During synaptogenesis in the CNS, the expression of growth-associated factor 43 (GAP-43) also known as B-50 or neuromodulin, which is a membrane-associated protein is upregulated in neuronal growth cones but is downregulated after synaptogenesis in most brain regions except in specific brain regions that retain plasticity. In the adult retina, GAP-43 is localized in the inner plexiform layer due to its expression by ganglion cells and a subset of amacrine cells (Ekstrom and Johansson, 2003). There is a transient increase of GAP-43 expression following retinal injury or optic nerve transaction (Coblentz et al., 2003), that suggests a structural remodeling in the inner plexiform layer following injury in order to preserve retinal function (Isenmann et al., 2003). GAP-43 immunostaining was increased in retinal ganglion cells at 3 and 7 days of reperfusion after retinal ischemia (Ju et al., 2002). A significant increase of GAP-43 mRNA levels was observed between 12 and 72 h reperfusion with a peak around 24 h (Dijh et al., 2007). Early research suggested that Thy-1, a member of the immunoglobulin supergene family, is expressed predominantly on the retinal ganglion cells (Beale and Osborne, 1982) and it has been widely used as a specific marker for these cells. However, Dabin and Barnstable (1995) found that Thy-1 was expressed by Muller cells but only in cultures denuded of neurons. Inspite of this, it still seems likely that Thy-1 is predominantly associated with RGCs, with only low-level expression in a limited number of other cells. Osborne and Larsen (1996) used anti-Thy-1 antibodies to observe RGC death after experimental ischemia/reperfusion in the rat retina and observed a dramatic decrease in the diffuse immunostaining over the inner plexiform layer and the nuclei present in the GCL after the insult. Moreover, Thy-1 immunoreactivity in the rat retina is dramatically decreased in a dose-dependent manner after NMDA-induced neurotoxicity suggesting that changes in Thy-1 levels can be successfully used as an indicator of ganglion cell death (Nash and Osborne, 1999). Hence, the measurement of total retinal Thy-1 mRNA levels can be a useful indicator of ganglion cell death especially when combined with

immunohistochemical localization of Thy-1. Both ischemic injury and ischemic preconditioning induce the expression of stress-associated genes that encode products such as the heat shock proteins (HSPs) in many cell types, as well as neurons and glial cells (Li et al., 2003). Previous studies reported that HSPs might be used as highly sensitive markers for cellular injury (Dwyer et al., 1989). Under hypoxic conditions, HSP's transcriptional control is associated with hypoxia-inducible factor (Whitlock et al., 2005), a hetero-dimeric transcription factor that is induced by restricted oxygen supply. In addition to the loss of ganglion cells after experimentally induced ischemia, many cells in the inner nuclear layer, presumably amacrine cells, also undergo apoptosis (Osborne et al., 1999) and some evidence has been presented that amacrine cells also undergo degeneration in glaucoma (Panda and Jonas, 1992), and in experimental models with chronic elevation of intraocular pressure (Wang et al., 2000b). Osborne et al. (1999) demonstrated that the major effect of prolonged 2VO is photoreceptor degeneration which is indicated by thinning of the outer nuclear layer and reduced Ret-P1 immunoreactivity (Osborne et al., 1999).

Apoptosis in Ischemic Diseases

There appear two major modes of cell death that participate in ischemic cell death: necrosis and apoptosis. While necrosis is more dominant in the core tissue, penumbral cells die by means of either mode, with apoptosis being more common for cells further away from the core.

Many neurodegenerative diseases such as stroke, brain trauma, spinal cord injury, amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, and Parkinson's disease, are characterized by neuronal cell death. These two mechanisms have discrete histological and biochemical features. Acute ischemia or traumatic injury to the brain or spinal cord leads to necrotic cell death in the CNS (Linnik et al., 1993). Free radicals and excitotoxins (e.g., glutamate, cytotoxic cytokines, and calcium) are generated in the tissue regions which are severely affected by an abrupt biochemical collapse. Mitochondrial and nuclear swelling, dissolution of organelles and condensation of chromatin around the nucleus are considered as histological features of necrotic cell death, which lead to rupture of nuclear and cytoplasmic membranes and the degradation of DNA by random enzymatic cuts in the molecule (Graham and Chen, 2001). Cellular signaling pathways behind the neuronal necrosis are characterized by three consecutive phases: initiation, propagation, and execution. However, our present knowledge of necrotic cell death does not enable us to clearly distinguish between these phases, particularly between propagation and execution events. Unraveling these signaling cascades contributing to necrotic cell death will permit us to develop tools to specifically interfere with necrosis at certain levels of signaling. Treatment or prevention of necrotic cell death is extremely difficult due to these mechanisms and the celerity with which the process occurs.

Apoptotic cell death or programmed cell death can be a characteristic of both acute and chronic neurological diseases (Martin-Villalba et al., 1999; Graham and Chen, 2001). Interestingly, in the last 10 years, several studies provided preliminary data, indicating that apoptosis is recognized as the key event in the number of important ophthalmic diseases such as glaucoma, retinitis pigmentosa, cataract formation, and retinoblastoma. Apoptosis is necessitated in both normal development and in the progression of many diseases. Moreover, apoptosis occurs in the tissue region around the injury which is not affected severely. For instance, after ischemic injury, necrotic cell death takes place in the core of lesion and apoptotic cell death in the penumbral region of the lesion where collateral blood flow reduces the intensity of hypoxia (Linnik et al., 1993; Li et al., 1995).

Many studies have explicitly demonstrated that apoptosis of cells in the retinal ganglion cells and the inner nuclear layer are induced after retinal ischemia-reperfusion by transient elevated IOP and suggested that the treatment with anti-apoptotic agents after ischemia-reperfusion is executable (Lam et al., 1999). Furthermore, Steffany et al. reported that chronic cerebral hypoperfusion induced apoptotic loss of pyramidal neurons which may have a pivotal role in memory impairment associated with clinical conditions of chronic cerebrovascular insufficiency (Bennett et al., 1998).

Role of Growth Factors

Numerous reports suggest that several growth factors and neurotrophic agents possess survival-promoting activity in the central and peripheral nervous systems. Several investigations were carried out to determine whether growth factors or neurotrophic agents prevent damage or degeneration in retinal diseases and neurodegenerative disorders. Pressure-induced retinal ischemia in the rat eye and focal cerebral ischemia in the rat brain may offer degenerative models to address this question because these insults result in alterations and degeneration in several retinal layers and brain, respectively, depending on the extent and duration of ischemic injury (Biichi et al., 1991). Most recently, investigators have demonstrated that basic fibroblast growth factor (bFGF) supports neuronal survival and neurite outgrowth and is essential for the maintenance of the neurons of the cerebral cortex and spinal cord (Westermann et al., 1990). Besides, bFGF is one of the most potent mitogens, inducing both mesodermal and neuroectodermal tissue regeneration, and it induced fiber outgrowth of ganglion cells in cultures of rat retina which roves its function in retina (Baehr et al., 1989). Furthermore, some evidence has been reported that bFGF delays the phenotypic degeneration of Royal College of Surgeons rat retinal photoreceptors (Faktorovich et al., 1990) since it may play an important role in the survival and maintenance of retinal neurons. It has been demonstrated that bFGF is efficient in rescuing the retinal ganglion cells and other inner retinal elements from retinal ischemic injury induced by elevating the intraocular pressure (Zhang et al., 1994). These findings indicate that some neurotrophic agents may have possible therapeutic roles in disorders that affect either photoreceptors or the retinal pigment epithelium. Several studies reported that brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) significantly protect photoreceptors from constant light damage (La Vail et al., 1992), and also demonstrate survival-promoting activity in ganglion cell cultures of chicken embryos (Lehwalder et al., 1989) and rat (Johnson et al., 1986).

Vascular endothelial growth factor (VEGF or VEGFA), an angiogenic peptide promotes endothelial-cell proliferation during development and in disease condition including hypoxia or ischemia. The expression of VEGFA is upregulated in neurons (Jin et al., 2000a) and astrocytesv (Krum and Rosenstein, 1998; Salhia et al., 2000), after focal cerebral ischemia in the rat (Jin et al., 2000a) and in patients after stroke (Issa et al., 1999). Moreover, an intracerebroventricular VEGFA helps to decrease infarct volume and brain edema after transient focal ischemia (Harrigan et al., 2002), and it protects cortical neurons and HN33 (mouse hippocampal neuron × neuroblastoma) cells from hypoxia and glucose deprivation (Jin et al., 2000c) and HN33 cells from serum withdrawal (Jin et al., 2000b) in vitro. These observations suggest that VEGFA has neurotrophic and neuroprotective as well as angiogenic properties; recent studies have explicitly demonstrated that VEGFA enhance the stimulation of

neurogenesis (Jin et al., 2002). VEGFA acts through the VEGFR2/Flk1 receptor tyrosine kinase. Unraveling the roles of other VEGF family members and receptors in cerebral ischemia may help identify new therapeutic targets in stroke. VEGFB, a heparin-binding growth factor (Grimmond et al., 1996) is having similar homology to VEGFA, but its distribution differs from VEGFA (Nag et al., 2002) and, is expressed most abundantly in heart, skeletal muscle, pancreas, and brain (Lagercrantz et al., 1998). The alternatively spliced VEGFB167 and VEGFB186 isoforms bind to at least two VEGF receptors: VEGFR1/Flt1 (Olofsson et al., 1998) and neuropilin-1 (Nrp1) (Makinen et al., 1999). It has been demonstrated that VEGFB promotes angiogenesis after surgically induced hind limb ischemia in mice (Silvestre et al., 2003) and may play a role in tumor angiogenesis because, like VEGFA, it is induced in tumors (Salven et al., 1998). Further VEGFB protects the brain from ischemic injury and cultured cerebral cortical neurons from hypoxic injury which indicates that its protective action is mediated at least in part through a direct effect on neurons (Sun et al., 2004). However, the physiologic role of VEGFB in brain is not fully understood.

Recently, neuroglobin (Ngb), a tissue globin is identified and it is having a high affinity for oxygen. It is widely expressed in neurons of vertebrate central and peripheral nervous systems, retina, and endocrine tissues (Awenius et al., 2001). Furthermore, it was demonstrated that Ngb was upregulated in both transcript and protein levels in cultured primary cortical neurons during the acute phases of hypoxia (Sun et al., 2001). Since it is a newly discovered member of the globin family, Ngb has been considered as the equivalent of brain or nerve tissue hemoglobin (Mammen et al., 2002). Its distribution represented a function of Ngb in metabolically active, oxygen-consuming cell types. But in general, tissue globins mediate multiple cellular and molecular responses to hypoxic/ischemic insults. For an instance, myoglobin in cardiomyocytes and oxidative skeletal myofibers supports oxygen transport, maintenance of nitric oxide homoeostasis, and scavenging activity of reactive oxygen species (Vasil'ieva et al., 1996). These findings proposed that Ngb might have the similar actions in brain. Furthermore, it has been demonstrated that overexpression of Ngb protects against hypoxic neuron injury in cell culture and reduces acute 1-day ischemic brain damage in vivo (Mammen et al., 2002), but the effects of Ngb overexpression on long-term neurological outcomes have not been validated and underlying neuroprotective mechanisms still remain to be elucidated.

Stem Cell Therapy

Stem cells have tremendous potential in medical research. Different therapeutic strategies, based on stem cells have been developed and studied (Kan et al., 2005). Acute ischemic stroke is the most important CNS disorder and a leading cause of death. Despite beneficial effects after thrombolysis, only very small numbers of patients get timely access to the treatment. Cell replacement strategies seek to replace infracted tissue and damaged neurons and sustain nerve conduction. The cell replacement facilitates secretion of growth factors and nutrients for survival, migration, and differentiation of precursor cells. Stem cell therapy is understood to work in two ways: firstly endogenous, that stimulates stem cells that are already present and exogenous, where cells are transplanted locally or systemically.

Stem cells are found throughout the development of the organism. And according to their differentiation capacity, stem cells are of different types. Totipotent stem cells are found from fertilisation to the 4- to 8-cell stage. These can develop into complete embryos including the extra-embryonic tissue. Pluripotent stem cells are known to differentiate into cells of three germ layers. With further cell division and development the ability to differentiate becomes inhibited. Thus, it can form limited number of cell types. Such multipotent stem cells are involved in regular renewal of cells in different tissues of the body—brain, bone marrow, liver, skin, and gastrointestinal tract. Stem cells can also be distinguished based on their origin, that is, embryonic, fetal, and adult. Embryonic stem cells (ESCs) are derived from the blastocysts and are totipotent in nature—can give rise to cells from different germ layers. Fetal stem cells have restricted differentiation capacity and are derived from the fetal organs. But both the embryonic and the fetal stem cells have attending ethical and legal issues. Both embryonic and adult stem cells hold much promise in the newly emerging field of regenerative medicine (Gerecht-Nir and Itskovitz-Eldor, 2004; Rajarathna, 2009; Lenka and Anand, 2010).

ESCs are pluripotent cells which have an unlimited proliferation capacity. They have the potential to differentiate into various cell types, such as hematopoietic cells, astrocytes, hepatocytes, neurons (Sharma, 2010), and have a tremendous capacity for expansion. ESCs have been demonstrated to replace and replenish various different types of cells in damaged tissues. The ESCs have been shown to differentiate into cells of cardiac phenotypes. Hodgson et al. studied ESCs as a source for regeneration of myocardial cells. The authors in this study transplanted ESCs in a rat model of myocardial infarction, which resulted in improvement in ventricular function and structure and also led to decrease in necrosis of the damaged tissue (Hodgson et al., 2004). Transplantation of human ESCs has led to functional as well as structural recovery in MCAO stroke model. Transplanted ESCs differentiated into neurons, astrocytes, oligodendrocytes, and endothelial cells (ECs) (Wei et al., 2005). ECs derived from the ESCs form vascular-like structures in vivo as well as in vitro and thus inducing angiogenesis (Levenberg et al., 2002). Alternative to ESCs are the induced pluripotent stem cells (iPSCs), which are the pluripotent stem cells, derived by inducing forced expression of specific genes in a nonpluripotent cell. These cells were first produced in 2006 from mouse and in 2007 in human cells. iPSCs share same features as the ESCs, that is, self-renewal and differentiation into three germ-layers and are derived from the host itself, hence there is no immune rejection. iPSCS are usually obtained by viral transfection of associated genes, such as *Oct-3/4*, *Sox-2*, *c-myc*, *Klf-4*, and *NANOG*. These cells have been shown to have potential for multi-lineage differentiation. iPSCs have the potential to differentiate into astroglial and neuron-like cells with functional properties. iPSCs have been transplanted in stroke model leading to reduction in infarct volumes as well as downregulation of pro-inflammatory molecules (Chen et al., 2010). The problem associated with iPSCs is that some of the genes involved are oncogenic and may form tumors.

Adult stem cell therapy may offer an accessible, therapeutic tool for damaged tissue replacement and tissue engineering that is free of ethical debate (Kan et al., 2005). The plasticity of the adult stem cells may offer treatment options for a broad spectrum of diseases, especially neurodegenerative disorders and traumatic brain injury. In these conditions the best therapeutic methods are limited due to their inability to repair or to replace the damaged tissue. Because mesenchymal stem cells (MSCs) and MSC-like cells are multipotent and easily expanded in culture, there has been much interest in their clinical potential for tissue repair and gene therapy and as a result, clinical trials are currently underway demonstrating efficacy of MSCs in humans for a range of disorders. In particular, promising results have been obtained using hMSCs in clinical trials for osteogenesis imperfecta, metachromatic leukodystrophy, and Hurler syndrome (Wei et al., 2005). Current clinical applications of adult stem cells include the use of allogenic MSCs to treat osteogenesis imperfecta, a disease with a defect in type I collagen (Horwitz et al., 1999). Several preclinical studies and clinical trials are now being conducted

using MSCs for fracture healing, tendon repair, cartilage regeneration, and support of engrafting after chemotherapy. Differentiation of adult bone marrow to brain cells elucidates a remarkable plasticity of adult tissues with potential clinical applications in treating a variety of CNS disorders, such as brain injury, stroke, Parkinson's disease, and other neurodegenerative disorders (Mezey et al., 2000). Other potential clinical applications of marrow stem cells include using them to treat hepatic failure and myocardial infarction (Wang et al., 2000a).

Cellular therapy represents two therapeutic strategies. One supports transplantation of undifferentiated cells into the tissue, hoping to achieve their integration, site-specific differentiation regulated by local signals, and if not differentiation then at least therapeutic benefits. Another approach supports differentiation, partial or maximal, of MSCs in vitro and transplantation of the differentiated cells, promoting adult stem cell therapy, and illustrating the potential of MSC differentiation (Kan et al., 2005). Rationale behind cell delivery to promote tissue repair is based on the belief that endogenous repair involving the mobilization of MSC from the BM, is insufficient in several pathological conditions. Therefore, cell transplantation or an enhanced mobilization of endogenous therapeutic reservoir may contribute to tissue functional recovery. Different studies have shown homing capability of artificially administered MSC and their site-specific differentiation (Prabhakar et al., 2010). These findings, along with the studies that showed MSC multiple-lineage differentiation potential in vitro, recommend adult MSC as a very promising prospective therapy. Yet, all these potential benefits will have to prove real and beneficial in actual tissue repair (Kan et al., 2005).

Recently it has been demonstrated that transplantation of marrow stem cells into fetal sheep early in gestation survived in multiple tissues, and these cells underwent site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma (Liechty et al., 2000). This data support the possibility of the transplantation of marrow stem cells and their potential utility in tissue engineering and cellular and gene therapy.

Various therapeutic strategies aim to protect the remaining neurons, to slow down the progression of damage, to replace the damaged tissue and to reduce the severity of the symptoms. Efficient neuroprotection has yet to be achieved and since the symptoms of most brain injuries and neurodegenerative diseases occur after major neuronal loss, neuroprotective benefits are limited. Moreover, pharmaceutical therapy is only partly effective, since it cannot replace the lost neurons or stop the progression of the damage and usually causes serious side effects with prolonged use. These limitations are not unique to neurodegenerative diseases and brain injuries, as in many diseases injured tissue repair or regeneration cannot be achieved and only symptomatic therapy is provided (Cao et al., 2002; English and Anand, 2010).

Stroke causes cell death through apoptosis and necrosis. Stroke also leads to generation and migration of new neurons in the damaged area. It causes proliferation of endogenous progenitor cells. Studies have shown that the ischemia induces proliferation of neural stem cells (NSCs) in the subventricular zone and also their migration to the damaged area. Transplantation of stem cells into the stroke-induced brains can reduce neuronal damage. Li et al. showed that the transplantation of nonhematopoietic adult bone marrow cells (BMCs) into the striatum of mice after the induction of stroke lead to functional recovery. The authors observed that these transplanted cells survived in the injured brain and expressed neuronal markers, NeuN, GFAP (Li et al., 2000).

To unravel the clinical use of stem cells, it is necessary to acquire an understanding of their proliferation, migration, differentiation, immunogenicity, and establishment of functional cell contacts (Stone, 1950). Since the knowledge about the critical factors affecting stem cell behavior remains limited. Rapid progress is being made, and some of the first applications of stem cells in human eyes have produced successes that offer hope for the use of stem cells in other ophthalmologic conditions.

Recently, it has been reported that BMCs differentiate into various cells including hepatocytes (Shi et al., 1998), ECs of the blood vessel (Krause et al., 2001), epithelial cells of the stomach, esophagus, small intestine, large intestine and bronchus (Orlic et al., 2001), cardiac muscle (Ferrari et al., 1998), and skeletal muscle (Woodbury et al., 2000). It has also been reported that BMCs differentiate into neural cells and astrocytes in vitro (Azizi et al., 1998) and also into astrocytes in vivo when transplanted into the normal (Eglitis et al., 1999) or ischemic brain (Chang et al., 2007). Moreover, the intravenous injection of BMCs into mice has been shown to induce neuronal differentiation in the brain (Mezey et al., 2000). The stem cells derived from bone-marrow are the MSCs and can produce fibrous tissue, bone, cartilage, and muscle. These cells have the capacity to pass through blood–brain barrier. The bone marrow-derived stromal cells when administered intravenously migrated to the brain and lead to decrease in neurological function deficits. Chen et al. subjected rats to MCA occlusion for 2 h and transplanted stromal cells in different doses. These cells survived and localized in ipsilateral ischemia hemisphere and were shown to express markers for neural cells—NeuN, GFAP, and MAP2 (Chen et al., 2001). Bone marrow-derived stem cells differentiate into other cells after stroke. The bone-marrow cells have been shown to differentiate into neuronal cells—neurons, oligodendrocytes, and astrocytes. The injury itself leads to increase in bone marrow plasticity. Under the pathological conditions, circulating bone-marrow cells enters brain and renews CNS tissue. The GFP-labeled BMCs from male mice were transplanted in female mice that underwent MCA occlusion after transplantation. As the process of neovascularisation and neurogenesis are linked to each other after stroke, the GFP—expressing cells were found to express markers for ECs as well as expressed NeuN, a neural cell marker (Hess et al., 2002). These results demonstrate that BMCs can differentiate into nerve cells with the appropriate stimulators. From the last decade, stem cell transplantation with a variety of cell types for stroke in the animal model was regularly tried for restoration of brain function. Graft survival and even evidence of connection with the host brain was demonstrated mostly in rodent model. ESCs have ethical limitations and some questions remain pertaining to their safety and efficacy. Autologous somatic stem cells are a very attractive source, since there are no ethical concerns and graft rejection is not an issue. However, it is not clear that somatic cells are plastic enough and can be safely induced to a neural fate. Stem cells migrate to areas of brain pathology such as ischemic regions. The homing potential of stem cells may be due to the involvement of molecular signaling pathways such as various chemokines, cytokines, and integrins. Among these, stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor-4 (CXCR4) signaling is required for the interaction of stem cells and ischemia-damaged host tissues (Civin et al., 1984). The process of angiogenesis and neurogenesis are linked to each other via different factors such as VEGF, BDNF. VEGF is already known to be involved in angiogenesis. It also shows neurotrophic and neuroprotective effects. The expression of VEGF receptor—Flk-1 has been shown in the neural progenitor cells of mouse retina (Sondell et al., 2000). It stimulates differentiation into amacrine neurons and photoreceptor cells (Silverman et al., 1999). The expression of VEGF also increases after cerebral ischemia. The ischemic conditions stimulate its expression in brain which

further stimulates formation of new blood vessels (Sun et al., 2003). Erythropoietin is another cytokine that is known to promote angiogenesis in vitro as well as in vivo. It is involved in proliferation and differentiation of erthyroid progenitors. It has been studied that the administration of erythropoietin (EPO) after induced stroke improved the functional recovery (Bernaudin et al., 1999). Erythropoietin is a cytokine that regulates production, proliferation, and differentiation of RBCs. It has been found to be neuroprotective and plays a role in neuroprotection. Tsai et al. demonstrated that the animals null for erythropoietin (Epo) and erythropoietin receptor (EpoR) gene show severe embryonic neurogenesis impairment. The deletion of EpoR in brain leads to decreases in cell proliferation in the SVZ and defective poststroke neurogenesis. Thus, EPO and EPOR are essential for neural development and neurogenesis in both embryonic and adult stages (Digicaylioglu et al., 1995; Tsai et al., 2006). Wang et al. treated rats with recombinant human erythropoietin after stroke and demonstrated functional recovery. The treatment led to increase in cells expressing doublecortin, nestin markers and also increased the expression of other growth factors in brain—VEGF, BDNF, and both of which are involved in angiogenesis and neurogenesis (Wang et al., 2004).

Traditionally, retina and brain damage has been considered irreversible in humans and animals due to the lack of the regenerative capacity of the mammalian nervous system. The retina, as a part of the CNS, is the target of many degenerative diseases with blindness as a final outcome. Retinal regeneration has been a field of interest for more than 50 years (Kim et al., 2002). Recent discoveries in the field of neural stem/progenitor cell biology offer new hope for treatment of incurable chronic ocular and neurodegenerative diseases (Cameron and McKay, 1998) and also potentially provide an alternative to the use of fetal tissue (Cogliati and Swaroop, 2009). In case of retinal neuronal degenerative diseases too, the use of stem cells can be a therapeutic option to restore vision. Stem cells will replace cells in damaged retina and/or decrease further degeneration in retinal neurons. Bone marrow-derived stem cells have been shown to differentiate into retinal neural cells in vivo and in vitro. Stem cells integrate into damaged retina and differentiate into retinal cells. DAPI—labeled bone-marrow stem cells when injected intravitreally in rat model of laser-induced retinal injury. These cells incorporated into the host retina and expressed markers showing neuronal differentiation—GFAP, rhodopsin (Castanheira et al., 2008; Singh et al., 2011). But still more research needs to be done on functional recovery and graft survival.

Traditionally, the mammalian CNS was considered to be a nonrenewable tissue, but this principle has been challenged in the past decade. Neurogenesis in adult mammals has been found in dentate gyrus of hippocampus, subventricular zone of lateral ventricles and olfactory bulb. Studies have demonstrated that NSCs exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans NSC are capable of undergoing expansion and differentiating into neurons, astrocytes, and oligodendrocytes in vitro and after transplantation in vivo. However, inaccessibility of NSC sources deep in the brain severely limits their clinical effectiveness. As to endogenous brain repair, neurogenesis in adult brain is restricted to certain brain regions, lessens with age and does not allow significant functional recovery of the damaged brain tissue. For that reason, neuronal death and functionally damaged or completely dysfunctional remaining tissue are the consequences of brain injury or neurodegenerative disease (Kan et al., 2005). Andres et al. isolated human neuronal progenitor cells from fetal cortical brain tissue, and demonstrated that when cultured and transplanted after stroke they led to functional recovery (Andres et al., 2011).

Neural stem or progenitor cells are primordial cells which have the ability to differentiate into cells of all CNS lineages (neurons, oligodendroglia, and astroglia), to give rise to new progenitors with similar potential, and to populate developing and/or degenerating CNS regions (Gage et al., 1995; Cogliati and Swaroop, 2009; Lagasse et al., 2000). These cells have been isolated from adult, developing and embryonic brain, and in vitro studies have revealed that they possess the ability to adopt a variety of cellular fates (English and Anand, 2010). Stem cells target the stroke-damaged region in brain. Kelly et al. showed that the fetal stem cells injected into the rat brains migrate to damaged region and differentiate into the neurons. Fetal stem cells form neurospheres which show an advantage over both adult and ESCs (Kelly et al., 2004).

Endothelial Precursor Cells

Interestingly, since 1980s, hematopoietic stem cell transplantation and clinical research have mainly focused on the cell surface proteins, CD34, used as a marker for positive selection of heterogeneous hematopoietic stem and progenitor cells (Ogawa, 1993). Hematopoietic stem cells are also capable of transdifferentiation into neural lineages. These stem cells are easily accessible and can be obtained from bone marrow, umbilical cord blood, and peripheral blood.

Although the true role of the CD34 molecule remains to be elucidated, $CD34^+$ HSPC have been functionally defined as capable of generating progenitor-derived clones in vitro and by their potential in reconstitution of the lymphomyelopoietic system in myelocompromised hosts (Yin et al., 1997). An alternative marker for primitive HSPC was CD133 and is enriched with CD34 bright cells (Forraz et al., 2002). In spite of the fact that $CD133^+$ cells had interesting ex vivo expansion potential, they still included cells at various stages of differentiation (Osawa et al., 1996). Several groups suggested that in the absence of CD34 and hematopoietic lineage markers ($Lin^-/CD34^-$), the cells could engraft immunocompromised animal hosts and sustain long-term in vivo hematopoiesis (Asahara et al., 1997).

The Lin^- population comprises a variety of progenitor cells including those capable of becoming vascular ECs (Kalka et al., 2000). Variety of signaling molecules induce these endothelial progenitor cells (EPCs) to mobilize from the bone marrow (Kocher et al., 2001) and can target sites of angiogenesis in ischemic peripheral vasculature (Kalka et al., 2000), myocardium (Scott et al., 1994; Grant et al., 2002), or induced ocular injury. Otani et al. (2002) reported that Lin^- HSCs injected directly into the eye can target activated astrocytes and participate in normal developmental angiogenesis in neonatal mice or injury-induced neovascularization in the adult.

Several studies reported the existence of circulating ECs in peripheral blood in various vascular diseases (Solovey et al., 1997; Khakoo and Finkel, 2005). Despite of these findings, awhile it was unclear about the role of these cells or their precursors in postnatal vascular growth. It has been demonstrated that the $CD34^+$/vascular endothelial growth factor receptor-2 $(VEGFR2)^+$ EPCs is observed in human peripheral blood and it was found that these cells differentiated into mature ECs in culture, which were capable of incorporating into the sites of active neovascularization in animal models (Kalka et al., 2000). This evidence supported the aspect that in adults, endothelial stem or precursor cells may contribute to the formation of new blood vessels by vasculogenesis. Since then, the EPCs' origin, phenotype and function gained significant insights. Emerging evidence demonstrated that the EPCs have a critical role in adult, postnatal endothelial repair and vasculogenesis that accompanies physiological and pathological conditions such as myocardial ischemia and infarction, limb ischemia, wound healing, atherosclerosis, endogenous

endothelial repair, and tumor vascularization (Gehling et al., 2000). But in EPCs research, the identification of EPCs still remains controversial. Previous studies showed that the cells co-expressing hematopoietic stem cell marker CD34 and endothelial marker VEGFR2 is delineated as EPCs (Orlic et al., 2001). Futhermore, it has been reported that some mature ECs also co-express CD34 and VEGFR2 which indicated that CD34 was not an exclusive marker for hematopoietic cells, a novel CD133 glycoprotein was accepted as a more appropriate marker for immature progenitor cells (Handgretinger et al., 2003). Hematopoietic stem cells express the glycosylated form of CD133 protein but not mature ECs, and it is recognized by AC133 monoclonal antibody (Peichev et al., 2000). A subset of circulating $CD34^+$ cells is positive for both VEGFR-2 and AC133 which represents a functional EPC population and plays a role in postnatal angiogenesis or vasculogenesis (Mizrak et al., 2008). Though EPCs share many cell surface markers with ECs and with stem/progenitor cells of different tissues, it is now customary to define EPCs as cells that are positive for AC133, CD34, and VEGFR2 markers, with the following distinction: $AC133^+/CD34^+/VEGFR2^+$ cells represent an immature, highly proliferative EPC population localized mainly in the bone marrow, while $AC133^-/CD34^+/VEGFR2^+$ cells are considered circulatory, more mature cells that are limited in their proliferative capacity (Gehling et al., 2000). Besides, some of the endothelial-specific antigens such as platelet EC adhesion molecule 1 (PECAM-1 or CD31), E-selectin (CD62E) and VE-cadherin (CD144), chemokine receptor CXCR-4 (CD184) is also expressed in these mature cells which have the ability to migrate due to the influence of CXCR-4 ligand, SDF-1, and VEGF. As such, it has been accepted that the new vessels can also be formed through recruitment of circulating EPCs. In recent years, it has been demonstrated that intravenously administered progenitors isolated from bone marrow, peripheral blood or cord blood can home to ischemic sites. Moreover, we have demonstrated the homing potential of intravenously administered bone marrow derived lin^{-ve} and mononuclear cells in laser injured retina and focal cerebral ischemia mouse model, respectively (Prabhakar et al., 2010). Though the significant amount of data available, controversy still remains on the identity and function of the putative EPC and it is functional significance and contribution to regeneration of ischemic injury. However, further identification and characterization of novel, more specific EPC markers is warranted.

For successful development of stem cell-based therapies, many issues need to be addressed—ethical, regulatory, and economical. Apart from these, types of cells suitable, how to control proliferation, survival, differentiation, development of procedures for cell delivery, and patient assessment also need to be taken into account.

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Recruitment of Stem Cells into the Injured Retina After Laser Injury

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Retinal degeneration is a devastating complication of diabetes and other disorders. Stem cell therapy for retinal degeneration has shown encouraging results but functional regeneration has not been yet achieved. Our study was undertaken to evaluate the localization of stem cells delivered to the retina by intravenous versus intravitreal infusion, because stem cell localization is a key factor in ultimate in vivo function. We used lineage-negative bone marrow–derived stem cells in a model wherein retina of mice was induced by precise and reproducible laser injury. Lin^{-ve} bone marrow cells (BMCs) were labeled with a tracking dye and their homing capacity was analyzed at time points after infusion. We found that Lin^{-ve} BMCs get incorporated into laser-injured retina when transplanted through either the intravitreal or intravenous route. The intravenous route resulted in optimal localization of donor cells at the site of injury. These cells incorporated into injured retina in a dose-dependent manner. The data presented in this study reflect the importance of dose and route for stem cell-based treatment designed to result in retinal regeneration.

Introduction

The retina provides a convenient locus to investigate the stem cell function and distribution in the nervous system as it can be manipulated with relative ease and is readily accessible. In addition, a number of degenerative conditions exist, such as retinitis pigmentosa and macular degeneration, which may be amenable to stem cell therapy. In fact, hematopoietic [1], mesenchymal [2,3], embryonic [4,5], neural [6,7], and retinal stem cells [8–11] have been reported to differentiate into neuronal lineages and may be able to regenerate neural retinal tissue. The use of stem cells for retinal regeneration, while widely heralded, should at this time be considered a potential use of stem cells therapeutically. No study to date has succeeded in even approaching retinal regeneration by stem cell or any other therapy. However, much hope has been directed to this possibility lately based on encouraging results dating since 2002 [4,6,7,10,12,13]. BMSCs have been tested for their neurogenic potential in various models of injury [12,14,15] besides transgenic animal models of retinal degeneration [1,2,16]. The mechanism of laser injury has been well studied [17,18], facilitating interpretation of results. Defining the route and dose for optimal localization of regenerative stem cell localization at the retina is of major importance at this time.

The present study was designed with the objective of investigating the recruitment of Lin^{-ve} bone marrow cells (BMCs) at the site of laser injury in retina when delivered through intravenous and intravitreal routes at varying doses and time points.

Materials and Methods

Animals

All the experiments in this study were performed according to the Institutional Animal Ethics Committee Guidelines. Animals used in this study were 6–8-week-old C57BL/6J female mice and efforts were made to minimize the number of animals and suffering. Animals were maintained in a 12-h light/dark cycle (LD 12:12) and fed on chow diet with free access to drinking water.

Study design

Animals were broadly divided into 3 groups—Group 1: intravitreal transplantation; Group 2: intravenous transplantation; and Group 3: laser injury control. In Group 1, Lin^{-ve} BMCs were transplanted through intravitreal route in mice with laser-injured retina at 3 different doses (50,000, 100,000, and 200,000 cells; $n=6$ eyes for each dose) and all animals were sacrificed after 4, 11, and 21 days of cell transplantation. In Group 2, Lin^{-ve} BMCs were intravenously transplanted (100,000 cells through tail vein route) in mice with laser-injured retina. The animals were sacrificed at 4, 11, and 21 days ($n=6$ for each time period). To study the role of injury in donor cell incorporation, 100,000 Lin^{-ve} BMCs were intravenously transplanted in mice with or without laser injury (Group 3, $n=6$). The uninjured contra lateral eye served as control for laser injury.

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Establishment of laser injury model of mouse retina

Argon green laser (532 nm; Iris Medical) was used to induce injury in retina. Briefly, mice were anesthetized with a cocktail of xylazine (10 mg/kg) and ketamine (100 mg/kg) and placed in front of a laser photocoagulator. Pupils were dilated with tropicamide (1% solution), and lignocaine (2% solution) was applied as local anesthetic to the eyes. Laser was imparted to the retina to produce 8 spots around the optic nerve in circular fashion, 2 each in a quadrant, maintaining a distance of 2 disc diameter from optic disc and employing 200 mW and 532 nm with a pulse of 100 ms, generating 100 μm spots.

Fluorescein angiography and fundus photography

Fluorescein angiography was performed after laser injury to visualize the laser spots on 7th day. Fluorescein dye leakage was observed at the sites of laser damage and images of laser injury spots were captured with a fundus camera.

Tissue sectioning

Mice were sacrificed with an overdose of anesthesia (ketamine–xylazine cocktail). The eyeballs were enucleated and either processed fresh or fixed with 4% *p*-formaldehyde solution overnight at 4°C. For sectioning, eyeballs were embedded in optimal cutting temperature compound (OCT) medium (Jung, Leica Microsystems) and 6-μm sections were obtained with a cryostat (CM 1510 S cryostat; Leica). The sections were laid on poly-L-lysine (Sigma Aldrich)–coated glass slides with frosted end (Heathrow Scientific). The whole eye ball was serially sectioned to locate laser injury spots and transplanted donor cells.

Histopathological analysis of laser-injured retina

Cryosections were stained with hematoxylin and eosin and examined under a microscope to study the morphological changes as a result of laser injury in the retina.

Immunohistochemistry

Immunostaining of rhodopsin was also performed to examine the laser-induced damage to photoreceptors. The sections were incubated with 5% goat serum for 1 h at room temperature followed by incubation with primary antibody (anti-mouse rhodopsin, 1:100; Santacruz) for overnight at 4°C in a humidified chamber. The next day, sections were washed 3 times with phosphate buffered saline tween-20 (PBST) buffer [0.01 M phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.05% Tween20] followed by incubation with secondary antibody for 2 h at room temperature (fluorescein isothiocyanate–labeled goat anti-rabbit IgG; Jacksons Immunoresearch). The sections were mounted with antifade mounting medium (FluorSave Reagent; Calbiochem), coverslipped, and examined under a fluorescence microscope (Leica DM-1000).

Isolation and enrichment of BMCs

Mouse femurs and tibias were flushed with culture medium (Invitrogen) to obtain BMCs. BMCs were depleted of RBCs using RBC lysis buffer (Gey's solution). Cells were then incubated with primary antibody (biotin-conjugated lineage antibody cocktail) (anti-CD45, CD5, TER-119, CD11b, and GR-1) for 10 min at 4°C followed by incubation with streptavidin-conjugated magnetic beads for 15 min at 4°C (Lineage Depletion Kit for mouse; Miltenyi Biotech). Unbound antibodies were washed away from cells with MACS buffer [PBS (pH 7.2) and 0.5% BSA]. The magnetic cell sorting (MACS) column was placed in the magnetic field of QuadroMACS separator and rinsed with 0.5 mL MACS buffer before loading cells. Cells were loaded into MACS column (MS column; Miltenyi Biotech) and allowed to pass through the column and the effluent was collected as a fraction enriched with Lin^{-ve} cells. The cell number for transplantation was adjusted with a hemocytometer. The method used was the same as recommended by the commercial kit and as used by previous studies [19,20].

CFDA-SE labeling of BMCs

Carboxyfluorescein diacetate succinimydal ester (CFDA-SE) (Vybrant CFDA cell tracer kit; Invitrogen) was used to track the recruitment of transplanted cells in laser-injured retina. Briefly, 2 mL of CFDA-SE solution (5–10 μM) was added to 2 mL cell suspension and incubated for 15 min at 37°C with dye. After 2 washings, these were reincubated with PBS for 30 min at 37°C. The cell number was adjusted for transplantation.

Transplantation of Lin^{-ve} BMCs

Intravitreal injection. About 2 μL of Lin^{-ve} BMCs with 3 different doses (50,000, 100,000, and 200,000 cells; $n = 6$ per dose group) were intravitreally injected using Hamilton syringes (Hamilton Company) immediately after laser injury. As a control, normal saline was injected into the contralateral eye of the same animal.

Intravenous injection. About 100,000 Lin^{-ve} BMCs suspended in 100 μL PBS were injected with a 1-mL syringe fitted with a 27-gauge needle into the tail vein immediately after laser injury.

All transplantation procedures were carried out under anesthesia as per Institute of Animal Ethics Guidelines.

Quantitative analysis of donor cells in laser injury. CFDA-SE–labeled donor Lin^{-ve} BMCs incorporated into laser injury were counted in both intravitreal and intravenous groups. Cell counts were performed on fluorescence images of cryosections of the laser-injured retina by a blinded observer. The whole eyes were serially sectioned for counting donor cells ($n = 6$ eyes each for intravitreal and intravenous groups).

Results

Validation of laser injury by fluorescein angiography and fundus photography

Laser-injured mouse eyes were imaged with a fundus camera to score the leakage of fluorescein dye injected through the intraperitoneal route on 7th day after injury (Supplementary Fig. S1a, b; Supplementary Data are available online at www.liebertonline.com/scd). The dose of laser radiation was optimized such that retinal damage was limited to the photoreceptor layer in a reproducible fashion unlike the one used for choroidal neocascularization (CNV).

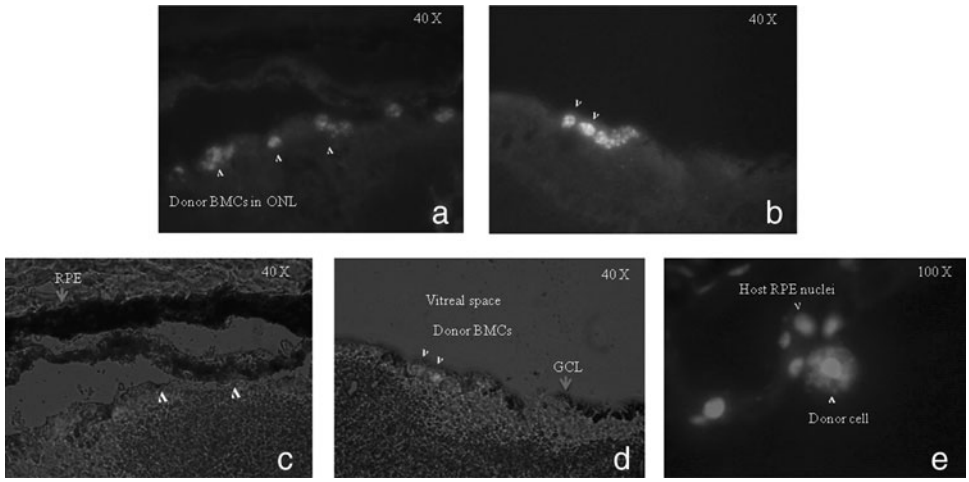

FIG. 1. Incorporation of donor Lin^{-ve} BMCs transplanted via intravitreal route in laser-injured retina. About 100,000 donor BMCs labeled with CFDA-SE (indicated by >) were intravitreaously transplanted in mice immediately after retinal laser injury. Cryosectioning of enucleated eyes at 21 days revealed incorporation of donor cells (indicated by arrows) in the photoreceptor layer **(a)** and GCL **(b)**. **(c, d)** Nomarski images of **a** and **b**. **(e)** A magnified image of a donor cell settled along host RPE cells at laser injury site (counterstained with propidium iodide and indicated by >). BMCs, bone marrow cells; CFDA-SE, carboxyfluorescein diacetate succinimydal ester; GCL, ganglion cell layer; RPE, retinal pigment epithelium.

Retinal pigment epithelium (RPE) cells of C57BL/6J mice have high melanin content that results in maximal absorption of laser light and consequent damage to the RPE cells. Hematoxylin and eosin staining of cryosectioned eyes revealed disrupted RPE and the outer nuclear layer (ONL) of the retina (Supplementary Fig. S1c, d).

Rhodopsin immunolabeling

Rhodopsin is a transmembrane protein present in the outer segments of rods and serves as an excellent immunohistochemical marker for rod cells in mammalian retina. Immunostaining intensity and pattern of rhodopsin in laser-injured eyes of mice, in comparison to the control eyes, confirmed the disruption of photoreceptors in these animals. Laser injury led to disruption of photoreceptor outer segments, resulting in a typical kink, which was immunolocalized by application of rhodopsin antibody (Supplementary Fig. S1e, f). This disruption was lacking in mice eyes that were not laser injured and there was no immunolocalization of rhodopsin like the one seen in laser-injured mice.

Enrichment of Lin^{-ve} BMCs

BMCs isolated from mouse femurs and tibias were enriched for Lin^{-ve} cells by MACS. Lin^{-ve} BMCs were stained with CFDA-SE and its incorporation was verified by fluorescence assisted cell sorting (FACS) analysis (Supplementary Fig. S2a, b).

CFDA-SE–labeled Lin^{-ve} BMCs were injected in the vitreal space of laser-injured eyes at 3 different doses, that is, 50,000, 100,000, and 200,000 cells/animal, which were sacrificed 21 days later. Cryosectioning of eyes revealed the incorporation of donor CFDA-labeled cells in various layers of the host retina. Donor cells were observed in the photoreceptor layer (Fig. 1a, c) as well as in the ganglion cell layer (GCL) (Fig. 1b, d) of injured retina.

The effect of dose on the incorporation of donor cells was studied by transplanting different doses of donor cells in the vitreous of laser-injured retina. The donor cells were found

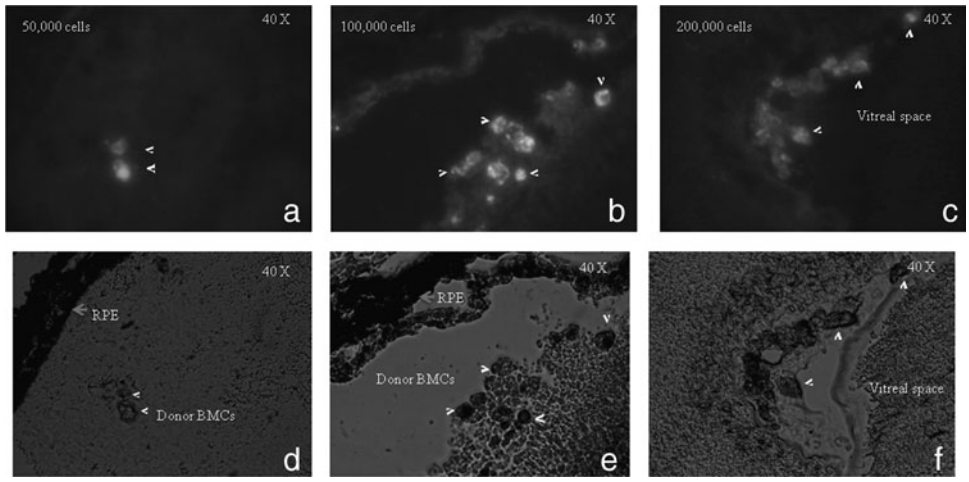

FIG. 2. Dose-dependent incorporation of donor cells in laser-injured retina (indicated by >) at 21 days with 3 different doses of Lin^{-ve} BMCs (50,000, 100,000, and 200,000) transplanted via intravitreal route. **(a, b)** Incorporation of donor cells into the host photoreceptor layer was observed in retina of eyes that received a dose of 50,000 and 100,000 donor BMCs. A dose of 100,000 cells showed more number of cells when compared with other doses (quantitative analysis shown in Fig. 5). **(c)** Donor cells remained in vitreal space in contact with GCL and did not show any incorporation in photoreceptor layer with a dose of 200,000 cells. **(d–f)** Nomarski images of **a–c**.

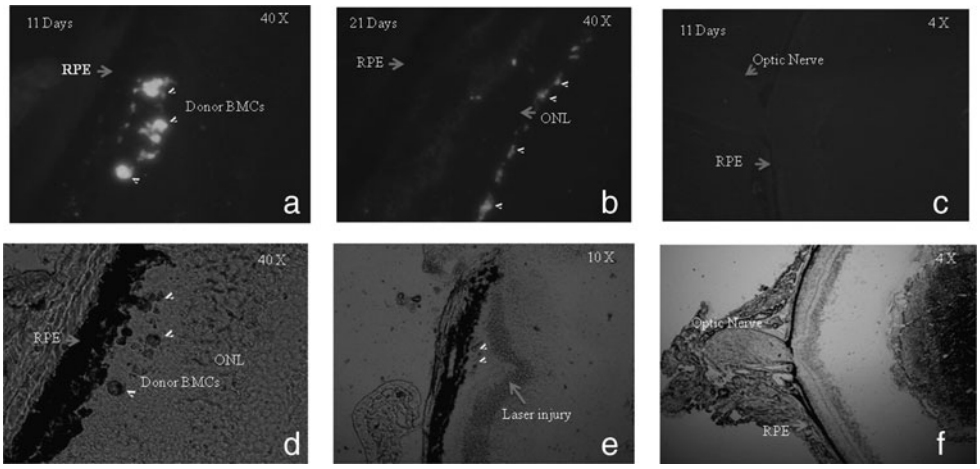

FIG. 3. Incorporation of Lin^{-ve} BMCs in injured retina by intravenous route. About 100,000 cells labeled with CFDA-SE were immediately transplanted after injury through tail vein and animals were sacrificed on 11th and 21st days. Donor cells were observed to be incorporated in the laser-injured retina at both time points and showed widespread incorporation and alignment along host ONL at 21st day **(b)** when compared with 11th day **(a)**. **(d)** Nomarski image of **a**. **(e)** A low-magnification Nomarski image of **a**, highlighting donor cells incorporated into laser injury site. **(c)** Donor cells were not observed in uninjured retina at 11th day, highlighting the role of injury in the engraftment of donor cells. **(f)** Nomarski image of **c**. < indicates CFDA-SE–labeled donor Lin^{-ve} BMCs. ONL, outer nuclear layer.

to reach the injury site at all the doses used in this study. Donor cells were observed in the injured retina with a dose of 50,000 cells (Fig. 2a) and 100,000 cells (Fig. 2b). However, the highest dose of 200,000 cells resulted in clumping of cells in the vitreous (Fig. 2c) with relatively poor migration in the outer retina. We observed that donor cells in the 200,000 dose group remained in the vitreal space as long as 21 days posttransplantation. The total number of cells observed in the host retina as well as in individual layers was counted to compare the effectiveness of different doses.

Incorporation of donor Lin^{-ve} BMCs by intravenous route

Approximately 100,000 Lin^{-ve} BMCs were immediately transplanted after laser injury via tail vein route in mice. These cells were found to be incorporated in RPE and photoreceptor layer, where they survived up to 21 days (Fig. 3b). RPE is seen as an autofluorescent layer that can be easily differentiated from other layers of retina. Donor cells in the 11 days group were mostly found to be clustered around laser injury site (Fig. 3a, d). However, the donor cells were found to be aligned along the host photoreceptor layer by 21st day (Fig. 3b). A low-magnification image of the same section as in Fig. 3a, b shows donor cells clustered into laser injury site (Fig. 3e). In our study, it was not possible to identify the localization of the injected stem cells in the specific areas of the retina.

A comparative quantitative analysis of total number of donor cells incorporated per eye via intravenous and intravitreal routes was made for the 100,000 cells dose group for the 21 days group. The intravenous route showed better incorporation of donor cells (802 ± 35 cells) than the intravitreal route (253 ± 16 cells; Fig. 4). The donor cells survived up to 21 days in injured retina when transplanted either through intravenous or intravitreal route.

Role of injury in incorporation of transplanted Lin^{-ve} BMCs

To evaluate the impact of injury on the donor cell incorporation in retina, CFDA-labeled donor Lin^{-ve} BMCs were injected through tail vein in mice with laser injury in 1 eye only. The other eye of the same animal was not injured with laser. Donor Lin^{-ve} BMCs were found to incorporate in the injured retina (Fig. 3a), whereas the uninjured fellow eye of the same animal did not show any incorporation of transplanted Lin^{-ve} BMCs upto 11 days (Fig. 3c).

Discussion

Laser injury model of retina has been previously used by many workers [14,21–25] and the mechanism and pathophysiology of laser-induced damage has been also well studied [18,26–28]. The laser-induced damage is known to occur by photocoagulation due to heat energy liberated

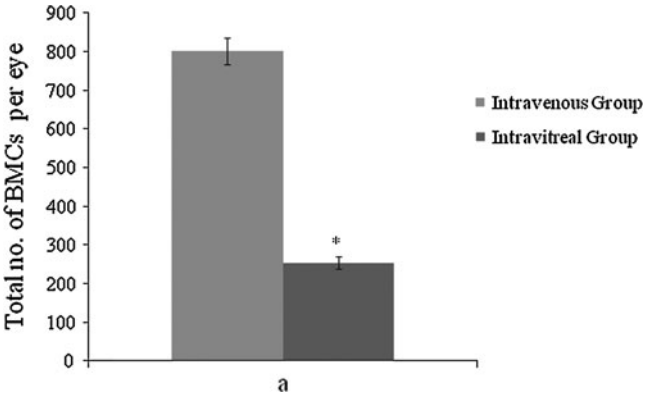

FIG. 4. Quantitative analysis of donor BMCs in laser-injured retina. CFDA-labeled Lin^{-ve} BMCs were transplanted through intravenous and/or intravitreal route (100,000 per animal) and cells were counted in serial sections throughout the retina at 21 days of transplantation. The total number of BMCs counted in intravenous transplantation group was found to be significantly higher than the intravitreal group ($P < 0.001$ for intravenous group vs. intravitreal group; $n = 6$). The data are presented as mean ± standard error of the mean. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered significant. *Statistically significant difference between two graph bars.

during interaction of laser with pigments such as melanin and hemoglobin in retina. As the BMCs have been earlier shown to migrate to site of injury [8,14], we tested whether Lin^{-ve} BMCs exhibit equally good recruitment and survival when transplanted via intravenous and intravitreal routes upon laser injury in retina. Our results show that Lin^{-ve} BMCs incorporated and migrated into various layers of laser-injured retina and survived up to 21 days upon transplantation by both the routes. This is consistent with reports wherein stem cells or retinal progenitors have been shown to integrate into various layers of degenerating retina of transgenic mice [4,9] or injured retina [7,15,29,30] and survive for even longer periods in retina [4,30] when compared with our analysis at 21 days.

The mammalian retina becomes somewhat resistant to transplanted cells after its complete development. Injury results in a microenvironment that is conducive for the recruitment, differentiation, and survival of stem cells. Ahmad and colleagues demonstrated that ocular stem cells do not incorporate in adult retina or uninjured retina but they do so when they are transplanted in animals with retinal injury [8,30,31]. Our study corroborates these reports as we did not observe any incorporation of transplanted cells in uninjured eye in this study. Wang et al. [32] as well as Castanheira et al. [33] have rather recently postulated the use of stem cell therapy for retinal degeneration and showed the incorporation of MSCs in injured retina of rats. Neither group showed any evidence of retinal regeneration, and neither groups' work led to any therapeutic advance. Both studies have, however, led to increased interest in this promising therapy for this devastating consequence of diabetes, a disease that is rampant in developed countries, and seems that it shall be for the foreseeable future. The need for retinal regeneration shall increase as diabetes continues to increase among members of a well-fed world.

The recruitment of BMCs to laser injury sites was observed in our study with both the routes studied, although tail vein administration showed superior homing of transplanted cells. The intravitreally transplanted BMCs settled in GCL, photoreceptor layer, and the RPE. Previous workers have also reported similar incorporation pattern of donor cells in mechanical and transgenic injury models [1,7,8]. BMCs transplanted in the vitreous cavity have to pass through different layers of retina while migrating toward the injury site in the RPE–photoreceptor junction, resulting in residual population of these cells in the GCL as observed in our study. According to recently published evidence from Jiang et al., a substantial number of retinal progenitors were subretinally injected, where they may well exist in vivo migrated into the ONL. In addition, a subpopulation of these cells develop morphological features reminiscent of mature photoreceptors, express photoreceptor specific proteins including synaptic protein, and appear to form synaptic connections with bipolar neurons [34]. Even though this observation has little bearing on our results, it underlines the importance of delivery routes in stem cell translational studies.

We have observed that the homing pattern of donor cells was notably different between 11 and 21 days intravenous groups. A widespread migration was observed in the 21 days group with a characteristic alignment of donor cells in a lateral fashion along the host photoreceptor nuclear layer. Such an arrangement of donor cells was not seen at 11 days posttransplantation. Donor cells in the 11 day group were found to be clustered around the injury site. The intravenous route provides easy access to RPE, the junction between choriocapillaris and outer retina. This partly explains why transplanted BMCs enter retina faster through choroidal vasculature, which is adjacent to RPE, the site of injury. Choroidal vasculature possesses the fastest blood supply than any other organ (about 1400 mL/min in humans), representing a focal point for transplanted BMCs to home to the injury site. Although it can be argued that our results did not provide new information facilitating stem cell treatment of retinal degeneration, to date no study has provided. No study to date has succeeded in even approaching retinal regeneration by stem cell or any other therapy. We have to start somewhere to reach this elusive goal. Thus, defining the route and dose for optimal localization of regenerative stem cell localization at the retina is of major importance at this time.

A quantitative comparison of total number of donor cells at 21 days posttransplantation (50,000 and 100,000 cells group showed an average of 82 ± 4 cells in 50,000 cells group and 253 ± 16 in the 100,000 group) showed better incorporation in the intravenous group as compared to intravitreal group. The highest dose of 200,000 cells delivered to the animals resulted in clumping of cells in the vitreal cavity in close contact with GCL, leading to poor migration in the retinal layers. Donor cells transplanted at a dose of 100,000 cells showed better migration, incorporation, and survival when compared with other dose groups.

Both the dose and route of the transplanted cells have clinical significance for the therapeutic applications in degenerative diseases of retina as most of the clinical trials suffer from lack of data on the optimal dose range as well as the optimal route of injecting stem cells. However, only carefully designed preclinical studies in appropriate models can provide vital clues for accurate extrapolation of results to humans. The results of these experiments can be useful for not only the cases of acute injury but also progressive disorders such as ARMD, which is characterized by breach in the Bruch's membrane, and glaucoma, in which the ganglion cell death occurs. Certain therapeutic strategies to rescue the experimental CNV in animals have been tested by delivering antiangiogenic factors via mesenchymal cells [35]. It is therefore a very attractive proposition to test BMCs for delivery of important molecules in retina. The faster incorporation of Lin^{-ve} BMCs in retina and RPE through intravenous route may play an important role in treating retinal diseases that demand timely recruitment of cells to replace damaged photoreceptors or RPE. The role of growth factors such as vascular endothelial growth factor (VEGF), stromal cell derived factor-1 (SDF-1), and other chemokine factors on recruitment of transplanted cells to the sites of tissue damage has been well established [36]. The differentiation potential of Lin^{-ve} BMCs observed in GCL, RPE, and photoreceptor layers in the intravitreal versus intravenous group will yield important information about the functional state of these injected cells, although several groups have earlier shown differentiation of cells analyzed at various time points and in different models. In this respect, it is pertinent to mention the work of Otani et al., who showed absence of stem cell-derived neurons in the ONL, ruling out the possibility that the injected cells are transforming into photoreceptors (PRs) [37]. This controversial study was offset by a report by Maclaren

et al., who showed successful integration of rod photoreceptors that were found to be immature postmitotic rod precursors and not proliferating progenitor or stem cells [10]. In the present study, we have not been able to evaluate the functional effect of Lin^{-ve} BMCs transplantation but assert that a side-to-side comparison between cell types, just like the comparison between delivery routes, is important to advance the field and resolve the problem. Although the study provides vital clues about effective mobilization of BMCs through the intravenous route, we have not been able to conduct side-to-side comparison of various doses of BMCs via different routes at same time points. It should be noted that there was no demonstrable incorporation of donor cells at 4 and 11 days in the intravitreal group, and because the best incorporation was observed at the 100,000 intravitreal dose group in the 21 days time point, the intravenous-related investigations and accompanying quantitative comparisons were limited for 21 days and 100,000 dose group. Further studies that compare the efficacy of transplantation of BMCs through various routes and doses of donor cells in other animal models of retinal injury or degeneration can provide insights into emerging area of biotherapeutics. The dose-dependent effect of donor cells on the incorporation pattern in injured retina emphasizes a need for standardization of dose in animal and human cell transplantation studies.

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Author Disclosure Statement

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Emergence of Chondrogenic Progenitor Stem Cells in Transplantation Biology—Prospects and Drawbacks

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ABSTRACT

Avascular tissues such as a cartilage contains a unique type of cell called as the chondrocyte. We, however, have not understood the origin of the chondrocyte population and how this population is maintained in the normal tissue. In spite of being considered to be a simple tissue, scientist had always faced difficulties to engineer this tissue. This is because different structural regions of the articular cartilage were never understood. In addition to this, the limited self-repair potential of cartilage tissue and lack of effective therapeutic options for the treatment of damaged cartilage has remained an unsolved problem. Mesenchymal stem cell based therapy may provide a solution for cartilage regeneration. This is due to their ability to differentiate into chondrogenic lineage when appropriate conditions are provided. An ideal cell source, a three-dimensional cell culture, a suitable scaffold material that accomplishes all the necessary properties and bioactive factors in specific amounts are required to induce chondrocyte differentiation and proliferation. Cartilage tissue engineering is a promising and rapidly expanding area of research that assures cartilage regeneration. However, many unsolved questions concerning the mechanism of engraftment of chondrocytes following transplantation in vivo, biological safety after transplantation and the retention of these cells for lifetime remain to be addressed that is possible only through years of extensive research. Further studies are therefore required to estimate the long-term sustainability of these cells in the native tissue, to identify well suited delivery materials and to have a thorough understanding of the mechanism of interaction between the chondrocytes and extracellular matrix and time is not far when this cell based therapy will provide a comprehensive cure to cartilage disease. J. Cell. Biochem. 113: 397–403, 2012.

KEY WORDS: CHONDROCYTES; SCAFFOLDS; ENGRAFTMENT; GROWTH FACTORS; CARTILAGE REPAIR; PROLIFERATION

Stem cells have recently evoked interest as a promising alternative cell source for treating articular cartilage defects. Due to the controversy that is associated with the use of embryonic stem cells, there was an urgent need to establish a viable alternative to embryonic stem cells that is characterized by its clonogenicity, multipotency and its migratory activity and above all they must be associated with a non-controversial source. A suitable alternative such as the stromal stem cells also called as mesenchymal stem cells (MSCs) is associated with the mesodermal lineage and it is known to differentiate into numerous cell types including adipocytes, osteocytes [Pittenger et al., 1999], myocytes [Negishi et al., 2000], astrocytes, and neurons [Woodbury et al., 2000]. Apart from these lineages, MSC's has also shown to have the potential to differentiate into chondrogenic lineage [Kafienah et al., 2006, 2007] and hence they are termed as chondrogenic stem cells (CSCs). The process by which these multipotent cells differentiate into chondrocytes is called as chondrogenesis.

MSCs are shown to be present in various tissues such as bone marrow [Friedenstein et al., 1970; Castro-Malaspina et al., 1980], adipose tissue [Zuk et al., 2001], synovial membrane [De Bari et al., 2001], trabecular bone [Noth et al., 2002], and other tissues. The high proliferation capacity, make them attractive as a distinguished cell substitute for chondrocytes in cartilage regeneration [Baksh et al., 2004]. MSCs can fulfill the requirements demanded by cells for tissue engineering of cartilage, as they can be conveniently manipulated in vitro to differentiate to chondrocytes for these purposes. MSC's were first isolated from the bone marrow and hence the name marrow stromal cells was coined [Bernardo et al., 2009]. However, due to the painful procedure involved in its isolation, risk-containing sampling procedure and limitations in the number of cells in older individuals made the isolation of stem cells from other sources an attractive alternative. An alternative source, however, is the Wharton's Jelly that is isolated from the Umbilical Cord Tissue [Nekanti et al., 2010]. These cells are naïve in nature as

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they originate from the extra embryonic tissue and thus considered as an attractive alternative. Adipose tissue is particularly considered to be an equally attractive source for MSC to bone marrow, as it is easily accessible in large quantities and adipose-derived MSCs show a proliferation and multilineage capacity comparable to sources that are isolated from other established sources [De Ugarte et al., 2003; Winter et al., 2003]. MSC from synovial membrane [De Bari et al., 2001], muscle [Bosch et al., 2000], periosteum [Nakahara et al., 1991], and many other mesenchymal tissues are in experimental use in the field of regenerative medicine. In this review we will discuss the current knowledge of MSCs and its differentiation into chondrocytes. We shall focus on the attempts undertaken in the isolation strategies, characteristic features, and its role in Transplantation Biology. Though this discussion attempts to identify key areas, further detailed research in the potentials of these cells in the areas of regenerative medicine is required.

ROLE OF SCAFFOLDS AND 3D CULTURE IN CHONDROGENIC INDUCTION

In order to induce chondrogenesis, a three-dimensional culture together with a proper scaffold material is required which can be comparable to the cartilage formation in physiological conditions. It has been reported that cells are not retained in the tissue if they are directly administered into the damaged site [Archer et al., 1990]. Therefore, cells are grown in a scaffold that helps to retain them after transplantation. Several publications have reported the chondrogenic differentiation potential of MSCs in vitro [Winter et al., 2003] and in vivo [Breinan et al., 2001; Madry et al., 2002]. Human umbilical cord-derived MSCs (hUCMSCs) cultured in a scaffold made of synthetic polymers polyglycolic acid (PGA) and poly-L-lactic acid (PLA) also differentiate into chondrogenic lineage and upregulates chondrocyte specific genes like collagen type I and II, aggrecan, etc. [Wang et al., 2010]. Similar results were obtained by others [Guilak et al., 2004] for demonstrating the potential of adipose tissue-derived stem cells differentiating into chondrogenic phenotype that synthesized collagens and proteoglycans (Fig. 1).

THE ORIGIN OF CHONDROCYTES DURING EMBRYONIC DEVELOPMENT

Stem cells are known to be classified based on the origin. They may be either of the embryonic or adult origin. Adult stem cells are thought to be present in a specific area of the tissue called a ''stem cell niche.'' In an organism, adult stem cells remain in a state of inactivity for long periods unless they are prompted to divide by some external or internal signals. Multipotent stem cells that are mainly of the hematopoietic or mesodermal lineage are found in many areas of the body such as bone marrow, umbilical cord tissue, adipose tissue, dental pulp, etc. These cells upon stimulation give rise to specialized cells which has created tremendous interest among researchers.

During the embryonic stages of development, MSCs is responsible in the differentiation into two different kinds of cartilage: permanent and transient [Kronenberg, 2003]. The permanent hyaline cartilage arises from MSCs that form the distal ends of the developing bones. After initial condensation, the stem cells differentiate towards stable chondrocytes that thereafter synthesize the typical hyaline extracellular matrix of articular cartilage. In addition to permanent cartilage, a second form also develops from MSCs: the transient cartilage. Prior to skeletal bone formation, chondrocytes originating from MSCs build up a transitional cartilaginous model of the skeleton that is later replaced by mineralized bone in a process called endochondral ossification. After the cartilaginous scaffold is formed, chondrocytes in the middle of the diaphysis cease to proliferate and become hypertrophic, implicating that they enlarge in size and start expressing the hypertrophy marker molecule collagen type X. After further differentiation, hypertrophic chondrocytes start calcifying the surrounding matrix and either transdifferentiate towards osteoblasts or undergo apoptosis [Adams and Shapiro, 2002]. When using MSCs for articular cartilage repair, the great challenge during chondrogenesis is to generate chondrocytes comparable to articular cartilage-derived chondrocytes that do not undergo hypertrophy as a terminal differentiation stage.

EPIGENETIC REGULATION OF CHONDROGENIC STEM CELLS

Chondrogenesis is believed to be a multistep process consisting of several stages: MSC condensation, chondrocyte proliferation, differentiation, maturation, and programmed cell death [Goldring et al., 2006]. The whole process of chondrocyte differentiation is controlled by several signaling molecules, bioactive factors, and transcription factors. These molecules have been reported to phosphorylate the transcription factor cAMP response element (CREB) which induces chondrogenic specific gene expression. Sox9 considered as a fundamental transcription factor plays a pivotal role in promoting chondrogenesis. It is thought to enhance the MSC condensation and stimulate chondrocyte differentiation [Akiyama, 2008]. Sox9 interacts with Sox9 binding sequences on promoters of chondrocyte specific genes like collagen typeII (Col2a1), aggrecan and cartilage oligomeric matrix protein (COMP) and initiates transcription [Bell et al., 1997; Bridgewater and Lefebvre, 1998; Kou and Ikegawa, 2004; Liu et al., 2007] The post-translational modification of Sox-9 gene alters it's functional activity and hence affects the Sox9-dependent transcription in chondrogenesis [Akiyama, 2008; Wegner, 2010]. Protein-kinase A mediated phosphorylation of Sox9 facilitates the Sox9-dependent transcription [Huang et al., 2000]. On the contrary, the transcriptional activity of Sox9 retards with PIAS1 mediated sumoylation [Oh et al., 2007]. Sox5 and Sox6 are members of Sox gene family and are also believed to promote chondrogenic differentiation together with Sox9. This indicates that the transciptional activity of Sox9 is controlled by many factors during the process of chondrogenesis. Other chondrogenic related genes like Runx2, transcription factor Scleraxis (Scx), and chondromodulin-I also contributes in the process of chondrogenesis. This process also involves many chromatin factors other than the transcription factors indicating

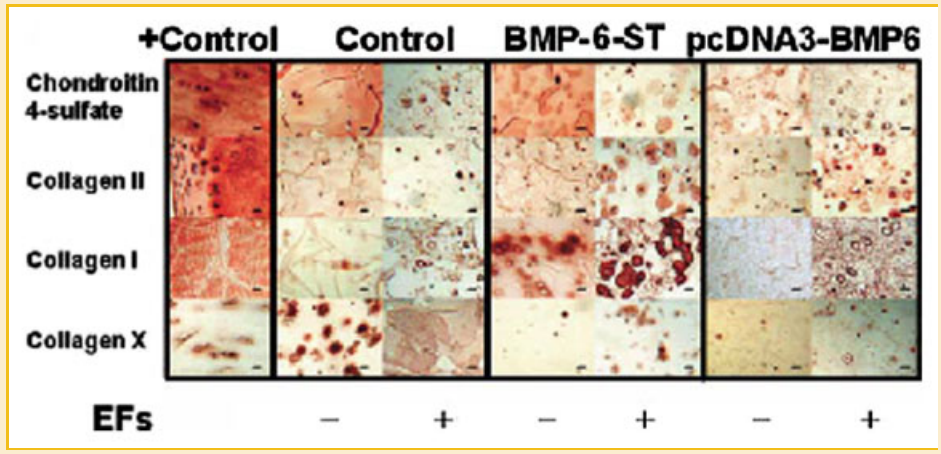

Fig. 1. Representative immunohistochemistry results for chondroitin-4-sulphate and types I, II, and X collagen for a typical experiment with ASCs encapsulated in alginate after 4 weeks in in vitro culture. Positive control: porcine cartilage for C-4-S, collagen II and collagen X; porcine ligament for collagen I. Control: incomplete chondrogenic medium supplemented with 10% FBS. Treated: incomplete chondrogenic medium supplemented with short-term exposure to BMP-6, in addition to continous exposure to rhEGF, ehFGF, and 10% FBS [Estes et al., 2010]. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

a crucial role played by them as well in chondrogenic differentiation.

Chondrogenic induction is initiated by the addition of growth factors like transforming growth factor-β(TGF-β) and bone morphogenetic protein (BMP-6) to the culture media. Many downstream pathways including Smad2, Smad3, and mitogen-activated protein kinase (MAPK) are thought to be activated after TGF-β binds to its receptor [Liu, 2003]. Furumatsu et al. [2005] showed that Smad3 pathway associates with Sox9 to induce chondrocyte differentiation. Other pathways like MAPK pathway also induces the expression of Sox9 and Col2a1 [Murakami et al., 2000; Stanton et al., 2003; Tuli et al., 2003]. It has been demonstrated that growth factors like fibroblast growth factor 1 and 2, insulin-like growth factor-1 (IGF-1) enhances the transcriptional activation of Sox9 [Shakibaei et al., 2006]. These findings suggest that that the process of chondrogenesis is regulated by several growth factors and transcription factors. However, it had remained a controversy that only specific genetic pathways are activated in the process though all cells contain the same genes. This has led to the discovery of another revolutionary field "epigenetics" although many unsolved questions need to be addressed.

The term epigenetics refers to mitotic or meiotic heritable changes in both phenotype and gene expression caused by a mechanism that is not coded in the DNA sequence. It also includes the modification of the histone protein core that includes acetylation, phosphorylation, methylation, and ubiquitation. Epigenetics involves two major modifications: (1) Post-translational modification of the histone proteins by certain enzymes like HAT, histone deacetylases (HDAC), and histone methyl transferases. (2) Methylation of DNA at CpG sites. DNA is wrapped around the histone protein core (consisting of two copies each of the core histone H2A, H2B, H3, and H4) and the structure is called as "nucleosome." Post-translational modifications like acetylation, phosphorylation, ubiquitination, and methylation occur at histone-N-terminals which are known as "histone tails" (Fig. 2). A balance between histone acetylation and deacetylation to regulate the epigenetic transcription is shown in Figure 3. HAT complexes such as coactivator p300 assists in the acetylation of histone protein while HDAC acts as a corepressor and is thought to deacetylate the histones resulting in reduced gene expression.

Highly methylated regions tend to be less transcriptionally active though the exact mechanism is not fully clear. It is believed that methylated areas prevent the binding of transcription factors to the DNA and hinder the process of chondrogenesis. Furumatsu et al. [2005] demonstrated that CREB posseses HAT activity and promotes gene expression after growth factors and other necessary bioactive molecules are added to the culture media. These studies reveals epigenetic activation of chondrogenic specific genes by histone acetylation but further studies are required that focus on methylation–demethylation of DNA. Other mechanisms that determine the epigenetic regulation in cartilage repair are still to be resolved.

LOCATION, CHARACTERISTIC FEATURES, AND ZONAL DISTRIBUTION OF THE MESENCHYMAL PROGENITOR CELLS (MPC's) IN THE NORMAL CARTILAGE

During embryonic skeletogenesis, there is a degradation of the hyaline cartilage. During osteogenesis, some of the remnant cartilage tissues serve as growth site between the ossified epiphyses (ends) and diaphysis (shaft) of the long bones until the completion of the longitudinal bone growth post-natally. In the adult, remnant hyaline cartilage serves as the principal skeletal tissue in the nose, trachea, bronchi, larynx. Remnant hyaline is also found within the rib cage (costal cartilage) and on the articular surfaces of diarthrodial joints [articular cartilage; Ross et al., 2003]. However, in the process of organogenesis of the vertebrate embryo, cells from three distinct mesenchymal lineages (sclerotome, somatopleure, and neural crest) independently undergo cartilage differentiation, or chondrogenesis, to form the multiple hyaline cartilages of the

Fig. 2. Histone modifications: Epigentics include modification of the histone protein core that includes accetylation, phosphorylation, methylation, and ubiquitation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

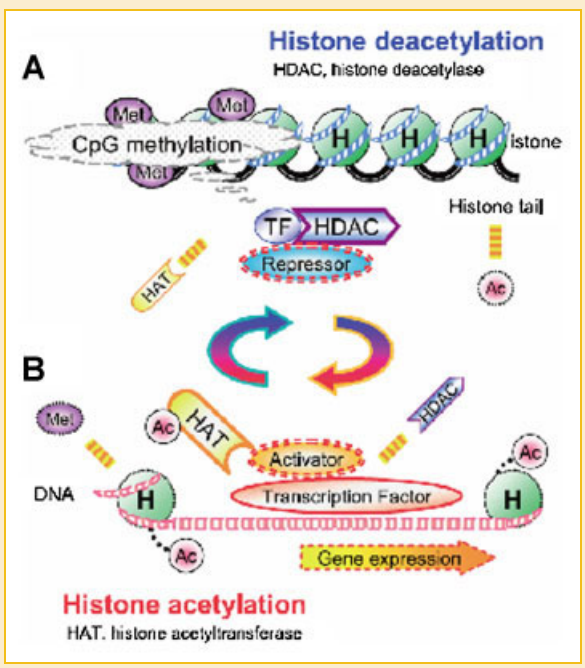

Fig. 3. Epigenetic regulation in the balance between histone acetylation and deacetylation. A: Schematic representation of a condensed heterochromatic structure. CpG islands are methylated in the promoter of the inactive genes on chromatin. In herterochromatic regions, the transcription factor (TF) cannot either recognize or associate with its DNA binding sequence. The repressing molecule and signal favorably associate with TF via the recruitment of corepressor HDAC. B: Schematic model of accessible euchromatic environment. TF, the coactivator HAT and activating molecule (e.g., Sox9, p300, and Smad3/4 trancscriptional complex) co-operatively induce histone acetylation. The chromatin structure changes from an inactive to accessible form by histone acetylation. H, histone; Ac, acetylation; Met, methylation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

primary skeleton. Despite the differences of the formation of chondrocytes from the cells of the mesenchymal origin and the anatomical location of chondrogenesis, it is noted that the formation of the various cartilage proceeds via a fundamentally analogous series of events [Cancedda et al., 2000; Olsen et al., 2000]. In the first stage, undifferentiated prechondrogenic mesenchyme cells migrate to the sites of the prospective skeletal elements, where they initially reside as a dispersed population of progenitor cells. This is followed by the subsequent assembly of the mesenchymal cells to form compact cellular aggregates or condensations—a process that is mediated by a specific combination of precartilage matrix and cell adhesion molecules that inhabit the extracellular space [DeLise et al., 2000]. In the next stage, the intimate juxtaposition of these aggregated mesenchymal cells enables crucial cell–cell surface interactions and signaling events that trigger overt chondrocyte differentiation. Specifically, the chondrogenic progenitor cells exchange their stellate, fibroblastic-like shape for the spherical morphology of hyaline chondrocytes and commence synthesis of cartilage-specific ECM molecules such as collagen types II, IX, and XI [Hoffman et al., 2003] and the highly sulfated proteoglycan aggrecan [Woods et al., 2005]. At this stage, the hyaline chondrocytes may either differentiate into hypertrophic chondrocytes and activate the expression of collagen type X, and contribute to the formation of the growth plate [Beier, 2005], or they may remain undifferentiated so as to maintain the ECM of a persistent hyaline cartilage that is retained throughout the lifespan of the organism.

WHAT MAKES CHONDROCYTES-DERIVED MESENCHYMAL STEM CELLS UNIQUE AND DIFFERENT FROM OTHER MESENCHYMAL STEM CELLS?

Although some similarities occur between chondrocyte-derived MSC and other MSCs, it has been shown [Bernstein et al., 2010] that differences between the two do exist. To confirm the hypothesis it

was imperative to depict gene expression differences between chondrocytes and MSCs during chondrogenic redifferentiation by Pellet Culture (PC). The similarities and differences are listed as follows:

- MSC-derived chondrocytes show inferior mechanical properties and produce less extracellular matrix proteins compared with primary chondrocytes.
- Experimental analysis using immunohistological and quantitative RT-PCR of SOX 9, collagen type I, II, and X using PC-conditioned chondrocytes and PC-conditioned MSC has shown that both chondrocytes and MSC showed a ready progression towards the chondrogenic lineage. Chondrocytes showed a higher SOX 9 expression although there was no significant differences in the collagen I and II mRNA expression.
- MSC's are less efficient at translating, processing, or incorporating collagen into the extracellular matrix.
- Chondrogenic arrest can be missed in both cell types and can proceed toward hypertrophy.
- Chondrocytes expressed significantly higher levels of aggrecan compared to MSC's.
- Increased glycano-anabolic activity is seen in chondrocytes as compared with MSCs due to chondrocytic upregulation of glycosaminyltransferase MGAT4B.
- Chondrocytes showed upregulated fatty acid metabolism that was validated for HMGCS1 and the cytochrome P450 oxidase CYP51A1, thus verifying the cholesterol-biosynthetic activity in chondrocytes.
- MSC's show an upregulation of the gene osteoprotegerin (TNFRSF11B) thus showing that it readily undergoes osseous differentiation as compared to chondrocytes.
- MSC's had a capability to remain in a sustained undifferentiated state and this was linked to the higher expression of the inhibitors of DNA binding ID3 and ID4 in MSC's.
- Downregulation of the eukaryotic initiation factor EIF2AK4 is linked to the increase of protein biosynthesis in MSC's.
- Chondrocytes expressed more of the intracellular chloride channel CLIC4, which is a conserved gene among vertebrates. CLIC4 is a mediator of TGF-b signaling via its translocation to the nucleus. It shows relevance in endothelial tubule formation, anti-apoptotic action, and fibroblast-to-myofibroblast transdifferentiation in cancer cells.

These evidences have shown that MSC's do not reach the same stage of chondrogenic differentiation as chondrocytes do, regardless of TGFb-supplementation and despite harvesting from younger and healthier donors.

ROLE OF CHONDROCYTE DIFFERENTIATION IN TISSUE ENGINEERING

Tissue engineering is an area of regenerative medicine that basically combines areas of cell biology, engineering, material sciences, and surgery to provide new functional tissues using living cells, biomatrices, and signaling molecules [Langer and Vacanti, 1993]. Recently, this discipline has greatly expanded, with numerous research groups focusing on the development of strategies for the repair and regeneration of a variety of tissues [Bonassar and Vacanti, 1998]. Many of these tissue-engineered approaches have targeted the musculoskeletal system in general, with special emphasis on articular cartilage [Temenoff and Mikos, 2000]. The vast majority of approaches that are focused to repair or regenerate articular cartilage are cell-based, thus aiming to provide a population of reparative cells to the injured site. There are various types of cells that are used to develop these strategies. They may be either differentiated chondrocytes isolated from unaffected areas of the joint surface [Risbud and Sittinger, 2002] or progenitor cells capable of differentiating into chondrocytes which can be isolated from a variety of tissues [Amiel et al., 1985]. As harvesting a tissue biopsy from valuable healthy articular cartilage will result in an additional injury, which ultimately cannot repair itself, this cell source might not be an ideal choice. Thus, considerable research efforts are directed to the isolation of progenitor cells and to understand the mechanisms involved in chondrogenic differentiation.

THE WAY FORWARD AND ITS POSSIBLE IMPLICATIONS IN FUTURE THERAPEUTIC INTERVENTIONS

Although in vitro studies and few clinical trials have demonstrated the effectiveness of chondrocyte transplantation in osteoarthritic patients, many challenges still need to be resolved. Scientists need to study in greater detail the mode of delivery of chondrocytes into the tissue, their long-term sustainability in the recipient after transplant, their integration into the damaged tissue and the biological safety these cells promise when administered must be addressed before bringing such treatments to the clinic. To conclude, chondrocyte stem cell therapy offers exciting promises for cartilage repair, but significant hurdles remain before it becomes an acceptable form of therapy in the years to come.

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The emerging role of stem cells in ocular neurodegeneration: hype or hope?

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Abstract Affecting over a hundred million individuals worldwide, retinal diseases are among the leading causes of irreversible visual impairment and blindness. Thus, an appropriate study models, especially animal models, are essential to furthering our understanding of the etiology, pathology, and progression of these diseases. In this review, we provide an overview of retinal disorders in the context of biotherapeutic approaches in these disorders.

Keywords Retinal degeneration · Stem cell · Regeneration · Transplantation

Introduction

The retina consists of complex neural circuit that transduces the light into the electrical signals which are then sent through the optic nerve to the higher centers in the brain for further processing, necessary for perception. In the outer plexiform layer (OPL), the synaptic terminals of rods and cones connect with horizontal cells and bipolar cells. These two cell types modify the incoming signals, and then relay them to the dendrites of the amacrine and ganglion cells via synapses in the inner plexiform layer (IPL). The amacrine cells further process the incoming signals, whereas the ganglion cells relay the visual information to the brain via their axons in the optic nerve (Fig. 1).

Early morphological investigations have revealed different glial cell types and their respective functions which can be discriminated with the help of immunological markers [1–3]. Muller cells have been identified as a distinct glial cell type specific for the neural retina [1]. Their cell bodies are located in the inner nuclear layer, extending radially oriented processes towards the most inner and outer layers of the retina.

The retina is subjected to degeneration from both genetic and acquired causes like other regions of the central nervous system. Once the photoreceptors or inner retinal neurons have degenerated, they are not spontaneously replaced in mammals.

Retinal diseases are characterized by progressive degeneration of retina due to malfunctioning of one or more types of cells involved in visual function, ultimately resulting in loss of vision. There are two major forms of retinal degenerations. Those that

1. Affect the outer retina and
2. Affect the inner retina

Outer retinal pathologies result in degeneration of photoreceptors. These are the primary sensory neurons responsible for detection of light. Signals received by photoreceptors are further processed by three types of interneurons: horizontal cells, bipolar cells, and amacrine cells. Through these interneurons, signals are further sent to retinal ganglion cells (RGCs). RGCs relay the visual information to the brain via the optic nerve. Inner retinal pathologies affect bipolar cells and retinal ganglion cells that disrupt the flow of visual signals to the brain.

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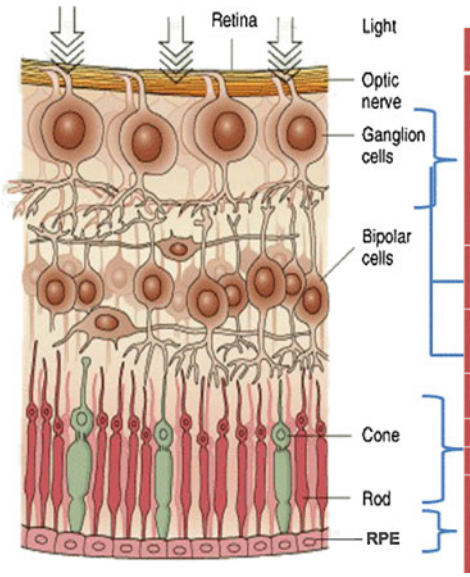

Models	Disease	References
IOP model Optic nerve crush model Optic nerve transection model NMDA induced injury model Laser induced ocular hypertension	Glaucoma	38,76,71
Ischemic/Reperfusion rat model	Retinal ischemia	39
Streptozotocin (STZ) injected mouse model	Diabetic Retinopathy	74
Oxygen induced retinopathy model	Retinopathy of prematurity (ROP)	75
RCS mouse/rat model	Retinal dystrophy	20
Light damage model	Retinitis pigmentosa	36
Laser-induced choroidal Neovascularization CCL2 and CCR2 knock-out mouse Light damage model	Age related macular degeneration	24, 72, 73

Fig. 1 *Line diagram* showing various animal models of retinal degeneration diseases

Age-related macular degeneration (ARMD) is characterized by the outer retinal pathology that causes vision loss in people over the age of 50 in both the developed world and the developing countries. It is a degenerative condition of the macula (the central retina). It begins with characteristic yellow deposits in the macula called drusen that deposit between the retinal pigment epithelium and the underlying choroid. In most advanced form of ARMD, there is abnormal blood vessel growth (choroidal neovascularization) in the choriocapillaries, through Bruch's membrane, ultimately leading to blood and protein leakage below the macula. It has been estimated that ARMD alone accounts for about 9% of global blindness [4]. In India, the prevalence of early ARMD is similar to western population but late ARMD is lower [5]. Glaucoma is another common inner retinal optic neuropathy with elevated intraocular pressure and progressive degeneration of retinal ganglion cells. Intraocular pressure (IOP) is a function of production of liquid aqueous humor by the ciliary processes of the eye and its drainage through the trabecular meshwork. Aqueous humor flows from the ciliary processes, then through the pupil of the iris into the anterior chamber. Then trabecular meshwork drains aqueous humor via Schlemm's canal into scleral plexuses. In glaucoma, IOP is increased due to reduced flow of aqueous humor through the trabecular meshwork or closure of iridocorneal angle. It affects 60.5 million people as on today and is predicted to rise to 79.6 million by 2020 [6]. Diabetic retinopathy is another disorder (damage to the retina) caused by complications of diabetes mellitus, which can eventually lead to blindness. It is an ocular manifestation that affects up to 80% diabetic patients who suffering from diabetes for more than 10 years [7].

Stem cells

Stem cells are undifferentiated cells that are able to self-renew and give rise to differentiated phenotypes [8]. Stem cells may originate from embryonic, fetal, or adult tissue and are broadly categorized accordingly. There are retinal progenitors that reside within retina, e.g., ciliary body epithelium [9], Iris pigment epithelium [10] and the peripheral margin of the postnatal retina [11]. However, conflicting reports dispute the ability of pigmented ciliary epithelium to differentiate into retinal neurons in vitro or in vivo conditions [12, 13]. Owing to limited evidence for endogenous repair in mammalian retina, investigators have attempted to rescue the vision by replacing the degenerated cells by transplantation of various kinds of stem cells. Stem cells have become the centre of so much attention because they turn into all the different cell types that make up complex organisms, and they promise to perform this remarkable feat on demand.

Some of the functions that are important to be achieved as a result of transplantation include:

1. Promotion of cell survival.
2. Secretion of cytokines and neurotrophic factors to modulate immune activity.
3. Transport of receptors to absorb excess unwanted proteins.
4. Effectiveness in sustaining vision without provoking abnormal pathology.

The retina is an excellent model for studying stem cell transplantation into the central nervous system (CNS). The retina arises from the same embryonic origin as the brain, but it is more easily accessible than other parts of the CNS. Many types of stem cell have been used in different models

Table 1 Studies showing transplantation of various types of stem cells with varying dos and route in different animal model of retinal degeneration

Type of stem cells	Specific cell types used	Model type	Dose of cells	Mode of transplantation	Time of sacrifice	Results	Retinal cells/markers expressed post-transplantation	References
Embryonic stem cells (ES cells)	YFP-labeled C2J ES cells	Rpe65rd12/Rpe65rd12 mice, mimics retinitis pigmentosa	10,000 cells/μl	Subretinal	7 months	25% of the mice showed increased ERG responses in the transplanted eyes	*RPE markers* RPE65, bestrophin *Tight junction marker* ZO-1	[18]
	B5 ES cell line derived from 129/Sv mouse strain	C57, Mnd mice strain	30,000 cells/μl	Intravitreal	16 weeks	Donor cells exhibited neuronal-like morphologies, showed expression of cell type–specific marker proteins	*Amacrine and horizontal cells marker* calretinin *Bipolar cells* cPKCα *Photoreceptors marker* rhodopsin	[19]
	Inner cell mass of the mouse blastocyst	20-day-old RCS rats	Not available	Subretinal	2 months	Delaying in photoreceptor cell degeneration	Not available	[20]
	hES cells derived retinal cells	Neonatal (P0–P3) mice	50,000–80,000 cells/μl	Intravitreal	1–6 weeks	No teratoma formation was seen. hESC-derived retinal cells migrated to different layers in retina after transplantation and expressed photoreceptor differentiation markers, synaptic marker	*Photoreceptor marker* Recoverin, S-opsin *Ganglion and Amacrine cells marker* Hu C/D *Bipolar cells* cPKCα *Synaptic marker* Synaptophysin	[21]
	hES cells derived retinal cells	Adult Crx-/- mice (a model of Leber's congenital amaurosis)	50,000–80,000 cells/μl	Subretinal	2–3 weeks	hESC-derived retinal cells expressed functional photoreceptor markers and restored of ERG responses	*Photoreceptor marker* Recoverin, Rhodopsin, Nrl *Synaptic marker* Synaptophysin, PSD95	[21]
	hES cell line WA09-RPE cells	Dystrophic RCS rats	20000 cells/eye)	Subretinal	2 weeks	ERG responses were seen in RPE-grafted animals. No tumor formation was seen	*RPE-specific markers* RPE65, Bestrophin *Photoreceptor marker* Recoverin	[70]
Bone marrow (BM) derived stem cells	Mouse BM-derived Lin^- hematopoietic stem cells	C3H/HeJ (*rd1/rd1*) mouse model	500,000 cells/0.5 μl	Intravitreal	2–6 months	Vasculopathic effect and neuroprotective effect lasts for 6 months	*Photoreceptor marker* red/green cone opsin, rod-specific rhodopsin	[23]
	BM-derived Lin-ve stem cells	Laser induced retinal degeneration in C57/BL6 mice	100,000 cells	Intravitreal and intravenous	21 days	More donor cells are incorporation in intravenous route than intravitreal route	No differentiation marker was studied	[24]
	h BM-derived somatic cells expressing CD90/CD49C markers	RCS rat Model	50,000 cells	Subretinal	2 weeks	Photoreceptor are rescuing with hABM-SC injection with or without cyclosporine	*Cone photoreceptor* Cone arrestin	[28]

Table 1 continued

Type of stem cells	Specific cell types used	Model type	Dose of cells	Mode of transplantation	Time of sacrifice	Results	Retinal cells/markers expressed post-transplantation	References
	BM-MSCs from Pcrx2 K-lacZ transgenic mice	RCS rat Model	50,000 cells	Subretinal	4–8 weeks	Secretion of several neurotrophic factors that promote photoreceptor cell survival	No expression of retinal markers	[35]
	Rat BMCs expressing CD44, CD166, and CD90	Light damage rat model (RP and AMD)	10^6 cells/ml	Subretinal	2 weeks	Release of neurotrophic factors slows down the retinal damage	No expression of GFAP, MAP2, nestin, rhodopsin or calretinin were observed. *Cytokine expression* BDNF	[36]
	BM-MSCs	Rhodopsin knockout mouse	60,000/µl	Subretinal space	35 days	Cells integrated into the RPE and displaying neuronal and glial morphologies Prolonged photoreceptor survival	*Tight junction marker* ZO1 *Retinal epithelial marker* Cytokeratin	[37]
	BM-MSCs expressing CD90 and CD44 but not of CD34 or CD45	Laser induced ocular hypertension, i.e., Glaucoma model	30,000 before 1 week of induction of hypertension	Intravitreal	5 weeks	RGC axon loss decreases significantly	No retinal markers	[38]
	BM-MSCs expressing CD90 and CD44 but not of CD34 or CD45	Laser induced ocular hypertension, i.e., Glaucoma model	5×10^6 after ocular hypertension induction.	Intravenous	5 weeks	No effect on Optic nerve damage	No retinal markers	[38]
	BM-MSCs expressing CD29, CD90, and CD44	Ischemic/ Reperfusion rat model	180,000 cells/µl	Intravitreal	2 or 4 weeks	Few cells showed integration in ganglion cell layer expressing various markers and neurotrophic factors	*Neurotrophic factors expression* bFGF, BDNF and CNTF *Neuronal marker* NSE, NF	[39]
	BM stromal cells expressing CD54	Chronic IOP elevated Glaucoma rat model	40,000 cells/µl	Intravitreal	2 and 4 weeks	BMCs showed neuroprotective effects in glaucomatous retina	*Neurotrophic factors expression* CNTF, GDNF, BDNF, bFGF, and HGFα	[71]
Induced pluripotent stem cells	Tail-tip mouse fibroblasts (TTFs)	Balb-c mice (5 week old)	10,000–20,000 cells/µl (Used Dkk1 + Noggin + DAPT + overexpression of Math-5)	Intravitreal	2–4 weeks	iPS-derived RG-cells were able to survive but unable to integrate into normal host retina	*RPC marker genes* Pax6, Rx, Otx2, Lhx2, and Nestin *RGC-related genes* Math5, Thy1.2, Islet-1, Brn3b RGC fate regulatory gene Hes1/ Hes5, Ath5	[53]

Table 1 continued

Type of stem cells	Specific cell types used	Model type	Dose of cells	Mode of transplantation	Time of sacrifice	Results	Retinal cells/markers expressed post-transplantation	References
	Mouse fibroblast cells	Eyes of PN1 C57Bl6 mouse pups	iPSC-derived retinal progenitors preincubated in PN1CM	Intravitreal	4 weeks	No teratoma formation, few transplanted cells were incorporated in the host retinas, expressing immunoreactivities towards rhodopsin	*Retinal Progenitor genes* Rx, Pax6, Six6, Sox2, Lhx2, Chx10 *RGC differentiation genes* Math-5, Ath5, Wt1, Brn3b, Rpf1, and Irx2) *Photoreceptor genes* Crx, Nrl *Rod-specific genes* Rhodopsin, Rhodopsin kinase *Cone photoreceptor genes* Gnat2, s-opsin *Amacrine cells marker*-syntaxin, *Bipolar cells* mGlur6, *Muller cells* Glutamine synthetase	[54]
	Adult mouse dermal fibroblasts	Rhodopsin null mice (8–10 week)	2.5×10^6 cells (SSEA1 depleted differentiated iPSCs)	Subretinal	28 days	No teratoma formation observed after 16 week post-transplantation Transplanted cells survived Differentiated cell integrated in outer nuclear layer Electro-retinal function were also increased	*Retinal progenitor cell genes* Chx10,Lhx2 *Photoreceptor cell genes* CRX, recoverin, rhodopsin, blueopsin, red/green-opsin, B opsin,ROM-1 and NRL *Retinal cell markers* Pax6, RX. *Synapse markers* synaptophysin, bassoon *Bipolar cell marker* PKCα	[55]

of retinal degeneration using different routes and doses but head to head comparisons are virtually lacking in the field particularly with reference to the type of stem cells. We are trying to summarize some of studies in the Table 1 and discussed in this review.

Types of stem cells

Embryonic stem cells

ES cells are derived from the inner cell mass of blastocyst-stage embryos, with self renewal capabilities as well as the ability to differentiate into various cell types such as hematopoietic cells, astrocytes, hepatocytes, and neurons [14]. Human ES cells have unlimited proliferation potential and maintain normal karyotype, telomere length and pluripotency [15]. For the first time in January 23, 2009, Phase I clinical trials were pursued for transplantation of human ES cells-derived oligodendrocytes (a cell type of the brain and spinal cord) into spinal cord-injured individual patients [16]. Previous experiments had shown an improvement in locomotor recovery in spinal cord-injured rats after a 7-day delayed transplantation of human ES cells that had been differentiated into an oligodendrocytic lineage [17]. Many groups have tried to use embryonic stem cells to generate retinal neurons. Yellow fluorescent protein (YFP)-labeled C2J ES cells were induced to differentiate into RPE-like structures. Differentiated cells expressed RPE-specific markers in vitro. After differentiation, ES cell-derived RPE-like cells were transplanted into the subretinal space of postnatal day 5 Rpe65rd12/Rpe65rd12 mice which mimics the disease of retinitis pigmentosa. The functional outcomes of transplantation were assessed by electroretinograms. YFP-labeled cells can be tracked with live imaging for 7 months. More than half of the mice showed retinal detachments or tumor development, only one-fourth of the mice showed increased electroretinogram responses in the transplanted eyes [18]. Neuralized ES cells at a density of 45,000 cells/1.5 µl were transplanted into the vitreous of each eye of mnd mice (strain B6.KB2-Cln8mnd/MsrJ, C57 mouse strain). These mice have an inherited lysosomal storage disease characterized by retinal and CNS degeneration. Sixteen weeks after transplantation, many of the donor cells exhibited neuronal-like morphologies and possessed numerous branched processes with varicosities and they showed the expression of cell type–specific marker proteins [19]. Embryonic stem cells from the inner cell mass of the mouse blastocyst were allowed to differentiate to neural precursor cells in vitro and were then transplanted into the subretinal space of 20-day-old RCS rats. Transplanted rats were sacrificed 2 months following cell transplantation. Transplantation of embryonic stem cells delayed photoreceptor cell degeneration in RCS rats [20]. Embryonic stem cells have potential to be used as cell replacement therapy. Human ES cells were cultured in media containing retinal determinants for 3 weeks following transfection with lentivirus expressing GFP. These GFP-expressing human ES cell-derived retinal cells were then transplanted intravitreally into new born mice and subretinally into adult Crx deficient mice. In new born mice, transplanted cells showed migration into ganglion cell layer, inner nuclear layer and outer nuclear layer. Expression of retinal cell markers, e.g., PKCα, recoverin, synaptophysin were seen. In case of Crx deficient mouse, these transplanted cells were able to form functional photoreceptors that can respond to light and evoke visual responses in electroretinogram (ERG) analysis without any teratoma formation [21]. Also, there are interesting reports including that of Maclaren et al. [22] who have shown that the rod photoreceptors expressing Nrl derived from immature post-mitotic rod precursors and not stem cells were found to be successful in transplantation studies. These findings emphasize the ontogenetic stage of donor cells for successful transplantation.

Bone marrow-derived stem cells

Many cells upon transplantation may result in undesirable characteristics, e.g., undergoing uncontrolled cell division, showing tendency towards unstable phenotype. They may promote inflammatory responses or provoke immune reaction to the host system. Bone marrow-derived hematopoietic stem cells generally consist of Lineage negative and Lineage positive subpopulations with the former consisting of various progenitor cells depleted of CD5, TER-119, CD2, CD3, CD14, CD15, CD16, CD19, CD56, CD123, CD11b and GR-1 antigens. Vasculopathic and neuroprotective effect was seen in rd1/rd1 mouse received adult bone marrow-derived Lin^- cells, intravitreally. Above effects persist for 6 months of transplantation [23]. Dose- and route-dependent effect of bone marrow-derived Lin^- cells was shown in laser injured mouse retina [24]. It was shown that more number of transplanted cells was incorporated when administered via intravenous route than intravitreal route and donor cells were seen to survive up to 21 days of administration by both routes.

Bone marrow-derived mesenchymal stem cells

Mesenchymal stem cells (*MSCs*) are non-immunogenic, effective at low doses, maintain a stable phenotype and represent a substantially homogenous population of non-hematopoietic somatic cells. These cells secrete various trophic factors and cytokines known to modulate inflammation, augment tissue repair and enhance regeneration [25, 26]. MSCs can also be differentiated into various

lineages including adipocytes, chondrocytes, osteocytes, astrocytes, and neurons [27]. These cells present specific advantages for interventional therapy to the eye. Human MSCs are isolated by the aspiration of bone marrow. These non-hematopoietic somatic cells co-express cell surface markers CD90 and CD49C, but lack expression of the pan-hematopoietic marker CD45. Lu et al. [28] subretinally injected 5×10^4 MSCs in retinal degeneration model of RCS rat. Quantitative analysis showed rescue of photoreceptors in these mice with or without cyclosporine immunosuppression. In CNS, MSCs showed significant functional recovery of forelimb in MCAO rat model of stroke after transplantation of 1.5×10^6 cells into three sites (5×10^5 cells per site) in the brain [29]. Earlier, there was no delineation of non-hematopoietic and mesenchymal cells because all experiments were done with mononuclear bone marrow. The term mesenchymal stem cell (MSC) was proposed by Caplan in 1991 [30], alternative to 'stromal' stem cell. Many questions have been raised over the usage of the term "mesenchymal stem cells" [31, 32]. The use of MSCs has an advantage because

1. These cells can be easily isolated and expanded from adult bone aspirates.
2. These cells have multipotent differentiation potential.
3. These cells enable autogenic transplantation that circumvent the immune related problems and prolongs the effect of cell-based therapy.

The Royal College of Surgeons (RCS) rat is a well-characterized model of retinal degeneration. In RCS rats, functional deficit due to the mutation of a gene encoding the tyrosine kinase, MERTK, causes retinal degeneration quickly [33, 34]. Inoue et al. [35] transplanted 50,000 MSCs subretinally in the RCS rat model. It was reported that MSCs secrete several neurotrophic factors that promote survival of photoreceptor cells. Recently, a study by Zhang and Wang [36], demonstrated that MSCs offered neuronal protection by inhibiting apoptosis in a light damage model after 2 weeks of transplantation. Authors used green light to produce retinal light damage to Sprague–Dawley (SD) rats, which primarily caused photoreceptor apoptosis similar to RP and ARMD. They transplanted 5,000 MSCs in suspension of 5 µl into the subretinal space that detached nearly half the retina. Transplantation of bone marrow-derived mesenchymal stem cells rescued photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse [37].

In 2010, Johnson et al. [38], created a laser induced ocular hypertension, i.e., Glaucoma model. In one group, 3×10^4 MSCs were transplanted intravitreally into eye before 1 week of induction of hypertension. In other group, 5×10^6 MSCs were injected intravenously through the tail vein immediately after the induction of ocular hypertension. Five weeks after transplantation, optic nerve damage quantification demonstrated that intravitreal MSC transplantation was significantly neuroprotective in experimental glaucoma. MSCs were observed to survive well within the recipient eye after intravitreal transplantation, at least for the 5-week study duration, despite using any form of immunosuppression. MSCs at a dose of 180,000 cells/10 µl were transplanted into the vitreous cavity of rats injured by ischemia/reperfusion (I/R). Two or four weeks after transplantation to normal retina, most of the cells were present in the vitreous cavity. But in I/R eyes, most of them were present along the inner limiting membrane. Few cells were integrated into ganglion cells layer and expressed markers like neurofilament (NF), neuron specific enolase (NSE) and neurotrophic factors [39].

Induced pluripotent stem cells (iPS)

iPSCs are pluripotent stem cells artificially derived from somatic cells by inducing a "forced" expression of specific transcription factors and could differentiate into cell types of three germ layers [40, 41]. They are similar to embryonic stem cells in terms of expression of certain pluripotent stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and propensity of differentiation [42]. Shinya Yamanaka's team at Kyoto University were the first group that generated ES-like induced pluripotent stem (iPS) cells from somatic cells by introducing four transcription factors Oct-3/4, Sox2, c-Myc, and Klf4 through retroviral transfection. Cells were isolated by antibiotic selection of $Fbx15^+$ cells (protein expressed in undifferentiated embryonic stem cells). This cell line showed DNA methylation errors and failed to produce viable chimeras when injected into developing embryos [42]. In June 2007, the same group came up with breakthrough study along with two other independent research groups from Harvard, MIT, and the University of California, Los Angeles, showing successful reprogramming of mouse fibroblasts into iPS cells and even producing viable chimera. This time researchers used Nanog for selection of iPS cells (major determinant of cellular pluripotency) [43, 44]. Further, Yamanaka [45] and James Thomson (2007) independently created iPSCs from adult human cell [41].

Recently, many reports have shown that iPS cells can be differentiated into neurons, [46, 47] hematopoietic progenitors, [48] cardiomyocytes [49, 50], and retinal pigment epithelial cells [51, 52].

Chen and its group recently generated iPS cells from tail-tip fibroblasts, induced by the four factors (Oct3/4, Sox2, Klf4, and c-Myc) inherently express RPC-related genes, making them a valuable source of cells for retina

regeneration research [53]. Over-expression of Math5, in combination with DN (DKK + Noggin) and DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester, γ-secretase inhibitor), activated retinal ganglion cell downstream genes and directly differentiated iPS cells into Retinal ganglion-like cells. This study showed a strategy of artificial change of cell fate by transcription factors. Also induced iPS cells responds according to microenvironments specific to different stages of retinal development having the potential to generate early- and late-born retinal cells. This was supported by the results of Parameswaran et al. [54] in the same year. Both retinal ganglion cells and photoreceptors were generated from iPS cells that provides therapeutic implications in degenerative changes in glaucoma and age-related macular degeneration. In 2011, Tucker et al. [55] showed the restoration of retinal structure and function in degenerative mice by adult mouse derived iPS cells. iPS cells were generated using adult dsRed mouse dermal fibroblasts via retroviral induction of the transcription factors Oct4, Sox2, Klf4 and c-Myc. On Day 33, authors found 30% of SSEA1 (stage-specific embryonic antigen 1-enriched) expression among undifferentiated pluripotent cells in the differentiated population possibly resulting in teratoma formation upon transplantation. Twenty-one days after subretinal transplantation into rhodopsin null mice showed that differentiated iPS cells (SSEA1 depleted) took up residence in the retinal outer nuclear layer and gave rise to increased electro retinal function as determined by ERG and functional anatomy. Discovery of human-induced pluripotent stem (hiPS) cells has, thus opened new avenues for the treatment of degenerative diseases using patient-specific stem cells to generate tissues and cells for autologous cell-based therapy. RPE comprises of a monolayer of pigmented cells and plays a crucial role in the formation of blood/retina barrier in retina by tight junctions. It helps in transportation of nutrients such as glucose or vitamin A from blood to the photoreceptors, a constant ion composition in the subretinal space, light absorption and isomerization of the retinal in the visual cycle, secretion of growth factors, and phagocytosis of the outer segments of the photoreceptors. Retinal pigment epithelium (RPE) cells derived from human induced pluripotent stem cells (IMR90-4) exhibit ion transport, membrane potential, polarized vascular endothelial growth factor secretion, and gene expression pattern similar to native RPE [56], showing that hiPS-RPE is a promising candidate for retinal regeneration therapies in ARMD.

The use of defined reprogramming factors for the generation of specific iPS offers:

(1) Treatment regimen that does not require the use of immunosuppressive drugs to prevent rejection
(2) Ability to expand a desired cell type in vitro and
(3) Absence of ethical problems faced when using ES cells.

However, major issues of using iPS cells are risk of viral integrations and oncogene expression during derivation of the cells [57]. Pros and cons of using particular stem cell are listed in Table 2.

Proposed mechanism of retinal restoration by stem cells

Cellular differentiation

It has been reported that BMSCs are able to "transdifferentiate" or change commitment into other cells that express early cell markers [58]. Initially, it was presumed that the repair seen in damaged host tissues following stem cell transplantation or homing was due to incorporation and transdifferentiation of the BMSCs at the sites of damage. However, a number of studies have challenged this concept, providing evidence that BMSCs may instead incorporate

Table 2 Characteristics, advantages and disadvantages of various sources of stem cells used in retinal regeneration

Type of stem cells	Sources	Advantages	Disadvantages	References
Embryonic stem cells	Blastocyst inner cell mass.	Pluripotent in nature, i.e., they can develop into any cell types including retinal cells	Serious ethical considerations	[18, 54, 55]
			Rejection risks due to allogenic source	
		Unlimited differentiation and stable karyotype	Can be tumorigenic	
Bone marrow-derived stem cells		Multipotent in nature	Restricted potential to differentiate	[27]
		No serious ethical considerations	Painful procedure	
			In vitro manipulation is challenging	
		No immune rejection or infections likely in a allogenic source	Difficulty in long term preservation	
			Very short life when cultured in vitro as compared to embryonic cells	
		Easy to isolate the cells		
Induced pluripotent stem cells	Adult somatic cells	Complex ethical considerations	Limited patient specific application	[55, 56]
		Genetically matched cell lines	Recently reported to form teratomas (using retroviral approach)	
		Easier to generate		

into host tissues via fusion [59]. Probably, many cytokines are involved in the recruitment of BM-derived cells into the degenerating retina. For example, stromal derived factor (SDF-1) is a recruitment factor in many retinal diseases such as diabetic retinopathy [60] and age-related macular degeneration [61]. Other factors are Granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-3 (MCP-3), stem cell factor (SCF) or IL-8 [62, 63]. It was hypothesized that microglia phagocytose cellular debris and clear the degenerative environment [64]. Another possible mechanism is that microglia secrete neurotrophic factors (e.g., NGF or CNTF) to promote residual cell survival and also modulate secondary neurotrophic factor expression in Muller glia, contributing to the protection of cells [65].

Paracrine effects

Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and fibroblast growth factor-2 (FGF2) may be important paracrine signaling molecules in stem cell-mediated angiogenesis, protection, and survival [66–68]. Also stem cells attenuate the injury by modulating local inflammation by anti-inflammatory paracrine factors (e.g., TGF-β1) [67]. Exogenous stem cell transplantation may activate neighboring resident tissue stem cells.

Translation from preclinical to clinical trials: where are the road blocks?

Limited use of primate model

Presently most of the transplantation studies are carried on mouse and rat models. There are many differences in the structures of their optic nerve and retina, from those of humans. Also, the eyes of rats and mice do not have maculae or foveae and 85–90% of their optic nerve axons intersect to the other side of the brain [39]. Being a nonhuman primate, Monkey has close phylogeny and high homology with humans. They have retinal and optic nerve anatomy nearly identical to human eyes. Thus, primate model is considered as having the closest compatible model for conducting research with the goal of understanding human diseases [68]. The validity of the experimental model depends upon the degree to which it resembles the human condition. Thus, preclinical investigations in monkey models as well as direct human intervention with appropriate informed consents should be considered before undertaking larger clinical trials in humans.

Functional measurement of transplanted cells in retina

There are limited electrophysiological options available to evaluate the electrical and functional aspects of transplanted stem cell in retina. Three clinically relevant measures could be used to assess visual function–visual acuity, luminance thresholds across the visual field and electroretinogram (ERG) [69].

1. Long duration studies are lacking and could greatly enable analysis of survival, stability and safety of transplanted cells in the retina.
2. Lack of blinding in experimental studies is another problem confronted in stem cell research thus affecting the outcome.

Hype or hope?

It remains to be determined whether the hype created by rapid increase in stem cell investigations using public funding is translated successfully in the bed side. Efforts to regenerate the retina need to be tempered with appropriate animal models knowing that occurrence of tumors can easily demolish the hype held by these studies. The domain of retinal stem cell research has been besieged by the hype generated from the anticipated gain to individuals desperate for cure of fatal diseases. Biotech companies and doctors have further propelled the field even though the results have not been reproducible in most of the studies. Attention has recently been shifted to use of non-embryonic cells with characteristics of an embryonic cells induced by viral infection as investigators in this group published defining reports that provided poof of principles that had to hold in order to attain the therapeutic endpoints anticipated. And while these papers are highly cited as proof of principle, the fundamental findings that they present individually have never been reproduced. Even as established experts at institutes known for their expertise in hematopoietic stem cell transplant successes were unable to validate these claims, they were widely endorsed. Not only could cells of the marrow stroma provide cells that were able to repair a damaged retina, the terrible waste of a brain was certain to become a problem of the past, as the hematopoietic stem cell, or some cells within hematopoietic lineage can be transdifferentiated into progenitors of cells of ectodermal and endodermal origin as well as into cells which were able to provide progeny that would regenerate virtually every component of the ectoderm. The only method that succeeded is surprisingly simple; sell hope. Aging individuals have avidly endorsed the therapeutic endpoints that these institutes promise without following central dogma (Fig. 2).

Conclusion

Like other neurodegenerative diseases, retinal pathologies require neuroprotective therapies. Stem cell-based regenerative

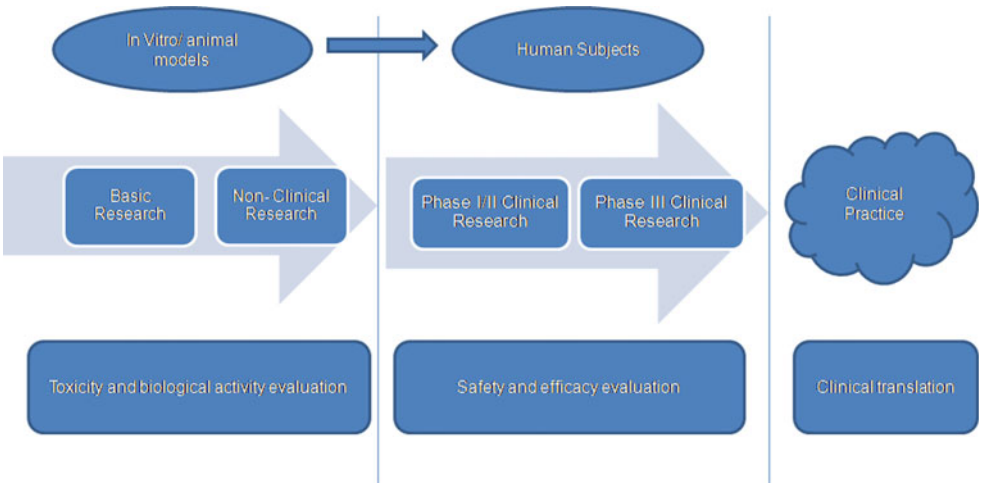

Fig. 2 Road map of stem cell from animal models to clinical level

therapies hold potential but a biological and immunological aspect of these cells needs further elucidation before translation. The level of quality, efficacy, and safety standards need to be consistent in clinical trials. Stem cell transplantation is successful in some diseases at least at preclinical level (e.g., diseases related to skin, etc.) but retina (like brain) related investigations are not conclusive due to difficulties in estimating the functional revival.

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ORIGINAL ARTICLE

Analysis of homing potential of marrow-derived mononuclear cells in an experimentally-induced brain stroke mouse model

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Abstract

Primary objective: To analyse the efficacy of bone marrow-derived mononuclear cells (MNCs) in traversing the blood–brain barrier (BBB) in an experimental model of stroke.

Methods and procedures: The middle cerebral artery occlusion (MCAo) mouse model was established and behavioural and histological analysis was performed, subsequently the carboxyfluorescein diacetate (CFDA)-labelled MNCs were transplanted through the tail vein immediately after 23 hours of reperfusion. The fluorescence microscopic analysis of the brain sections was analysed in both acute and sub-acute phases of transplantation.

Results: The neurological deficit was confirmed by TTC staining and contra lateral turning behaviour. After 2 and 7 days of transplantation, the CFDA-labelled MNCs were observed in the infarcted regions along the line of cortex.

Conclusion: The presence of the CFDA-labelled cells in the ischaemic injured brain lesions proved homing of the implanted MNCs in the infarcted regions of the brain. The successful homing of MNCs may pave way for future clinical trials using MNC in stroke.

Keywords: *Ischemic stroke, MCAO model, stem cell transplantation, marrow-derived mononuclear cells*

Introduction

Stroke is the third most common cause of death worldwide, a neurological syndrome, clinically defined and characterized by rapidly progressing symptoms and signs of focal loss of cerebral function [1]. Approximately 80% of stroke comprises of variant ischemia which results from either thrombotic or embolic occlusion of a major cerebral artery, especially the middle cerebral artery (MCA). Consequently, blood flow reduction occurs which triggers a series of multi-step pathophysiologic events characterized by ischemic cascade [2]. Asians have a higher prevalence of stroke when compared to coronary heart disease. Epidemiological survey conducted on an Indian population has revealed that there was a higher prevalence of cerebral haemorrhage in the community compared to that reported from Western countries [3].

Despite the increase in research investigations, no specific medical therapy has been developed for stroke, even though many promising experimental compounds have reached the Phase III clinical trials [1]. More than 700 drugs have been studied to date which are found to be effective in animal

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stroke models, but none has been proven useful except for a few free radical tapering agents and the thrombolytics [2].

Despite tremendous research in utilizing various sources of stem cells like mesenchymal stem cells, umbilical cord blood cells and amniotic epithelial cells, the homing potential has not been adequately investigated. Presently, the Indian government has initiated several studies using mononuclear cells for various clinical trials. In order to understand the therapeutic potential of bone marrow-derived mononuclear cells in the therapeutic management of stroke, this study analysed the homing efficiency of bone marrow mononuclear cells after experimentally-induced stroke in mouse. It has been believed that the changes in the molecules responsible for cellular trafficking to hypoxic site are responsible for the trapping of mononuclear cells in the ischaemic site [4]. The nerve growth factor of bone marrow mononuclear cells tends to increase peripheral nerve regeneration by its secreting potential [5]. This is possible if there is successful homing of bone marrow-derived mononuclear cells after which efforts can be focused on the signalling cascade involved in the regulation of matrix metalloproteinases or chemokine/chemokine receptors, which participate in the homing of bone marrow mononuclear cells to ischemic site.

Materials and methods

Animals

This study was approved by and performed in accordance with the guidelines of the Institutional Animal Ethical Committee, Post Graduate Institute of Medical Education and Research, Chandigarh, India. Healthy adult male Swiss albino mice ($n = 15$) with an average weight of 25–30 grams and an age of 6–8 weeks were randomly divided into four groups: (1) Sham group; (2) MCAO group; (3) Transplantation group, 2 days and (4) 7 days. The mice were barrier housed two per cage in a temperature ($24 \pm 2^{\circ}$C) and light controlled environment with a 12 : 12 hour light–dark cycle and provided free access to food and water.

Surgical procedure

An experimental stroke mouse model was established by intraluminal suture method, adapted to rat MCAo developed by Zea-Longa et al. [6]. For induction and maintenance of anaesthesia, a ketamine (80 mg/kg) and xylazine (10 mg/kg) cocktail was diluted in normal saline in the ratio of 1 : 10. After placing the animals in the supine position, the skin incision was made in the neck region, following which the muscle was retracted laterally. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated from the vagus nerve. The arteriotomy was made in the ECA to facilitate the insertion of monofilament. A poly-L-lysine coated 6-0 monofilament was carefully advanced up to 11 mm [7] from the carotid artery bifurcation or until resistance was felt. After 60 min of the occlusion period, a loose knot was placed on the ECA stump, below the arteriotomy, and the filament was then carefully removed. Finally, the knot was tightened and reperfusion was confirmed before closing the incision. For sham control, the right CCA, ECA and ICA were exposed via midline incision, but not disrupted, the suture was not inserted and, after 60 min, the incision was closed. Duration of anaesthesia was similar in both MCAo and sham groups.

Behavioural analysis

The neurological status of each animal was evaluated by confirming the turning behaviour towards the side contra-lateral to the ischemic hemisphere, immediately after recovery from anaesthesia, which persisted up to 4 hours after MCAo. This was an acute behavioural response after MCAo and was considered as a precocious index of neurological deficit and neuronal damage.

Histological analysis

After 23 hours of reperfusion, the animals were euthanized by administration of a high dose of ketamine and xylazine and the brain was dissected out carefully and was frozen at -80°C for 15 minutes. The frozen brain was sliced into coronal sections with 1 millimetre thickness and stained at 37°C for 20 minutes with saline solution containing 1% of 2, 3, 5 triphenyltetrazolium chloride (TTC). The cross-sectional area of infarction in each brain slice was examined under a dissection microscope. The stained slices then were transferred into 10% formaldehyde solution for fixation. Images of the brain sections were captured.

Isolation of mononuclear cells

The bone marrow cells were collected by flushing out the aspirates from the tibiae and femur of the mouse with the help of low serum-containing medium using 26G needles. RBCs were lysed using RBC lysis buffer, following which these cells were subjected to density gradient isolation of mononuclear cells. An isolation phase of mononuclear cells requires several manipulation and centrifugation phases of bone marrow aspirates [8]. One millilitre of Ficoll solution was placed in a

15 millilitre centrifuge tube and the bone marrow cells were layered on it carefully and centrifuged at 1800 rpm for 30 minutes in order to isolate low density mononuclear cells. Mononuclear cells were transferred and washed twice with culture medium at 3500 rpm for 10 minutes. The cells were then suspended in 500 microlitres of PBS for further use. Trypan blue dye was used to confirm the viability of transplanted cells which were counted using an haemocytometer. Flow cytometric analysis of mononuclear cells was performed to examine the purity and the presence of the mononuclear cell population before transplantation.

Transplantation

For transplantation, CFDA-labelled mononuclear cells were used to track the transplanted cells in the brain. The mononuclear cells were suspended in phosphate buffered saline (PBS) containing CFDA solution and incubated at 37°C for 15 minutes, accompanied with mild shaking. After 15 minutes, the cells were pelleted at 2000 rpm at 4°C and, subsequently, suspended in pre-warmed PBS at 37°C for 30 minutes. Cells were then washed with PBS at 2000 rpm at 4°C before transplantation. Approximately 2×10^5 to 5×10^5 labelled cells in 500 microlitres total fluid volume were injected with a 26G needle into a tail vein after 24 hours of MCAo.

Immunohistochemical analysis

The mice were sacrificed after 2 and 7 days of transplantation. The brains were removed and cryosectioned at a thickness of 8–10 micrometres. The sections were mounted on poly-L-lysine coated slides and then fixed in Histochoice for 10–15 minutes. The coronal sections were examined under fluorescence microscopy.

Results

Establishment of model: Behavioural analysis

In the present study, after creating the transient ischemia through middle cerebral artery occlusion, the behavioural analysis of contra-lateral turning confirmed the lesion was performed. The intraluminal thread method of ischemia in mice did not produce subarachnoid haemorrhage and seizures.

Histological analysis

The coronal brain sections of MCA-occluded animals showed a consistent pattern of ischemic damage, which was characterized by reduced or absence of TTC stain (Figure 1), whereas the sham-operated animals were devoid of ischemia. Significant correlation between infarction size and neurological deficit was observed.

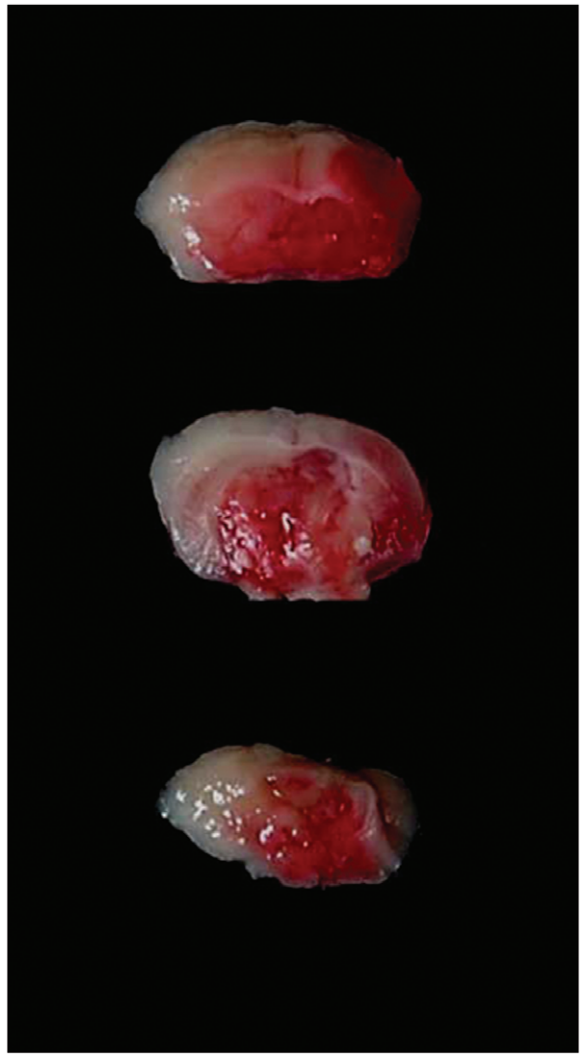

Figure 1. The images of TTC stained coronal sections of brain showing infarction after 60 minutes of occlusion and 23 hours of reperfusion. The white portion represents the ischemic injury in MCAO mice.

MNCs home to infarcted region

The pure population of mononuclear cells were confirmed by FACS analysis (Figure 2). The mononuclear cells equivalent to the 200–400 forward scatter correlates to the cell volume and 200–400 side scatter corresponding to the complexity of the molecule. The CFDA-labelled mononuclear cells were observed in the infarcted regions along the line of cortex after a period of 2 days (Figures 3(*a*) and (*b*)) and 7 days (Figures 3(*c*) and (*d*)) corresponding to acute and sub-acute phase. This is an index to determine the short-term fate

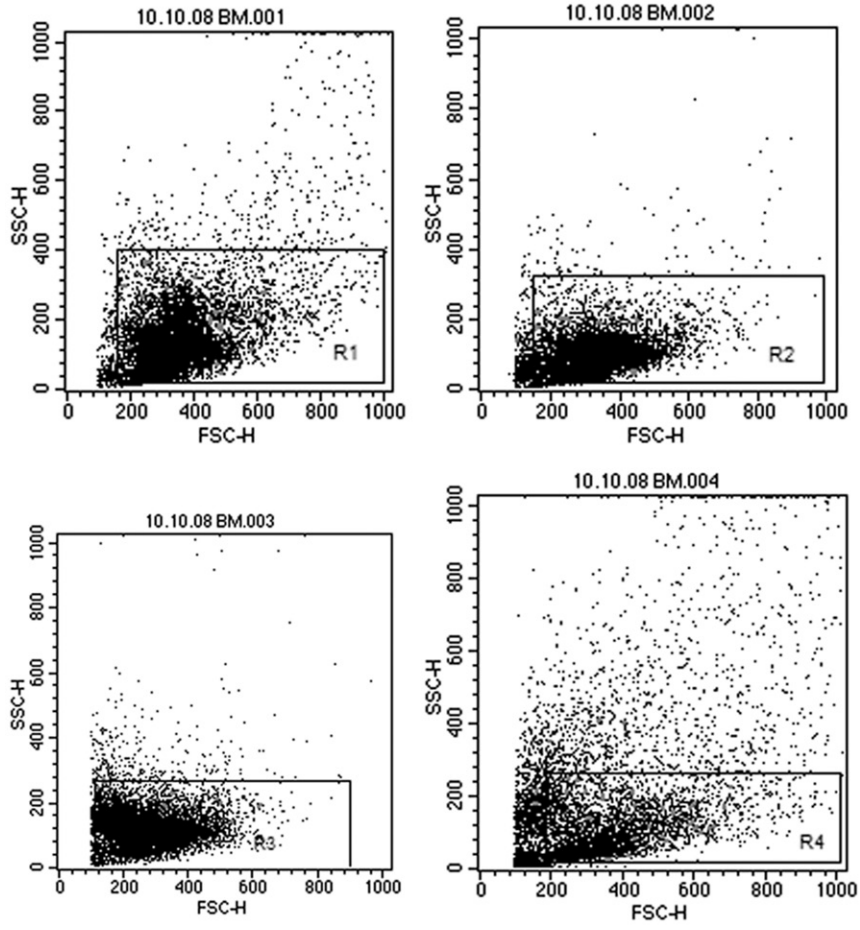

Figure 2. FACS analysis corroborated the purity and presence of mononuclear cells derived from mouse bone marrow.

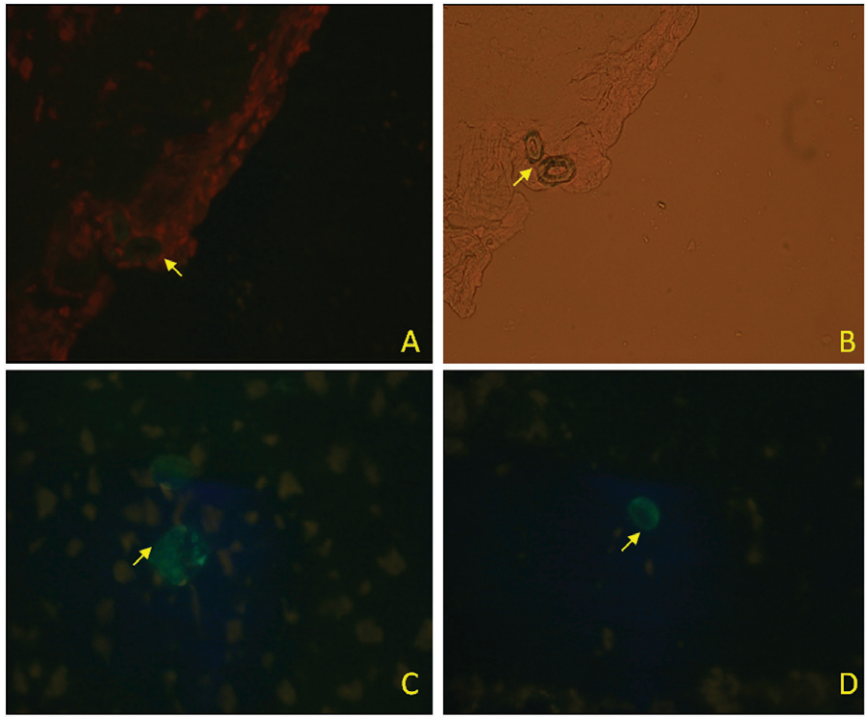

Figure 3. CFDA-labelled cells were detected in the coronal brain sections under fluorescence microscopy after 2 (*a* and *b*) and 7 (*c* and *d*) days of transplantation.

of mononuclear cells, proving the homing behaviour of mononuclear cells to the ischemic site.

Discussion

Although various sources of stem cells have been discussed recently in the current literature for neural therapy and repair, yet mononuclear cells are the most attractive type that have been used and led to several clinical trials for neurodegenerative diseases which have been initiated in this country. Techniques for inducing transient MCAo by an intraluminal thread method have been extensively used in experimental models of ischemic stroke since it induces relatively bigger and reproducible infarct volumes. A reliable focal stroke model of transient regional ischemia in rats reported in the literature [6] was less invasive than conventional methods. Several variations and applications of this technique have been reported in order to refine and validate this technique. The aim of the present study was to establish a transient MCAo model in mice and to evaluate the efficacy of homing of MNCs to the ischemic site in the acute phase of the experimental stroke mouse model. The advantages of this model are its reproducibility and ease of reperfusion. However, it is sometimes difficult to detect neurological deficits after cerebral ischemia in rodents [9]. In the present study, mice showed contra-lateral turning behaviour, immediately after surgery, which was considered as a marker of behavioural deficit and was distinctly correlated with infarction evaluated later. In contrast, previous reports have analysed the correlation between the severity of behavioural deficits and histological findings. Various tests to monitor the behaviour include average forelimb and hind limb placing scores, asymmetric limb use for wall movement, Morris water-maze test and probe trial performance and infarct volume measurement. It was found that the infarct size significantly correlated with only two behavioural measures (forelimb placing performance correlated with subcortical infarct volume) while performance on the water-maze test also correlated with subcortical infarct volume [10]. Hence, further investigations will reveal the importance of behavioural deficit in bringing infarct volume changes in experimental stroke.

This study confirmed the infarcted site with the staining of TTC, an oxidation-reduction indicator. Therefore, the tissue with normal levels of the enzyme is stained red, whereas ischemic and infarcted tissue remains unstained owing to loss of the enzyme [11]. The present study demonstrates the homing behaviour of bone marrow-derived mononuclear cells caused by successful trafficking through the BBB which may have been driven by chemotaxis. The detailed molecular mechanism needs further investigation. This study shows that intravenous infusion of bone marrow-derived mononuclear cells, 24 hours after transient MCAo in mice, results in accumulation of CFDA-labelled cells in the infarcted region of the brain. This illustrates that mononuclear cells are not only able to cross the blood–brain barrier but also survive there for up to a week. The mechanism of homing and cell cycle kinetics has been investigated [12] and molecular imaging of bone marrow mononuclear cell homing in ischemic myocardium has been analysed [13].

The data presented here has some limitations, like absence of MCAo validation by laser Doppler flow meter coupled with a limited choice of time points analysed for study. However, this study was still able to demonstrate that bone marrow MNCs can successfully migrate to the infarcted area across the blood–brain barrier in mice.

Conclusion

Since there is a sudden increase in MNC-based clinical trials, a dose-dependent investigation along with side-to-side comparison of various routes of delivery is warranted. As the majority of the studies have been done in rats, this is a rare study conducted in mice which is important because extrapolation of data is easy with the availability of several mice knock outs. This study establishes the proof that MNCs can indeed cross the mice BBB and reach the infarcted area at early time points. Further studies can investigate the biology of MNCs in neuronal repair with analysis of behavioural outcomes at other time points using additional validation tools including laser doppler.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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stem cell entrepreneurship in India: trends and advances-I

India's growing investment in stem cell research is powered by its economic growth and vision to acquire global leadership in biotherapeutics. The recent economic boom in country has been mildly shaken by its overdependence on outsourced service industry and investment from multinational companies which is unlikely to continue indefinitely. It is therefore prepared to overcoming the challenges once represented by the absence of consortium of IP attorneys, clinician-scientists and venture capitalists, the key elements in biotechnology entrepreneurship, so that the economic progress is sustainable. The signs of reversal of brain drain, which once affected value creation, are now becoming evident induced by schemes such as the DBT-Wellcome alliance. However, there are a few academic problems such as authority patterns, lack of performance based incentives and seniority over merit dogma, once an accepted part of Indian academics, that is now being reviewed so that the institutional and faculty support systems become more vibrant and responsive to innovation systems. In this article, the historical and socio-cultural influences that have shaped lifesciences in India have been discussed which include the contemporary academic challenges confronting the biomedical entrepreneurs. We also provide a projection of future growth prospects of stem research translation in India in the context of existing regulations. The framing of ICMR-DBT guidelines for stem cell research and therapy in India is pivotal to the future stem cell therapy is likely to hold for this country and the world. The health and science managers in India realize that India must rely on its own capacity for innovation than depend on MNC outsourced model. This will require more drastic policy changes in the manner that facilitate scientific temper, output and entrepreneurship.

India has a unique combination of ancient knowledge base and modern technology in order to foray into science business. The traditional system of imparting knowledge in India always consisted of free education in *Gurukul* (free residential school).[1] Some thinkers believe that the genesis of the problem of dissociation of wealth creation efforts from knowledge lies in the known relationship problems between *Saraswati* and *Lakshmi.* The idea of science entrepreneurship has thus never visited the political thinkers, policy makers or the biomedical scientists until very recently. In addition, majority of the research in India has continued to be supported by only public funds, because of which the licensing and protection of patents has remained under government control and commercialization of scientific enterprises required bureaucratic clearances. The absence of strong domestic academic pressure groups and private sponsored research entities has contributed to lack of awareness about the business prospects of the scientific ideas.

Policy reforms

The advancement of science, particularly stem cell therapeutics has coincided with the shared thrust of national leaders such as Ex President scientist Dr Abdul Kalam and economist Prime Minister Dr Manmohan Singh for pushing knowledge economy. Rapid scientist awareness campaigns have been launched in the country, medical tourism being promoted apart from increasing the R&D outlay. The recent example is the Indian Council of Medical Research (ICMR) which spearheads stem cell research activities in India. It was recently upgraded as a Department of Ministry of Science and Technology, almost doubling the budget allocation for medical research. Several knowledge parks, technology incubators, medi cities and Public-Private Partnership (PPP) projects are being initiated. A technology development board, a national body which oversees value creation projects has been actively promoting science entrepreneurship. The situation at the medical institutes is particularly alarming where there is a need for integration of health and science policy for potential research. The plea for optimal patient care is often justified as an excuse for neglecting the importance of innovation in such Institutes. As a result, the opportunity of translating the developments in stem cell research are lost because of separating clinical practice from basic research. In view of the current over-restrictive policies of US and Europe, there are opportunities for India to maximize the utilization of its human resource and encourage biomedical scientists to participate in generation of new clusters of stem cell research and therapy so that the unmet global demand for such services in the country can be provided in a careful and regulated manner. In order to understand the context in which the current innovation policies are placed in India it is pertinent to review the cultural background of Indian society which shapes the local intellect. Indian civilization is more than five thousand years old and pursuit of knowledge has always been considered central to most Indian households. Indian history of science began in 2000 BC when treatises in astronomy, mathematics, logic, medicine and linguistics were documented. However, at the heart of India's poor record at patenting and science entrepreneurship lies such profound disdain for the idea of commercializing knowledge. The first idea of commercializing knowledge changed when Chanakya, an erudite scholar from medieval period propagated "*Arth Kevichaye Vidya*" (wealth from knowledge). Yet most of the modern scientists such as JC Bose or CV Raman continued to refrain from accepting financial gains from their research. Today, there is growing realization that India is paying a huge cost for becoming only a user of IP than its generator. It has, therefore, decided to make huge investments in development of stem cell technology and serve local, national and International demand in the field. Fortunately, harvesting Embryonic Stem (ES) cells from *in vitro* fertilized egg for possible treatment is not considered as seriously problematic as the use of biologic science as the commercialization tool. Interestingly, none of the national or regional political parties have voiced any reservation against the use of this technology. However, for any model to serve these markets successfully, it is important if experimental therapy is not

slipped to those who have scant knowledge about stem cell biology, instead such centers should be carefully regulated with the help of qualified professionals that build the critical mass of medical specialists and biotechnologists who could finally participate in such form of therapy.

The flurry of activity that followed the 2004 success of Korean scientists led Canadian parliament to approve the use of excess embryos. Sweden followed by announcing that it would allow cloning of embryos for therapeutic purposes and UK approved a private firm to carry out the generation of ES cell lines. Similarly, Singapore earmarked $300 million for a technology park centered around exploitation of stem cells. Stunned by the Korean fraud, India took a cautious step by quickly promulgating ICMR-DBT draft, which awaits revision, on stem cell research and therapy so that the research is adequately regulated in this country. The regulation pronounces its expectation by stating that such research and clinical trials are conducted in a responsible and ethical manner, which comply with all regulatory requirements. The provision for separate mechanism for review and monitoring of stem cell research and therapy in the field of human stem cells, one at the National level as National apex committee for stem cell research and therapy (NAC-SCRT) and the other at the Institutional level called Institute Committee for Stem Cell Research And Therapy (IC-SCRT) has been made. The guidelines set out that all clinical trials with stem cells shall have prior approval of IC-SCRT and Drug Controller of India. These regulations provide for stamping out small clinics that promise big hopes without credibility and infrastructure and skilled stem cell scientists and pave way for registration mechanism with central bodies. There are some banks being run from homes and these regulations call for all cord banks to be registered with DCGI as per guidelines applicable to Blood banks. Separate mechanisms for setting up International collaborations for stem cell technology have been outlined and serve to be a big boon for those stem cell entrepreneurs and their western partners that possess requisite expertise and venture capital. There are several mechanisms for soft funding available in the form of Biotechnology Industry Partnership Program (BIPP) scheme launched by the Department of Biotechnology that funds upto $2 million. Such level of investment is happening despite the major expenditure being earmaked for defence in the face of cross border hostilities and rising global terrorism. For past two decades the R&D expenditure had never crossed 0.84 % (Das, 2004) of GDP with food and primary health care being the only goal of successive governments, however, it is now expected to go up at least 3 times in the next fiscal year. One of the determinants of the success of such stem cell business is the human resources. A special effort for attracting new brains in key sectors has been proposed by the sixth pay commission, the central body that reviews salaries for all government employees every decade. Govt has planned to expand the lifescience research by adding more institutions. A stem cell research center is coming up in Bangalore that will carry out research employing about 40 faculty members and participate in clinical trials in collaboration with Christian Medical College, Vellore. The Indian Institute of Science Education and Research have been opened at several places across India. Many think it is the exposure of graduate and undergraduate students to the institutes that may bridge the physical gap that existed between the colleges and Institutes. The govt's policy to promote stem cell research in the manner that ensures safety and efficacy of stem cells is likely to facilitate the unparalleled growth of stem cell industry that is likely to serve International clients.

Social and cultural factors

Indian life science labs and companies are run by graduate students with very few post docs. Young people are assaulted daily with symbols of India's emerging middle and upper class wealth advertisements of luxury apartments, brand new shopping malls, new cars, International vacations etc. Young people and their families want to be part of this new economic prosperity, and the surest path for a bright student to achieve a comfortable life style is to seek a IIT-MBA program or obtain a job in the IT sectors but this does not include biological sciences. Indian parents continue to hold the keys of their children's future by aspiring through their kids.[2] A majority of them actively participate in planning their future course of studies irrespective of their aptitude. Untill IT-MBA boom, it was fashionable to secure the careers of their kids by pushing them either into medicine or engineering careers. Due to competitive nature of IT, MBA, medicine and engineering courses (which require huge infrastructure but limited seats), only the most meritorious students with better resources and opportunities were able to compete successfully, leaving the others with alternative careers. As a result, a second tier of brains entered life sciences and hence the quality of research output in medical institutes declined. There are comparable trends in some countries but the problem is more acute in India. These challenges remain to be addressed by the policy managers and can be achieved by revamping the organization and education structure and by making research more lucrative, rewarding and interdisciplinary.

Today, the hype created by stem cell research can be positively translated into dream stem cell centers where research and clinical trials can go hand in hand and by not only catering to a very large segment of incurable degenerative diseases but also by the attracting first tier brains into the field. The linkages between investment in research and rationalization of treatment costs on one hand and its expansion through entrepreneurship models needs to be highlighted by science managers. The stem cell research regulations in India boasts of far more liberal policy when compared to USA or Europe and hence provides a unique opportunity of acquiring leadership in the world. Medical tourism which is being actively promoted by the government may benefit from this branch of medical therapeutics. Fortunately, there is no social, religious or cultural barriers that can halt the stupendous advancement in stem cell therapeutics in this region. Such an environment is conducive to build an International team so that the liberalism of Indian laws could accelerate the advancements in stem cell therapies.

Economics of Reductionism

Wealth creation from Biotechnology,

particularly stem cell technology involves heavy investment in the form of infrastructure, equipments and biologicals for which India pays a heavy price as IP user. Even the cost of accessing electronic knowledge resources is very high apart from the cost of patenting itself.[3,4] It is estimated that only 5-10 % of patents are eventually commercialized and hence recovering the costs requires capital. It is seldom realized that the huge cost involved in buying research consumables are directly proportional to the IP value of the product. National development research council (NDRC), Technology Development Board (TDB), ICICI science park and technology incubators are fast coming up to facilitate technology commercialization with the help of venture capitalists. This is important because lack of indigenous IP increases the cost of technology that the country will end up paying escalating the investments in research. Therefore, in effect, the costs of research itself continues to grow driven by continuous import of equipments of research. As majority of the scientists work in public funded centers, a vast majority of innovative scientists remain clueless about concepts of science entrepreneurship as there is no visible provision to create spin offs while being in government service. As the government conduct rules do not allow holding of two simultaneous jobs, forming of spin off company until recently was an impossible task. A mechanism of encouraging in-service scientists to form a company either by proceeding on sabbatical leave or by providing consulting services can change the landscape of innovative efficiency. The Indian PM has recently signed a regulation allowing in-service government scientists to hold equity in a company directed by him. This is being recognized as a major paradigm shift in Indian sciences.[5]

Until recently, India has witnessed a huge cash inflow and funds are not difficult to obtain. The multinational companies are also investing in knowledge parks and collaborating with Indian institutes, establishing their own manufacturing plants in India and hiring Indian scientists at good salaries. The range of opportunities the stem cell application is likely to create in future will depend on local investors and by serving the International patients who desire to obtain stem cell therapy but cant obtain it their own country due to stringent laws. Such profits will stay as long as those governments do not reverse their over-restrictive policies.

Academic Challenges

Whether the rapid brain drain from the country is affecting wealth creation in the country is hotly debated. On one hand the Non Resident Indians are able to transmit money back to their home country enhancing the foreign exchange while the research centers are not able to exploit their talent for national growth. The onus lies on the academic institutes and the policy makers to retain this intellect. There are now attractive schemes such as those floated by Department of Biotechnology (DBT) to recall the scientists who have left the country. DBT is offering them attractive incentives and facilities through Ramalingaswamy fellowships. Provisions for joint faculty between US and India and between the various departments within the country can greatly boost such efforts because this not only promotes scientific collaboration but also enhances innovation capacities by networking. The dual citizenship scheme propagated by Ministry of External affairs has become very famous for this reason, particularly among Non Resident Indians. Academic challenges lie ahead in bringing organizational changes. These include establishment of business development offices to encourage science entrepreneurship, especially in medical Institutes. This can save the Institutes from high rates of attrition. The lack of appreciation of the value of inter-disciplinary research is another challenge, which the medical Institutions are finding very difficult to recognize. Many of these challenges can be addressed at the root level by promoting science fairs in schools, enabling mentoring opportunities for young kids and establishing science museums in as many cities as possible. This can instill scientific temperament among kids. This is central to stimulating the young minds at the right time. DBT recently organized a business proposal development competition for science scholars, which created a lot of enthusiasm among young minds indicating that there is potential for engaging scientists towards market-oriented research.

The organization of awareness fairs, workshops and training programs will be the key in overcoming the political, organizational, social and economic hurdles towards shaping research efforts by science entrepreneurship model. The sustained change in paradigm of its education, innovation and commercialization policies with enhanced outlay in R&D can promote the growth of IP generation and commercialization for economic growth. Concomitant investments from private institutions and/or alliance with medical institutions can catalyse the generation of spin offs by the biomedical scientists. Since Indian economy is growing at a good rate of 9.4 % there is huge potential for both commercialization of IP and its consumption. A colloquium of lawyers, scientists and policy makers is a requirement that can strengthen the current innovation policy. This can facilitate the exploitation of the intellectual capital lying untapped in this sub continent. A lot of people believe that lack of accountability is the heart of inefficiency of scientists. It may be pertinent to stamp out the permanency of government jobs and enhance accountability through regular science audits. At such point, it is pertinent to make the academic institutes financially autonomous by allowing them to generate and survive on their funds. This will enhance accountability and only the meritorious faculty would be able to lead such Institutions.[6] The need for some sort of distinction between the hard working faculty and mediocre workforce needs urgent attention. The rapid advancements in the area of stem cell research has the potential to make India as an international capital of stem cell research therapy.

(To be continued...)

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ANNALS
EDITORIAL

neural stem cell therapy

how the hype began

Stem cell therapy has been held as a promising avenue to restore tissues lost to chronic diseases of aging, including those of the brain. However, despite reports using animal models, no therapeutic advance associated with neural stem cells has reached the clinic, since no advance has been reproducible or effective. However, many think that the only obstruction to our ability to cure diseases such as Alzheimer's and Parkinson's result not from failure of stem cell therapy but from regulatory impasse. There was wide and rampant speculation that when this impasse is lifted, cures for chronic diseases of aging shall be immediately available. Many individuals called for an end to the ban on embryonic stem cell research. In large part, this call stems from the failure of adult stem cells to reach the promise stem cell investigators held out a decade ago and from early demonstrations that embryonic stem cells could apparently mend a severed spinal cord. If embryonic stem cells could effect this result, it would be logical to assume that they may be beneficial in chronic diseases of the brain. While stem cell therapeutic advances judged in animal models has now for the most part been attributed to the release of cytokines and growth factors, there is no reason to conclude that stem cells, embryonic or otherwise, restore functional neural tissues or connections of the central nervous system. However, many believe that they do. The questions are why this belief is strongly held, one, and second, do the facts and passions provide any rationale to deliver now that the ban on embryonic stem cell research is thing of the past?

First, we postulate that embryonic stem cell research holds potential problems that transcend religious, ethical or moral principles. Changing the fundamental components of life as we know it may end life as we know it. While science has enjoyed a very liberal scope through the years, certain items perhaps should not be altered by investigation. Nature has a way of ensuring its own survival and the survival of nature was in fact threatened by our exploration of atomic nuclei, an exploration that resulted in the discovery of ways to unleash unimaginable energy that was first used for destruction. While no living being or component thereof is immortal, the fundamental law of nature holds mankind to be immortal, and in this, we survived eminent termination as nature must have known we would and always will. In doing so it allowed us to develop nuclear destructive devices, if only to illustrate their potential to immortalize mankind, an accomplishment Nature shall never allow. But while Nature may well have considered our ability to release the forces of atomic nuclei, and our wisdom in keeping these forces at bay, it is not at all clear that Nature similarly anticipated our drive to understand the basis of life itself would lead to our ability to alter its fundamentals. Embryonic stem cells may well represent an item with which we should not alter; because if we do, we have no idea of the forces we may release or our ability to contain them. Yet we persist, and scientists demand cloning of engineered nuclei of life, an undertaking that could surpass the threat of atomic extinction and to which Nature did not anticipate. Before this research reaches a state that is essentially irreversible, we should contemplate the results, and what we may attain in further studies. Diseases of aging are part and parcel to Nature's grand design. No one lives forever, and no one ever shall, but mankind will unless we put a premature end to our own existence. Stem cells of the embryonic kind fuel concern that we may be approaching that goal, if indeed this is the goal.

This report shall offer two diverse perspectives on the reported success of stem cell therapy for neural reconstitution. I shall provide evidence that no neural reconstruction has been achieved in any system while Dr. Akshay shall provide evidence that reconstruction has been achieved. In addition, this report shall provide a historical perspective of the hype that has driven many to accept the notion that CNS repair can (and has) been achieved with stem cell therapy. The history of the field is unprecedented in Science and has led to dogma prematurely assumed to be correct, while therapy has not been available for patients with neural disorders.

Although the primary goal of medical research has been to alleviate suffering, immortality has never been the espoused objective of either evolutionary biologists or stem cell researchers. Understanding the complexity of life, particularly unmasking of the mechanism of rescue of function effect of stem or progenitor cells remains a challenge that, many believe, will take time, hype (read hope) and multidisciplinary effort to resolve. The hype that has been created in hope of stem cell therapy reminds one of the hype that once existed when the prospects of gene therapy were being debated several decades ago. The era was characterised by a similar overexcitement of physicians who went ahead and carried out an FDA approved clinical trial which led to the death of an 18 year old volunteer, Gelsinger. The whole pack of cards came crashing down, threatening future funding and research in gene therapy. The incident also killed, in a way, not only the hopes that had been kindled by the prospects of gene therapy but also the 400 planned clinical trials at that time. However, it taught us several lessons, one, that clinical trials should be funded and planned with active involvement of basic scientists and not until proof of principle has been reasonably well established. The enormous publicity received by stem cells is partly due to controversies of regulating (or not) stem cell research and partly due to the publicity given to cloning of Dolly. The success of current stem cell trials being carried out today hinges on dominant role of basic scientists. In this context, it is worth contemplating that every jump in technology takes time and incremental advancements before it settles down successfully, for instance it almost took more than a century for an antibiotic like Pencillin to be discovered and enter the market[1] and another half

century for insulin to hit the clinics.[2] While a few may term stem cell therapy hype as dangerous, many counter argue that it is very good for raising the expectations and delivery standards from stem cell investigators. There have been several controversies that marked the launch of antiepileptic drugs,[3] but these could not deter the researchers from abandoning their plans for further advancement. Today, there are half a dozen variants of such drugs available in the market. Similarly, the lack of visible clinical benefit from stem cells should not serve to discourage the scientists. On the contrary, it should catapult them to intensify their efforts further until the incremental advances in the field lead to fulfillment of hopes held by the hype.

The prospect of repairing the damaged brain has provoked excitement, controversy and conflicting scientific claims. The brain was considered unchangeable as postulated by Cajal several decades ago. Altman later showed that neurons can regenerate by H thymidine incorporation studies.[4,5] Many reports have provided interesting leads using disease models where satisfactory functional recovery of cultured neurons has been shown.[6] There have also been several reports demonstrating the functional revival of damaged brain when embryonic stem cells, including neural stem/progenitor cells were implanted in various animal models of neurological disorders. These reports have demonstrated that the stem cells not only repair the damaged portion of the rodent brain but they also promote survival and delay neuronal cell death.[6]

There are populations of proliferating progenitor cells which are now believed to give rise to new neurons in sub ventricular zone (SVZ) of the lateral ventricles[7,8] and in subgranular layer of hippocampus[9,10] raising hopes of curing degenerative diseases. The group of Alvarez-Buyla[11] has shown that GFAP positive astrocytes are the source of neurogenesis in SVZ and sub granular region of hippocampus, the seat of spatial memory. Such advancements are critical in laying the road map for ultimate goal to repair the damaged or degenerate brain. Nakatomi *et al* even showed that infusion of epidermal growth factor (EGF) and fibroblast growth factor2 (FGF 2) into the lateral ventricle of the rat model of ischemia, in which CA1 neurons are selectively lost, leads to recovery of memory and learning function with concomitant regeneration of pyramidal neurons due to neurogenesis.[12] This has facilitated the discovery of factors that would enable desirable neurogenesis. For example, Gage's group even showed that enriched environment and exercise improves neurogenesis and learning[11,13] lending credence to the hope that environment can greatly influence the rate of neurogenesis. From the time when neurological disorders were left with limited treatment we are entering an era where cellular therapy may finally be able to reverse the disorders of brain. Similarly, there are reports that discuss the functional recovery of animals models when dopamine neurons derived from ES cells were implanted,[14,15] however, a very detailed analysis and further investigations can only lead to mapping of the cues that influence incorporation and differentiation of stem cells. In another development, Harris *et al* recently showed that intravitreally injected CD 133 progenitor cells from bone marrow can even regenerate retinal pigment epithelium (RPE) cells and improve retinal function, as evaluated by ERG, providing functional recovery of the visual cycle.[16] This is one of the findings that has implications for treatment of age related macular degeneration. One of the challenging tasks confronted with researchers today is the problem of translating the stem cell technology to clinic. As bulk of stem cell translation is happening in the Eastern part of the world, it is important to understand the socio-cultural factors which influence the dimension of scientific output. Most of the countries such as Korea, Japan, Singapore, China or India do not have MD-PhD programs in their Institutes or Universities. Many argue this to be one of the key determinants of stem cell translation to clinic. The lack of scientifically designed trials or absence of double blind placebo controls in such important studies may have consequences that can irreversibly alter the pace of clinical translation. Sandhya Srinivasan argues about the state of stem cell clinical trials in Indian subcontinent, in her article, 'Rogue research in the guise of Stem cell therapy' citing the experience of former Chief Minister, Mr Jogi from Chhatisgarh, who noted 'significant improvement' upon stem cell transplantation after his spinal injury.[17] Even though no conclusive evidence of such therapy was previously available, such small commercial clinics are openly endangering the hope stem cell therapy holds for us today. Even though there has been concerted effort to reconstruct the neurons using stem cell therapy, such unregulated trials such as those happening in one of the top medical institutes such as AIIMS, New Delhi[18] have the potential to create the same fear that gene therapy trial once did several years ago. Maintaining linkages with basic scientists is thus an urgent requirement, no longer a choice, in order to avoid unscientific trials, achieve homogeneity in the quality of stem cells to be implanted, maintaining the correct doses and devising the best route of administration to be followed. Only when these issues are addressed by parallel animal experimentation, the leads can be consolidated by clinical studies. It is being increasingly felt among scientists that there is rampant and sudden urge among the physicians to glorify themselves in the name of providing stem cell therapy to patients even though the studies are only at experimental state. People have suggested several ways of curbing this, one of which is to employ the skilled researchers who are trained to understand the value of a common transplantation SOP, one who understands the value of bench work and the drivers of biotherapeutics. This problem has been well addressed in US, where, despite the ES cell research being under scanner, the scientific community is replete with clinical-scientist entities. There are some who believe that it is the difficulties in intellectual property of stem cells which is actually hindering the pace of clinical translation than the stem cells themselves.

Therefore, one can argue that the failure of stem cells to quickly yield desirable clinical end points may not necessarily be a result of failure of stem cells to deliver. Instead, this could be due to the inability to plan the animal studies in a reproducible and meticulous manner coupled with lack of blinding of researchers when the experimental

groups are planned. Infact, the generality of need to urgently address such problems in any animal and clinical study can determine the pace of discovery. It is pertinent to note that the lackadaisical approach in translation of neural stem cell therapy is conspicuous when compared to fields such as physics, material science, space science or chemistry where the progress is more visible and rapid. It is possible that a large number of animal models often do not simulate the disease being investigated and extrapolation of results therefore becomes difficult. The appropriate translation dynamics for validating therapies in patients requires rational scaling from rodents-primate-humans, an approach that is abysmally deficient in collaborative effort amongst clinicians and scientists. The regulatory impasse in the use of non human primates coupled with unregulated exploitation of stem cells in the name of patient benefit may not bode well for terminally ill and unsuspecting patients; this has the potential to derail the tremendous impact that the stem cell research is worthy of making.

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India as the potential headquarters of International stem cell research

Stem Cells and Policy was adopted as the focus of the Journal to fill a specific need. The identification of human embryonic stem cells by Jamie Thompson in 1998 ignited an almost unbelievable series of events that at that time and encapsulated the stem cell scientific community in hype propelled to the status of dogma with little if any experimental evidence. This hype resulted from 2 key events. One, the failure of genetic engineering left a void that scientific entrepreneurs rushed to fill. The time was ripe, as the aging population of the United States and other countries sought endlessly for longer, healthy lives. Many of these individuals are and remain very wealthy, and are willing to spend enormous sums of money on any hope of avoidance or cure of disease. Second, the connotations of the meaning of the word "stem cell" was not at all clear, so this cell was elevated to the status of a cell that could restore any lost cell, regenerate any tissue and cure most diseases. This thought seemed to derive directly from the basic qualities of a "stem cell", that quality being that the embryonic stem cell, derived directly from fertilized embryos, indeed generated during gestation all of the component parts of the human organism. This being the case, surely the cell would regenerate parts lost to disease of assault after birth and provide a cure for diseases of aging. These thoughts were not unsupported. Shortly after Dr. Thompson's identification of the human stem cell, Dr. John Gearheart led individuals to believe that these cells could restore intact function of a severed spinal cord. The literature became replete with anecdotal as well as preliminary supportive data showing utility of stem cells in diseases ranging from cardiomyopathy to diabetes. No disease was immune; stem cells could do it all.

Surveying the literature and failed clinical trials, the focus for *Annals of Neurosciences* was developed to provide a different forum, one where dogma developing too rapidly could be challenged;[1,2] one where reasoned investigations and interpretation would lead to advance, albeit not overnight. However, in the United States and elsewhere, profit motivated corporations began to sprout and advertised the potential of stem cell therapy to an overly receptive audience, including members of the press. No one was particularly interested in hearing reality; a frenzy developed wherein individuals almost unaminously held dear the promise of stem cell research, and many invested their careers as well as capital in this potential, thought to be assured. When ethical considerations led to deterrence of embryonic stem cell research, investigators actually used this as an excuse for not quickly reaching the goals they promised, and soon developed alternative sources of stem cells which they held out as equivalent cells to attain these goals. Lost in all the hype, fury commotion and fame bestowed upon stem cell researchers was the simple fact that in no case did stem cells restore or regenerate any tissue in adults, nor did they cure any disease. Every advancement was hailed as a remarkable achievement absent the promised attainment of any clinically applicable therapeutic goals.

As investors questioned the attainment of promises, advances were sought with a fury unprecedented in the history of science, Many of these were said to become clinical realities soon, and an eager public kept the field active. We found many studies of stem cells indeed afforded new thoughts and models of development, and highlighted these as we questioned studies that claimed therapeutic success. The latter have not been realized and the reason for this is the haste of investigators to make claims based on flimsy and irreproducible data, claims that were highlighted in high profile journals. Amidst all this fury was one simple fact; the tremendous motive to attain success obscured interpretation of data of key trials and led to a shotgun approach, rather than a well thought out plan to alter conditions and interpret data accurately. Of all the manuscripts we received *Stem Cells and Development,* the manuscripts received from India presented a striking exception to this rule. These manuscripts from only a few centers were reliable, not over interpreted and offered new approaches that we thought may lead to success, but not overnight. These facts and others led us to propose India as the center of International stem cell research. This proposal was greeted with surprising enthusiasm by many investigators throughout the world. Recent therapeutic successes in India support our reasoning in this regard and we stand by our hope that this proposal is successfully adopted throughout the world as failures due to rapid and over interpreted experiments still predominate the field. Recent results from India show that a rigorous, scientifically based and carefully interpreted approach will lead to further success.

Economics of Investment

Wealth creation from technology--- one of only a few exportable industries-- involves heavy investment in the form of infrastructure, equipment and reagents. While India has set the standard for economic development as a result of their investment in technology, the cost of accessing electronic resources is very high when initially compared to the revenue initially gained. For example, the cost of patenting and deploying new developments in electronic technology reveals that as few as 5-10 % of patents are eventually commercialized; hence recovering the costs requires capital and it requires manpower and time. With regard to the latter, India in collaboration with US firms very quickly developed an electronic infrastructure which indeed sets the example that serves as a model for development of other technologies. In addition, as outlined by one of us, the costs of research itself grows as it continues,[3] this growth being driven by the continuous need to import state of the art facilities and equipment. However, in the end, the investment has paid off in a major way as India's economy outsteps many economies of developed countries. Thus Prime Minister of India recently signed a regulation allowing in-service government scientists to hold equity in companies under their purview. This is

recognized as a major paradigm shift in Indian science, and sets the stage for a similar investment in stem cell research. However, the medical institutions are still left clueless as to how to implement this regulation that has seen cabinet approval. A new order of leadership in these Institutions akin to one conceived by famous Valliathan report is urgently required to propel India to lead stem cell translation. Interdisciplinary leadership such as one seen in Department of Biotechnology (DBT), the major funding agency for stem cell research, and National Brain Research Centre (NBRC) could be extended to medical Institutions.

Until recently, India has witnessed a huge cash inflow taking place, funds were not difficult to obtain. Most of these funds are derived from multinational companies investing in knowledge parks and collaborating with Indian Institutes to establishing manufacturing plants in India. These corporations hired many Indian scientists at good salaries. The range of opportunities the stem cell application, if correctly developed, is likely to create will depend on providing health services national and International patients who desire to obtain stem cell therapy but can't obtain it in their own country due to over-regulation. Such profits will stay as long as those governments do not reverse their over-restrictive policies.

Political considerations

Despite India's booming economy, the major expenditure continues to be reserved in India for defence. With rising global violence in the form of terrorism, India is constrained to earmark a big share of its resources in defence and for fighting terrorism. The Indian government's policy to promote stem cell research despite other national priorities in the manner that ensures safety and efficacy of stem cells is likely to facilitate the unparalleled growth of health industry, results of which are starting to make headlines in the world.

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Advancements in Stem Cell Research – An Indian Perspective

Stem cell research is a promising frontier in biomedical research and therapeutics that will permanently transform the way medicine is being practised. It has been more than half a decade since India ventured into stem cell research arena in a much extensive scale. Undoubtedly, India possesses the initiative, resources and pragmatism to acquire leadership in this important area of biology. The zeal exuded by the Government of India has led to the prompt identification of stem cells as a niche area requiring intense focus. Its translation in real-time is evidenced by its ever-increasing patronage in the form of grants towards infrastructure development and operational activities. In fact, a dedicated task force has been established under the auspices of Department of Biotechnology (DBT), Government of India to recognize the priority areas and to provide financial support to promote this area.

India is poised to acquire leadership in this area by exploiting both the embryonic and adult stem cells systems since neither alone is likely to meet all therapeutic needs. In fact, a lot of thrust has been given to understanding the basic and clinical biology, which are being pursued at various centres (both government and private alike) within the country. Fortunately the religious concerns especially with respect to embryonic stem cells derivation and usage are relatively non-existent in India. Indeed, the fundamental research on embryonic stem cells that could provide clues about developing understanding of the processes of cell differentiation and dedifferentiation would further enable harnessing of the potential of both embryonic and adult stem cells to their fullest extent. Moreover, since the stem cells from most of the lineages in adult retain astonishing level of plasticity, these could help in exploring their transdifferentiation potential, along with the lineage committed embryonic stem cells derived multipotent stem/progenitors, with a view to having possible application in therapeutic transplantations. Countries like India that possess rapidly growing population can exploit the power of stem cells in establishing authentic human embryonic stem cell lines as well as pioneering regenerative medicine. Establishment of stem cell lines using surplus IVF human embryos and maintaining those in an undifferentiated state in prolonged cultures is of immense significance for fundamental research on stem cells. This would serve as a great source in order to explore our own intellectual potential. Scientists at NCCS, Pune (Dr. Lenka); NIRRH, Mumbai (Dr. Bhartiya); NCBS (Dr. Panicker) and JNCASR (Dr. Inamdar), Bangalore; NBRC, Gurgaon (Dr. Mani, Dr. Seth); CDFD, Hyderabad (Dr. Khosla), RGCB, Trivandrum (Dr. James) and the private organization like Reliance Life Sciences, Mumbai, among others, are actively involved in embryonic stem cell research from sources of both murine and human origin. Some of the recently published reports on partially/fully characterized human embryonic stem cell lines (Mandal et al., 2006; Lenka and Ramasamy, 2007; Inamdar et al., 2009; Kumar et al., 2009) indicate the progress accomplished in this direction. Moreover, attempts have also been made at NIRRH, Mumbai (Dr. Bhartiya) to establish somatic cell nuclear transfer (SCNT)/ therapeutic cloning in primates as well as in human species. Further characterization of these reprogrammed cells into various lineages as well as developing strategy for the enrichment and purification of lineage specific stem cells would supplement their clinical relevance in cell replacement therapies in treating various degenerating diseases. The recent revolution in the stem cell arena, the establishment of induced pluripotent stem cells has also been initiated by a couple of groups within the country and will bear fruit as institutional support mechanism work in tandem with enhanced outlay for stem cell research.

Keeping pace with the global thrust, scientists in India are also actively engaged in various aspects of tissue specific stem cell research – fetal and adult origins. While work on neural stem cells is a major focus at NBRC (Dr. Mani), NCCS (Dr. Shastry, Dr. Shiras and Dr. Lenka), NCBS (Dr. Panicker), TIFR (Dr. Tule and Dr. Vaidya), PGI (Dr. Anand) and LVPEI (Dr. Kashyap), the group at CCMB studies stem cell quiescence using skeletal muscle cell lines (Dr. Dhawan). Major strides have been made in the hemato-poietic stem cell research by NCCS (Dr. Kale), NII (Dr. Mukhopadhyay) and INMAS (Dr. Gurudatta) among others, in basic biology, maintenance, and signaling cues underlying hematopoiesis. More-over, successful endeavour of NCCS scientists (Dr. Limaye) in devising the technology for efficient cryopreservation of umbilical cord blood and bone marrow derived stem cells has resulted in transferring the know-how to nearby hospitals with a view to venturing into therapeutic exploration. In fact, in NCCS alone a number of groups are already pursuing investigations on stem cells from hematopoietic, neural, cardiovascular and pancreatic origin with active collaboration with clinicians. Efforts are also ongoing in understanding the biology and significance of cancer stem cells at NCCS (Dr. Bapat, Dr. Shiras) and IISc. (Dr. Rangarajan). The future course will be determined by the pace of integration of nanotechnology and biomaterial scaffold for engineering tissues in a 3D platform, that can also boost interdisciplinary research.

Several research institutions and biotech companies have been formed with the mandate on bringing stem cell research outcomes from bench to bedside. Some of the major medical institutions like PGI, Chandigarh; AIIMS, New Delhi; SGPGI, Lucknow; CMC, Vellore; AFMC, Pune, Manipal Hospital, Bangalore; CLRI, Hyderabad have either already initiated or are in the process of venturing into stem cell transplantations using sources like umbilical cord blood and bone marrow in treating neurological, hematological, hepatic and cardiac disorders. LVPEI, Hyderabad and Shankar Netralaya, Chennai have successfully carried out limbal stem cell transplantations and restoring vision to many. Among the private companies, Reliance life sciences, Mumbai and Stempeutics, Bangalore are the key players in conducting stem cell