CHAPTER: IV

OBJECTIVE 2

To Study the Neuroprotective Efficacy of Transplanted hDPSCs/hBM-MSCs and hDPSCs/hBM-MSCs Derived CM Treatment Against Kainic Acid Induced Hippocampal Neurodegeneration in an In Vivo Condition.

OBJECTIVE 3

To Study the Hippocampal Functional Recovery following hDPSCs/hBM-MSCs Transplantation and hDPSCs/hBM-MSCs derived CM Treatment.

OBJECTIVE 4

To Decipher the Molecular Mechanisms of Neuroprotection Mediated by hDPSCs/hBM-MSCs Transplantation and hDPSCs/hBM-MSCs Derived CM Treatment Against Kainic Acid Induced Hippocampal Neurodegeneration in an In Vivo Condition.

1. INTRODUCTION

The hippocampal formation is important for spatial learning and memory function and exhibits a high level of plasticity in response to behavioural changes as well as injury. Dysfunction of the hippocampus is one of the hallmark features of neurodegenerative diseases such as TLE and AD (1). Glutamate mediated excitotoxicity plays a major role in neuronal death in these disease conditions. To date, there is no identifiable treatment available for most of the neurodegenerative diseases. Cell therapy is emerging as an alternate therapeutic option for treating neurodegenerative diseases (2–4). Several studies have demonstrated the beneficial effects of BM-MSCs in treating various neuronal and non-neuronal diseases (5,6). However, the disadvantages of using BM-MSCs are that bone marrow isolation is a painful and invasive procedure with fewer yields of MSCs, lower proliferation properties and differentiation capacity that correlates with the age of the donor (7). In this context, DPSCs originated from neural crest can be considered as an excellent alternative for BM-MSCs. Dental pulp stem cells holds several advantages over BM-MSCs including easy availability, higher proliferation index and their inherent differentiation propensity towards neural lineage (8,9). However, as compared to BM-MSCs, till date there is no study that investigated the neuroprotective ability
of DPSCs against excitotoxicity mediated hippocampal neurodegeneration. Thus, a detailed analysis is essential to estimate the neuroprotective efficacy and to understand the molecular mechanisms of neuroprotection mediated by DPSCs as compared to BM-MSCs. Furthermore, as it is becoming more consensuses that the secretome released by grafted cells are contributing to host tissue regeneration (10–14), it is also important to investigate whether the CM derived from hDPSCs/hBM-MSCs per se can protect hippocampal neurons against excitotoxicity in an in vivo condition. Accordingly, in this Chapter-IV we investigated the neuroprotective efficacy (Objective 2), hippocampal functional recovery (Objective 3) and the molecular mechanisms of neuroprotection (Objective 4) mediated by hDPSCs/hBM-MSCs and its CM in an animal model of hippocampal neurodegeneration.
2. MATERIALS AND METHODS

2.1 Subjects

Male CF-1 mice of 2-3 months are the subjects for the present work. These adult mice were housed 3-4 in a cage at Central Animal Research Facility, Manipal Academy of Higher Education, Manipal in a climate and light dark cycle-controlled room with food and water provided ad libitum. All animal experiments were carried out in accordance with the guidance provided by Institutional Animal Ethical Committee (Protocol # IAEC/KMC/53/2013). Mice were grouped as follows:

Experimental Groups:

1. Normal Control Group (NC; intrahippocampal injection of 0.9% saline and serum free DMEM)
2. Kainic Acid Group (KA; mice received intrahippocampal injection of Kainic Acid)
3. Kainic Acid + hDPSC Group (KA + hDPSC; intrahippocampal transplantation of hDPSCs in KA mice)
4. Kainic Acid + hBM-MSC Group (KA + hBM-MSC; intrahippocampal transplantation of hBM-MSCs in KA mice)
5. Kainic Acid + hDPSC-conditioned medium Group (KA + hDPSC-CM; intrahippocampal injection of hDPSC-CM in KA mice)
6. Kainic Acid + hBM-MSC-conditioned medium Group (KA + hBM-MSC-CM; intrahippocampal injection of hBM-MSC-CM in KA mice)

2.2 Intrahippocampal Injection of Kainic Acid

Adult CF-1 male mice (n=6/group) were anesthetized with intra-peritoneal injection of ketamine (10 mg/kg; Ketamine Hcl, Harman FinoChem Limited, Aurangabad, Maharashtra, India) and xylazine (100 mg/kg; Xylo-B, Brilliant Bio Pharma Private Limited, Medak, Telangana, India) and positioned on a stereotaxic instrument. The head was positioned straight and firmly on the stereotaxic instrument. The stereotactic co-ordinates for the CA3 sub-field of the hippocampus was adapted from the open access Allen mouse brain atlas (http://mouse.brain-map.org/). Kainic acid (0.05 µg/µl Enzo Life Sciences; East Farmingdale,
New York) was injected bilaterally into the CA3 sub-field stereotaxically at a rate of 0.05 µl/min using a Hamilton syringe (Hamilton Company, Reno, Nevada, USA). The KA dose was standardized to induce hippocampal cell death without causing any behavioural seizure. The sham control mice were given a vehicle injection of 0.9% saline. Towards the end of the surgery, a cannula was placed, positioned bilaterally at the lesion site for further cell transplantation and CM treatment purpose. The cannula was secured by dental cement.

2.3 Intrahippocampal Transplantation of hDPSCs/hBM-MSCs

One day following KA injection, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and positioned on a stereotactic instrument. Four microliter of hDPSCs/hBM-MSCs suspension (~1,00,000 cells) labelled with Hoechst dye (Sigma-Aldrich, St. Louis, Missouri, US) were transplanted into the lesion site through the cannula bilaterally at a rate of 0.5 µl/min using a 10 µl Hamilton syringe (Hamilton Company, Reno, Nevada, USA). The needle was left in place for 5 minutes and slowly withdrawn to avoid backflow. The sham control group received 0.9% saline. Following cell transplantation, mice were housed under standard housing conditions for 10 days. On the 11th day, mice from different groups (n=6/group) were subjected to behavioural analysis and were later perfused and their brain sections were used to assess the extent of neuroinflammation, neuroprotection and neurogenesis in the hippocampus.

2.4 Intrahippocampal Injection of hDPSCs/hBM-MSCs Derived CM

In a sub-group of mice (n=6/group) that had undergone intra-hippocampal KA injection, CM derived from hDPSCs/hBM-MSCs was injected (4 µl/hemisphere) through the cannula to the lesion site. The stereotactic surgery procedure for KA injection and cannula implantation was similar to that explained in the previous section. Following CM injection, mice were housed under standard housing conditions for 10 days. On the 11th day, mice from different treatment groups (n=6/group) were subjected to behavioural analysis and were later perfused and their brain sections were used to assess the extent of neuroinflammation, neuroprotection and neurogenesis in the hippocampus.

2.5 Animal Perfusion and Brain Sectioning

Mice were perfused transcardially under isoflurane anaesthesia, first with physiological saline, followed by 4% PFA. Brain was removed and post fixed in the same fixative for 24 hours at 4°C. Following graded sucrose treatment (10-30%), brain was blocked coronally and 30 µm
sections taken from the entire brain using cryostat (Leica Biosystems, Wetzlar, Germany) and the sections were collected serially in a 24 well plate with PBS. Thus collected brain sections were stored at 4°C and were used for assessing neuroinflammation, neuroprotection and neurogenesis in the hippocampus.

2.6 Assessing Hippocampal Neuroinflammation Following hDPSCs/hBM-MSCs Transplantation and hDPSC-CM/hBM-MSC-CM Treatment

In order to assess the extent of neuroinflammation, coronal sections were processed with antibody against reactive astrocyte marker, i.e. GFAP. Briefly, free-floating sections were incubated in 20% methanol plus 3% hydrogen peroxide in PBS for 30 minutes and rinsed thoroughly in PBS. The sections were then incubated in PBS containing 10% normal serum plus 0.1% Triton X-100 for 30 minutes, followed by overnight incubation at 4°C with primary antibody against GFAP (1:200, Goat polyclonal, SC-6170, Santa Cruz Biotechnology Inc Texas, USA). Next day, sections were rinsed thrice in PBS and incubated for an hour in HRP conjugated secondary antibody (Donkey anti-goat, SC- 2020, Santa Cruz Biotechnology Inc Texas, USA) at room temperature. The immunohistochemical reaction was visualized using 3-3’-Diaminobenzidine (DAB) (CAS 91-95-2, Santa Cruz Biotechnology Inc, Texas, USA) as a colour developing agent. The sections were mounted on slides, air-dried and processed for microscopic observation (Nikon Eclipse, TE 200-U, Minato, Tokyo, Japan).

Additionally, as microglia activation is one of the hallmark features in neuroinflammation, we estimated the protein expression of IBA-1, a protein that is specifically expressed in microglia and is upregulated during microglial activation. Towards this, the brain tissue lysate was prepared by homogenizing the tissue in cold lysis buffer (50 mM Tris Base pH 8, 150 mM NaCl, 0.1% Triton X-100, 0.5 mM Sodium Molybdate, 0.5 mM Sodium Orthovanadate, 10 mM Sodium fluoride, 10 % Glycerol and 1X Protease inhibitor). The tissue lysates were centrifuged at 2000 x g for 5 minutes and the supernatant was used to estimate the protein concentration using Bradford reagent (Bio-Rad Laboratories, California, USA). The proteins were separated on SDS-polyacrylamide gels consisting of 10% resolving gel and 5% stacking gel along with the pre-stained protein ladder (~10-180 kDa; Puregene, Genetix Biotech, New Delhi, India). The proteins were then transferred onto the PVDF membrane (Millipore, Bedford, MA) and the blots were blocked with 5% BSA in 1X Tris-buffered saline and tween-20 for 1 hour at room temperature. Subsequently the blots were incubated overnight with rabbit polyclonal anti-β-actin (1:1000; Santa Cruz Biotechnology, Inc, Texas, USA) and goat
polyclonal anti-IBA-1 (1:500; Santa Cruz Biotechnology, Inc, Texas, USA) at 4°C. Following several washes, the blots were incubated with HRP-conjugated mouse anti-goat IgG and anti-rabbit IgG (Santa Cruz Biotechnology, Inc, Texas, USA) for 2 hours at room temperature. The blots were washed and developed using the 1-Step Ultra TMB-blotting solution (ThermoFisher Scientific, Waltham, MA, USA).

2.7 Assessing Hippocampal Neuroprotection Following hDPSCs/hBM-MSCs Transplantation and hDPSC-CM/hBM-MSC-CM Treatment

To determine the extent of neuroprotection, coronal sections (n=6/group) were processed with antibody against specific nuclear antigen marker FOX-3 (NeuN). Briefly, free-floating sections were incubated in 20% methanol (Hi-Media, L.B.S Marg, Mumbai, India) plus 3% hydrogen peroxide (Hi-Media, L.B.S Marg, Mumbai, India) in PBS for 30 minutes and rinsed thoroughly in PBS. The sections were then incubated in PBS containing 10% normal goat serum (Sigma-Aldrich, St. Louis, Missouri, US) plus 0.1% Triton X-100 for 30 minutes, followed by overnight incubation at 4°C with primary antibody against FOX-3 (1:200, Goat polyclonal, SC-246957, Santa Cruz Biotechnology, Inc Texas, USA). Next day, sections were rinsed thrice in PBS and incubated for an hour in HRP conjugated secondary antibody (Donkey anti-goat, SC-2020, Santa Cruz Biotechnology, Inc Texas, USA) at room temperature. The immunohistochemical reaction was visualized using DAB (CAS 91-95-2, Santa Cruz Biotechnology, Inc Texas, USA) as a colour developing agent. The sections were mounted on slides, air-dried and processed for microscopic (Nikon Eclipse, TE 200-U, Minato, Tokyo, Japan) observation and cell counting using ImageJ Software (Version 1.46; National Institute of Health, Bethesda, USA). Briefly, all the slides were blind-coded to circumvent any bias by the experimenter, and the cell density in CA1, CA3 and DH between the bregma levels of -2.15 and -2.35 mm was quantified by means of direct visual counting of FOX-3+ neurons in CA1, CA3 and DH over the length of 120 µm. The number of FOX3+ neurons from each group was averaged, and the final value was expressed a mean ± standard error of the mean (SEM) of FOX-3+ neurons per 120 µM.

2.8 Assessing Hippocampal Neurogenesis Following hDPSCs/hBM-MSCs Transplantation and hDPSC-CM/hBM-MSC-CM Treatment

In order to assess the amount of neurogenesis, coronal sections were processed with antibody against doublecortin (DCX). Briefly, free-floating sections were incubated in 20% methanol plus 3% hydrogen peroxide in PBS for 30 minutes and rinsed thoroughly in PBS. The sections
were then incubated in PBS containing 10% normal serum plus 0.1% Triton X-100 for 30 minutes, followed by overnight incubation at 4°C with primary antibody against DCX (1:200 Goat polyclonal, SC-8066, Santa Cruz Biotechnology, Inc Texas, USA). Next day, sections were rinsed thrice in PBS and incubated for an hour in HRP conjugated secondary antibody (Donkey anti-goat, SC- 2020, Santa Cruz Biotechnology, Inc Texas, USA) at room temperature. The immunohistochemical reaction was visualized using DAB (CAS 91-95-2, Santa Cruz, Texas, USA) as a colour developing agent. The sections were mounted on slides, air-dried and processed for microscopic (Nikon Eclipse, TE 200-U, Minato, Tokyo, Japan) observation and cell counting using ImageJ software (Version 1.46; National Institute of Health, Bethesda, USA). Briefly, all the slides were blind-coded to circumvent any bias by the experimenter, and the cell counting was carried out and the number of DCX positive cells were expressed as fold change.

2.9 Assessment of Hippocampal Dependent Spatial Learning and Memory

The eight-arm radial maze is commonly used for assessing hippocampal dependent spatial learning and memory functions in rodents. It consists of eight equally spaced arms radiating from an octagonal central platform.

2.9.1 Acquisition of Spatial Task

Hippocampal dependent spatial learning and memory was assessed using eight arm radial maze. The original body weight was reduced by 85% by semi-starving mice for 48 hours before behavioural assessment. These mice were allowed to habituate with the radial maze. All the eight arms of the radial maze were baited with food pellets before each trail. The mouse was allowed to freely explore the maze by placing them at the centre of the maze. The mice were trained to take the food pellets from each arm without making a re-entry into previously visited arm. When the animal had taken the food rewards from all the eight arms or after 10 minutes, if all the eight arms were not visited, the trail was concluded. The radial arms were wiped with 50% alcohol prior to each session to avoid olfactory cues. The animal’s performance was monitored during the trail by the experimenter, standing at a marked location in the room. The experimenter recorded the number of entries into the arms and the total time taken to visit eight arms. Two trials per day were conducted to each animal. The average criteria for acquisition is attending 7 of 8 correct choices. If the animal re-enters into an already visited arm, it was considered as an error. The performance of the animal was scored by calculating the percentage
of correct responses divided by the total number of entries made by the animal. Retention test was carried out ten days following acquisition.

2.9.2 Retention Test

Following learning of the eight-arm radial task, mice were retained in their respective cages for 10 days without training. In order to assess whether the mice retained the learned task, their performance in the eight-arm radial maze was assessed for a single trial and the scores were noted.

2.10 Gene Expression of Endogenous Neuronal Survival Factors Following hDPSCs/hBM-MSCs Transplantation and Their CM Treatment

To investigate whether hDPSCs/hBM-MSCs transplantation or their respective CM stimulates the host’s endogenous neuronal survival factors, brain tissue extracts from different treatment groups (n=3/group) were analysed for an array of neuronal survival factors such as BDNF, GDNF, CNTF, VEGF, PDGF, NGF, NT3, bFGF and EPO using qRT–PCR (Kapa SYBR fast qPCR kit, Kapa Biosystems, Wilmington, MA) with the following thermal profile: Segment 1–1 cycle: 95°C for 10 minutes, Segment 2–40 cycles: 95°C for 15 seconds followed by 60°C for 1 minute, Segment 3 (dissociation curve) - 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds. Total RNA was extracted from different treatment groups with RNAiso Plus reagent (Takara Bio Inc., CA, USA) and the purity and concentration of RNA was assessed using a Nano Drop spectrophotometer (ND-1000 spectrophotometer, Nano drop Technologies Inc, Wilmington, USA). Single stranded cDNA was synthesized using a Verso cDNA synthesis kit (Thermo Scientific; Waltham, MA, USA). GAPDH was used as an internal control.

2.11 Caspase 3/7 Activity Assay

The brain tissue (n=3/group) was lysed using lysis buffer (50 mM Tris Base pH 8, 150 mM NaCl, 0.1% Triton X-100, 0.5 mM Sodium Molybdate, 0.5 mM Sodium Orthovanadate, 10 mM Sodium fluoride, 10% Glycerol and 1X Protease inhibitor). The Caspase-Glo 3/7 (Promega, Promega Corporation, Cat no: G8091, WI, USA) assay was performed to measure the caspase-3/7 activities as explained in Chapter III. Before initiation of the assay, the Caspase-Glo 3/7 reagent was prepared and was allowed to equilibrate to room temperature. The 96-well plates containing brain tissue lysate was allowed to equilibrate to room
temperature. 100 µl of Caspase-Glo 3/7 reagent was added to each well of a 96-well plate containing 100 µl of blank and brain tissue lysate. The plate was covered with a plate sealer. The contents of the wells were gently mixed using a plate shaker at 125-208 x g for 30 seconds and incubated at room temperature for 1 hour. The luminescence was measured for each sample in a plate-reading luminometer (Perkin Elmer multimode plate reader, St. Waltham, Massachusetts).

2.12 Protein Expression of Pro-Cell Survival Factors Following hDPSCs/hBM-MSCs Transplantation and CM Treatment

To estimate the protein expression of anti-apoptotic factors like phosphorylated PI3Kinase and Bcl-2 following various treatment regimens, the brain tissue lysate prepared by homogenizing the tissue in cold lysis buffer (50 mM Tris Base pH 8, 150 mM NaCl, 0.1% Triton X-100, 0.5 mM Sodium Molybdate, 0.5 mM Sodium Orthovanadate, 10 mM Sodium fluoride, 10 % Glycerol and 1X Protease inhibitor). The tissue lysates were centrifuged at 2000 x g for 5 minutes and the supernatant was transferred to a fresh tube. The protein concentration was estimated using Bradford reagent (Bio-Rad Laboratories, California, USA). Proteins were separated on SDS-polyacrylamide gels consisting of 10% resolving gel and 5% stacking gel along with the pre-stained protein ladder (~10-180 kDa; Puregene, Genetix Biotech, New Delhi, India). Proteins were then transferred onto the PVDF membrane (Millipore, Bedford, MA) and the blots were blocked with 5% BSA in 1X Tris-buffered saline and tween-20 for 1 hour at room temperature. Subsequently the blots were incubated overnight with rabbit polyclonal anti-β-actin (1:1000; Santa Cruz Biotechnology, Inc, Texas, USA), rabbit polyclonal anti-phosp-PI3K (1:500; Santa Cruz Biotechnology, Inc, Texas, USA) and rabbit polyclonal anti-Bcl-2 (1:1000; Novus Biologicals, LLC South park way, USA) at 4°C. Following several washes, the blots were incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc, Texas, USA) for 2 hours at room temperature. The blots were washed and developed using the 1-Step Ultra TMB-blotting solution (ThermoFisher Scientific, Waltham, MA, USA).

2.13 Statistical Analysis

Data are presented as mean ± SEM. One-way analysis of variance followed by Tukey's multiple comparison tests was used to compare the treatment effects among groups. For behavioural assessments, two-way ANOVA with repeated measures followed by a Bonferroni post-test was carried out. P value less than 0.05 (p< 0.05) was considered as statistically significant.
3. RESULTS

3.1 Survival of hDPSCs/hBM-MSCs in Hippocampus Following Intrahippocampal Transplantation

Intrahippocampally transplanted hDPSCs/hBM-MSCs were detected in the transplantation site when assessed 51 days following transplantation (Fig. 1). The transplanted hDPSCs (Fig. 1C) appear to disseminate from the transplantation site and migrate to different hippocampal sub-fields. In contrast, transplanted hBM-MSCs (Fig. 1B) appeared to be clustered and the cells migrate from these clusters.

Figure 1: Graft survival and migration in hippocampus. (A) Hippocampal coronal sections of normal control group without no cell transplantation. (B) Hippocampal coronal section of KA+hBM-MSC group with hBM-MSCs transplanted into CA3 sub-field of hippocampus. Note that the grafted cells were clustered. (C) Hippocampal coronal sections of KA+hDPSC group with hDPSCs transplanted into CA3 sub-field of hippocampus.

3.2 Intrahippocampal Transplantation of hDPSCs/hBM-MSCs and hDPSC-CM/hBM-MSC-CM Treatment Decrease Hippocampal Neuroinflammation

To understand the status of neuroinflammation following KA treatment, hDPSCs/hBM-MSCs transplantation and their respective CM treatment, mouse coronal sections were processed for immunohistochemical detection of reactive astrocytes by using antibody against GFAP. Qualitative observation revealed that the KA injection into mouse hippocampus induced prominent neuroinflammation as KA group demonstrated a noticeable increase in reactive astrocytes in the CA3 sub-field as compared to NC group (Fig. 2). In contrast, KA+DPSC or
KA+BM-MSC groups revealed a substantial decrease in reactive astrocytes suggesting that the neuroprotective effect of hDPSCs and hBM-MSCs might be partially attributed to their immunomodulatory effects (Fig. 2). Similarly, intrahippocampal injection of hDPSC-CM in hippocampal damaged mice substantially decreased the hippocampal neuroinflammation (Fig. 2). In contrast, intrahippocampal injection of CM derived from hBM-MSCs did not reduce the hippocampal neuroinflammation (Fig. 2). In order to substantiate the anti-inflammatory effects of hDPSC/hBM-MSC and hDPSC-CM, we analysed the expression of microglia markers IBA-1 in different treatment groups using the western blot method. Our study revealed that KA injection into mouse hippocampus significantly increased the protein expression of IBA-1 as compared to NC group (p<0.001; Fig. 3A and 3B). Whereas, treatment with hDPSCs/hBM-MSCs and their respective CM significantly decreased the protein expression of IBA-1 (KA+DPSC-p<0.01; KA+DPSC-CM-p<0.001; KA+BM-MSC-p<0.01; KA+BM-MSC-CM-p<0.01; Fig. 3A and 3B). Though, there was a significant reduction in IBA-1 expression in KA+BM-MSC and KA+BM-MSC-CM groups, the IBA-1 expression was still significantly higher in these groups (p<0.001) as compared to KA+DPSC and KA+BM-MSC groups (Fig. 3A and 3B).
Figure 2: GFAP Immunohistochemistry; GFAP immunostained mouse brain coronal sections demonstrating the reactive astrocytes in mouse hippocampus from different treatment groups in the CA3 region. Intrahippocampal injection of KA induced significant reactive astrocytes in the CA3 region of hippocampus. In KA+DPSC and KA+BM-MSC groups there was substantial decrease in the reactive astrocytes as compared to KA group in the CA3 region of hippocampus. Interestingly, in KA+DPSC-CM group, there was a notable reduction in the reactive astrocytes in the CA3 region of hippocampus as compared to KA, KA+BM-MSC and KA+BM-MSC-CM groups. Scale bar: 50 μm.

Abbreviations:
- Normal Control (NC)
- Kainic Acid treated mice (KA)
- KA injected mice transplanted with human bone-marrow mesenchymal stem cells (KA+BM-MSC)
- KA injected mice transplanted with human dental pulp stem cells (KA+DPSC)
- KA mice injected with conditioned medium derived from hBM-MSCs (KA+BM-MSC-CM)
- KA mice injected with conditioned medium derived from hDPSCs (KA+DPSC-CM)
3.3 Intrahippocampal Transplantation of hDPSCs/hBM-MSCs and hDPSC-CM/hBM-MSC-CM Protects Hippocampal Neurons Against Kainic Acid Mediated Excitotoxicity

Given the significant reduction in neuroinflammation in hippocampus following intervention with hDPSCs/hBM-MSCs transplantation or hDPSCs-CM/hBM-MSCs-CM treatment to hippocampal damaged mice, we next ascertained to verify whether any neuroprotection could be observed in various hippocampal subfield following hDPSCs/hBM-MSCs transplantation or hDPSCs/hBM-MSCs CM treatment. FOX-3 immunohistochemistry from KA group revealed prominent neurodegeneration in the CA3 sub-field of hippocampus (Fig. 4; KA). Interestingly, intrahippocampal transplantation of hDPSCs/hBM-MSCs protected the hippocampal neurons against KA induced excitotoxicity. The hippocampal cytoarchitecture was well preserved in KA+BM-MSC and KA+DPSC transplant groups (Fig. 4), suggesting that transplantation of hBM-MSCs or hDPSCs could confer hippocampal neuroprotection against excitotoxicity. Quantification of FOX-3+ neurons in CA1, CA3 and DH in KA group (Fig. 5A, B & C) confirmed significant neuronal loss in these fields. In CA1, CA3 and DH sub-field, there were 48% (9 [mean] ±1.67 [SEM] viable cells/120 µM; p<0.001; Fig. 5A), 49% (10.5±0.83 viable cells/120 µM; p<0.001; Fig. 5B) and 25% (17.83±0.75 viable cells/120 µM; p<0.001; Fig. 5C)
of neuronal loss in the KA group respectively as compared to NC group. In NC groups the cell viability was 17.17±2.48 /120 µM, 20.67±2.7 /120 µM and 23.83±2.48 /120 µM in CA1, CA3 and DH respectively. Also, significant differences in the neuroprotective potential between hDPSCs and hBM-MSCs, both MSCs from two different tissue sources viz., bone marrow and dental pulp demonstrated neuroprotection against KA induced neurodegeneration. In KA+BM-MSC group, the neuronal loss was 22% (13.33±0.81 viable cells/120 µM; p<0.01; Fig. 5A), 19% (16.83±1.16 viable cells/120 µM; p<0.01; Fig. 5B) and 7% (22.17±1.16 viable cells/120 µM; Fig. 5C) in CA1, CA3 and DH subfields respectively, as compared to NC group. Likewise, in KA+DPSC group, the neuronal loss was 8% (15.83±1.32 viable cells/120 µM; Fig. 5A), 5% (19.67±1.50 viable cells/120 µM; Fig. 5B) and 3% (23.17±1.32 viable cells/120 µM; Fig. 5C) in CA1, CA3 and DH subfields respectively, which were not statistically significant when compared to NC group. Of note, as compared to KA group and KA+BM-MSC group, the neuronal loss was significantly less in KA+DPSC group. Thus, our study demonstrates for the first time that DPSCs could confer superior hippocampal neuroprotection than BM-MSCs in in vivo hippocampal neurodegenerative conditions. Interestingly, intrahippocampal injection of CM derived from hDPSCs/hBM-MSCs protected the hippocampal neurons against KA induced excitotoxicity that was comparable to that of hDPSCs/hBM-MSCs transplantation groups (Fig. 4) suggesting that CM per se could recapitulate the therapeutic effects of hDPSCs/hBM-MSCs transplantation. This observation highlights that the neuroprotective effects of MSCs transplantation could be partially due to secretome released by the stem cells upon transplantation. In KA+BM-MSC-CM group, the neuronal loss was 24% (13±1.41 viable cells/120 µM; p<0.01; Fig. 5A), 16% (17.33±1.36 viable cells/120 µM; Fig. 5B) and 3% (23.17±0.75 viable cells/120 µM; Fig. 5C) in CA1, CA3 and DH subfields respectively, as compared to NC group. Likewise, in KA+DPSC-CM group, the neuronal loss was 3% (16.67±2.58 viable cells/120 µM; Fig. 5A), 3% (20.17±1.47 viable cells/120 µM; Fig. 5B) and 4% (22.83±0.98 viable cells/120 µM; Fig. 5C) in CA1, CA3 and DH subfields respectively, which were not statistically significant when compared to NC group. Interestingly, a superior neuroprotective effect was observed in KA+DPSC-CM group in the CA1 sub-field as the neuronal loss was negligible in CA1 (3%) and CA3 (3%) sub-fields when compared to KA+BM-MSC-CM treatment groups wherein the neuronal loss was 24% in CA1 sub-field and 16% in CA3 sub-field suggesting that CM derived from neural crest originated DPSC possess superior hippocampal neuroprotective properties.
Figure 4: FOX-3 (Neu-N) Immunohistochemistry: FOX-3 immunostained mouse brain coronal sections demonstrating the cytoarchitecture of mouse hippocampus from different treatment groups. Intrahippocampal injection of KA induced significant neurodegeneration in the CA3 sub-field of hippocampus (arrow head). Whereas, in KA+DPSC, KA+BM-MSC, KA+DPSC-CM and KA+BM-MSC-CM groups, the cytoarchitecture of hippocampus was well preserved that was comparable to NC group. Scale Bar: 500 µm

Abbreviations:
Normal Control (NC); Kainic Acid treated mice (KA); KA injected mice transplanted with human bone-marrow mesenchymal stem cells (KA+BM-MSC), KA injected mice transplanted with human dental pulp stem cells (KA+DPSC); KA mice injected with conditioned medium derived from hBM-MSCs (KA+BM-MSC-CM) and KA mice injected with conditioned medium derived from hDPSCs (KA+DPSC-CM).
Figure 5: Bar diagram representing the number of FOX-3⁺ neurons in CA1 (A), CA3 (B) and DH (C) of hippocampus from different treatment groups. Data expressed as mean ± SEM. *comparison between NC vs treatment groups. +comparison between KA vs treatment groups. #comparison between KA+DPSC-CM vs KA+BM-MSC-CM. ***p<0.001; **p<0.01; +++p<0.001; #p<0.05; ##p<0.01.
3.4 Intrahippocampal Transplantation of hDPSCs/hBM-MSCs and hDPSC-CM/hBM-MSC-CM Enhances Hippocampal Neurogenesis

To estimate the neurogenic potential of hDPSCs/hBM-MSCs and their respective CM, mouse coronal sections were processed for immunohistochemical detection of DCX positive cells in the SGZ of the hippocampus. Doublecortin being a cytoskeleton protein, is expressed in neural progenitor cells and it is a marker for identifying newly born neurons in adult DG. Our data revealed that intrahippocampal transplantation of hDPSCs/hBM-MSCs significantly increased hippocampal neurogenesis as the number of DCX+ cells in these groups were several folds higher than NC or KA groups (Fig. 6 and Fig. 7). The KA+BM-MSC group displayed 15-fold increase (p<0.05; Fig. 7) in DCX+ cells in the SGZ as compared to NC group. Interestingly, the neurogenic potential of hDPSCs was much greater compared to hBM-MSCs as the KA+hDPSC group displayed 21-fold increase (p<0.01; Fig. 7) in DCX+ cells as compared to NC group. In the present study, we also observed a significant increase in DCX+ cells (Fig. 7; p<0.05) in the SGZ of KA treated mice. Interestingly, intrahippocampal injection of hDPSCs derived CM in KA treated mice (KA+DPSC-CM) significantly increased hippocampal neurogenesis as the numbers of DCX+ cells in these groups were (19 fold) higher than NC, KA or KA+BM-MSC-CM groups (p<0.01; Fig. 6 and Fig. 7). Similar to hDPSCs transplantation, hDPSC-CM treatment also induced better hippocampal neurogenesis as compared to hBM-MSC-CM (Fig. 6 and Fig. 7). Furthermore, as compared to KA+ BM-MSC or KA+BM-MSC-CM groups, the DCX+ cells in KA+DPSC and KA+DPSC-CM groups were more densely packed with noticeable dendritic processes oriented vertically into the granule cell layer (Fig. 6).
Figure 6: DCX Immunohistochemistry: DCX immunostained mouse brain coronal sections demonstrating the DCX⁺ cells (neurogenesis) in the mouse sub-granular zone of the hippocampus from different treatment groups. Intra-hippocampal injection of DPSCs and DPSC-CM induced significant neurogenesis in the sub-granular zone of hippocampus that was several fold higher than NC, KA, KA+BM-MSC, KA+BM-MSC-CM groups. Scale bar: 50 μm.

Abbreviations:

Normal Control (NC); Kainic Acid treated mice (KA); KA injected mice transplanted with human bone-marrow mesenchymal stem cells (KA+BM-MSC), KA injected mice transplanted with human dental pulp stem cells (KA+DPSC); KA mice injected with conditioned medium derived from hBM-MSCs (KA+BM-MSC-CM) and KA mice injected with conditioned medium derived from hDPSCs (KA+DPSC-CM)
3.5 Human DPSCs/BM-MSCs and Their Respective CM Improved Spatial Learning and Memory

Following various treatments, mice were subjected to spatial learning and memory assessment using eight arm radial maze. In KA treated mice, significant impairment in learning (Fig. 8A; p<0.001; KA group) was observed. On the other hand, hDPSCs/hBM-MSCs transplantation in KA treated mice significantly ameliorated the impairment in acquiring new spatial information (Fig. 8A; KA+DPSC; KA+BM-MSC). Likewise, treatment with CM derived from hDPSCs/hBM-MSCs mitigated the learning impairment in KA treated mice (Fig. 8A; KA+DPSC-CM; KA+BM-MSC-CM). Interestingly, transplantation of hDPSCs or hDPSC-CM treatment to the hippocampal lesioned mice demonstrated early acquisition of spatial learning as compared to KA+BM-MSC or KA+BM-MSC-CM groups (Fig. 8A). As seen in Fig. 8B, the KA treated mice showed impaired performance during the retention test as compared to NC group (p<0.001). The hDPSCs/hBM-MSCs transplantation and their CM treated mice showed almost 90% correct performance (Fig. 8B; p<0.001) as mice were able to make correct entries without any errors.
Figure 8: Eight arm radial maze task to assess spatial learning and memory function following various treatment. KA treated mice (KA group) demonstrated significant spatial learning (A) and memory impairment (B). Whereas, hDPSCs/hBM-MSCs and their respective conditioned medium treatment to KA mice significantly ameliorated learning (A) and memory (B) deficits. Data expressed as mean ± SEM. *comparison between NC vs treatment groups. +comparison between KA vs treatment groups. #comparison between KA+DPSC-CM vs KA+BM-MSC-CM. ^comparison between KA+DPSC vs KA+BM-MSC. **p<0.001; +++p<0.001; ##p<0.01; ###p<0.001; ^^^p<0.01; ^p<0.05.
3.6 Human DPSCs/hBM-MSCs and Their Respective CM Upregulates Host’s Endogenous Neuronal Survival Factors in In Vivo Conditions

In order to understand the effects of hDPSCs/BM-MSCs transplantation or their respective CM treatments on endogenous neurotrophic factor expression, the mice hippocampal samples were processed for qRT-PCR analysis. Various nerve growth factors like BDNF, GDNF, CNTF, VEGF, PDGF, NGF, NT3, bFGF and EPO expressions were analysed using qRT-PCR method. Our results revealed that direct transplantation of hDPSCs/hBM-MSCs or their respective CM treatment resulted in significant changes in the mRNA expression of endogenous growth factors in the hippocampus as explained below.

3.6.1 Intrahippocampal Injection of Kainic Acid to Adult Mice Reduces the mRNA Expression of Growth Factors

Following KA treatment to adult mice, there was a significant decrease in crucial growth factors like BDNF (p<0.001; Fig.9A; KA), GDNF (p<0.001; Fig.9B; KA), CNTF (p<0.001; Fig.9C; KA), VEGF (p<0.001; Fig.9D; KA), PDGF-B (p<0.01; Fig. 9F; KA), NGF (p<0.001; Fig.9G; KA), bFGF (p<0.001; Fig. 9H; KA), NT-3 (p<0.01; Fig. 9I; KA) and EPO (p<0.01; Fig.9L; KA) as compared to NC group.

3.6.2 Intrahippocampal Transplantation of hDPSCs/hBM-MSCs Post KA Injection Alters the mRNA Expression of Growth Factors

Following hDPSCs cell transplantation to hippocampus damaged mice, there was a significant increase in BDNF (p<0.001; Fig. 9A; KA+DPSC), GDNF (p<0.001; Fig. 9B; KA+DPSC), CNTF (p<0.001; Fig. 9C; KA+DPSC), VEGF (p<0.001; Fig. 9D; KA+DPSC), PDGF- B (p<0.001; Fig. 9F; KA+DPSC), NGF (p<0.001; Fig. 9G; KA+DPSC), NT-3 (p<0.01; Fig. 9I; KA+DPSC) and EPO (p<0.05; Fig. 9J; KA+DPSC) was observed as compared to the KA group. Non-significant changes were observed in the expression of PDGF-A (Fig. 9E; KA+DPSC) and b-FGF (Fig. 9H; KA+DPSC) as compared to KA group.

In case of hBM-MSCs transplantation to hippocampus damaged mice, enhanced the mRNA expression of BDNF (p<0.001; Fig. 9A; KA+BM-MSC), GDNF (p<0.001; Fig. 9B; KA+BM-MSC), CNTF (p<0.001; Fig. 9C; KA+BM-MSC), VEGF (p<0.001; Fig. 9D; KA+BM-MSC), PDGF-B (p<0.001; Fig. 9F; KA+BM-MSC), bFGF (p<0.05; Fig. 9H; KA+BM-MSC), NGF (p<0.001; Fig. 9G; KA+BM-MSC), NT-3 (p<0.05; Fig. 9I; KA+BM-MSC) and EPO (p<0.05; Fig. 9J; KA+BM-MSC) was observed as compared to KA group. There was no significant
change in PDGF-A (Fig. 9E; KA+BM-MSC) expression was observed following hBM-MSCs treatment to hippocampus damaged mice as compared to KA group.

3.6.3 Intrahippocampal Infusion of hDPSCs/hBM-MSCs Derived CM post KA Injection Alters the mRNA Expression of Growth Factors

Human DPSCs derived CM treatment to hippocampus damaged mice resulted in significant increase in expression of BDNF (p<0.001; Fig. 9A; KA+DPSC-CM), GDNF (p<0.001; Fig. 9B; KA+DPSC-CM), CNTF (p<0.001; Fig. 9C; KA+DPSC-CM), VEGF (p<0.001; Fig. 9D; KA+DPSC-CM), NGF (p<0.001; Fig. 9G; KA+DPSC-CM), b-FGF (p<0.05; Fig. 9H; KA+DPSC-CM), EPO (p<0.01; Fig. 9J; KA+DPSC-CM) and NT-3 (p<0.05; Fig. 9I; KA+DPSC-CM) as compared to KA group. There was no significant change in the mRNA expression of PDGF-A (Fig. 9E; KA+DPSC-CM) and PDGF-B (Fig. 9F; KA+DPSC-CM) as compared to KA group.

Human BM-MSCs derived CM treatment to hippocampus damaged mice resulted in a significant increase in expression of BDNF (p<0.001; Fig. 9A; KA+BM-MSC-CM), GDNF (p<0.001; Fig. 9B; KA+ BM-MSC-CM), CNTF (p<0.001; Fig. 9C; BM-MSC-CM), VEGF (p<0.01; Fig. 9D; KA+ BM-MSC-CM), PDGF-B (p<0.001; Fig. 9F; KA+ BM-MSC-CM), NGF (p<0.001; Fig. 9G; KA+ BM-MSC-CM), NT-3 (p<0.05; Fig. 9I; KA+BM-MSC-CM) and EPO (p<0.05; Fig. 9J; KA+BM-MSC-CM) as compared to KA group. There was no significant change in the mRNA expression of PDGF-A (Fig. 9E; KA+BM-MSC-CM) and b-FGF (Fig. 9H; KA+ BM-MSC-CM) as compared to KA group.
### Fig. 9A

**BDNF expression**

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- ↓ Indicates decreased expression of neuronal survival factors following KA treatment
- ↑ Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM

### Fig. 9B

**GDNF expression**

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- ↓ Indicates decreased expression of neuronal survival factors following KA treatment
- ↑ Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM
Fig. 9C

CNTF expression

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↓ Indicates decreased expression of neuronal survival factors following KA treatment

↑ Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM

Fig. 9D

VEGF expression

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↓ Indicates decreased expression of neuronal survival factors following KA treatment

↑ Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM
Indicates elevated expression of neuronal survival factors following KA treatment

Indicates decreased expression of neuronal survival factors following KA treatment

Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM
Fig. 9G

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🔻 Indicates decreased expression of neuronal survival factors following KA treatment
🔺 Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM

Fig. 9H

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🔻 Indicates decreased expression of neuronal survival factors following KA treatment
🔺 Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM
**Fig.9I**

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- **↓** Indicates decreased expression of neuronal survival factors following KA treatment
- **↑** Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM

**Fig.9J**

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- **↓** Indicates decreased expression of neuronal survival factors following KA treatment
- **↑** Indicates elevated expression of neuronal survival factors after treatment with hDPSC/hBM-MSC and their CM

*comparison between NC vs KA
+comparison between KA vs treatment groups
3.7 Anti-Apoptotic Effects of hDPSCs/hBM-MSCs Transplantation and Its Respective CM Treatment

Interestingly, intrahippocampal injection of hDPSCs/hBM-MSCs and their respective CM treatment decreased the activity of pro-apoptotic enzyme caspase-3/7 (Fig. 10). Following KA treatment, caspase-3/7 activity was significantly elevated (p<0.001; Fig. 10) as compared to NC group. On the other hand, the caspase-3/7 activity in hDPSCs transplantation group (KA+DPSC) or hDPSC-CM treatment group (KA+DPSC-CM) was comparable to that of NC group, suggesting that hDPSCs transplantation/hDPSC-CM treatment could protect hippocampal neurons through anti-apoptotic mechanisms by inhibiting the pro-apoptotic caspase 3/7 activity. Even though we observed a significant decrease (p<0.001; Fig. 10) in pro-apoptotic caspase 3/7 activity following hBM-MSCs/hBM-MSC-CM treatment as compared to KA group, the pro-apoptotic caspase 3/7 activity in KA+BM-MSC and KA+BM-MSC-CM was significantly higher as compared to NC group (p<0.001; Fig. 10). Interestingly, hDPSCs/hDPSC-CM exhibited superior anti-apoptotic property as compared to hBM-MSCs/hBM-MSC-CM treatment groups (p<0.001; Fig. 10). To substantiate the anti-apoptotic role of hDPSCs/hBM-MSCs and their CM treatment, we quantified the protein expression of anti-apoptotic factors such as Bcl-2 and phosphorylated PI3K by western blotting method. As Bcl-2 is best known for its roles in inhibiting apoptosis, we assessed the protein expression of Bcl-2 in different groups. We observed a decrease in Bcl-2 protein expression (p<0.001; Fig. 12A) following KA treatment. Whereas, hDPSCs/hBM-MSCs or their respective CM treatment significantly increased the protein expression of Bcl-2 (p<0.001; KA+DPSC; KA+BM-MSC; KA+DPSC-CM; KA+BM-MSC-CM; Fig. 12A) as compared to KA alone group. As PI3K pathway is involved in cell survival, we assessed the phosphorylated-PI3K expression in various treatment groups. We observed a decreased expression of phosphorylated PI3K expression in the KA lesion group (p<0.001; Fig. 12B) as compared to NC. Whereas, hDPSCs/hBM-MSCs or their respective CM treatment increased the expression of phosphorylated PI3K (p<0.001; KA+DPSC; KA+BM-MSC; KA+DPSC-CM; KA+BM-MSC-CM; Fig. 12B) suggesting that the cell transplantation or CM treatment would possibly act through PI3K pathways to protect neurons (Fig. 11). Thus, our study confirms that the treatment with hDPSCs/hBM-MSCs or their respective CM to hippocampal damaged mice could protect hippocampal neurodegeneration by activating PI3K-Bcl-2 cell survival pathway.
Figure 10: Caspase 3/7 activity Assay: Bar diagram representing the luminescence (RLU) in different treatment groups. Data represented as mean ± SEM. *comparison between NC vs treatment. +comparison between KA vs treatment. #comparison between KA+DPSC vs KA+BM-MSC. ^comparison between KA+DPSC-CM vs KA+BM-MSC-CM. ***p<0.001; +++p<0.001; ###p<0.001; ^^^p<0.001. RLU: Relative Light Units.

Figure 11: Representative western blot image showing the protein expression of p-PI3K, Bcl-2 and β-actin in different treatment groups.
Figure 12: Densitometric analysis of western blot for Bcl-2 (A) and p-PI3K (B) expression in different treatment groups expressed as fold change. *comparison between NC vs treatment. +comparison between KA vs treatment. ***p<0.001; +++p<0.001.
4. DISCUSSION

In this chapter, we investigated the neuroprotective potential of hDPSCs/hBM-MSCs and its CM in an animal model of hippocampal neurodegeneration. Kainic acid injection into the hippocampal CA3 sub-field induced significant neurodegeneration, neuroinflammation and prominent hippocampal dependent spatial learning and memory dysfunction. Of note, intrahippocampal transplantation of hDPSCs/hBM-MSCs one day post KA injection significantly attenuated the hippocampal neurodegeneration, neuroinflammation and spatial learning and memory impairments. Interestingly, the neuroprotective potential of hDPSCs/hBM-MSCs could be recapitulated with hDPSCs/hBM-MSCs CM per se highlighting the role of adult stem cell secretome in conferring neuroprotection in a neurodegenerative condition.

Hippocampus play a pivotal role in acquisition of new information, memory consolidation, locomotor and mood stability. The hippocampus is commonly referred to hippocampus proper which includes DG, CA1-CA4 subfield, subicular regions and EC. The hippocampus is a region of research interest for its simplified yet well organised structures and for its extreme vulnerability in progressive neurodegenerative diseases like AD and TLE. Hippocampal neurons are highly susceptible to excitotoxicity in such disease conditions and therapeutic strategies to mitigate hippocampal neurodegeneration in AD and TLE is a primary focus of research. In order to develop therapies for neurodegenerative diseases, animal prototype that mimics particular neurodegenerative disease is highly essential. In the current study, we utilized KA as a neurotoxin to induce hippocampal neurodegeneration in mice. Micro injection of KA, a glutamate analogue into CA3 subfield of hippocampus resulted in significant loss of CA3 neurons. Besides, neuronal losses were also observed in CA1 subfield of hippocampus and DH region. The CA1 and DH neuronal loss observed in the current study might be due to retrograde and anterograde degeneration as a result of KA induced neurodegeneration in CA3 subfield. Such retrograde and anterograde neuronal losses have been reported following injection of other neurotoxins like ibotenic acid into the hippocampal subfields (15,16). Kainic acid injections into the hippocampal CA3 subfield resulted in significant hippocampal neuroinflammation evidenced by reactive astrocytes in various subfields of the hippocampus as reported by others (17–19). Furthermore, the microglial marker IBA-1 one was highly elevated in hippocampus following KA mediated neuronal damage. Of note, KA injection into the hippocampus resulted in a significant increase in hippocampal neurogenesis. Previous studies have also demonstrated significant upregulation of hippocampal neurogenesis.
following intrahippocampal/ intraperitoneal injection of high dose of KA which resulted in status epilepticus (20,21). Though in the current study the KA dosage was minimized to not have any behavioural seizure at the time of KA injection, it cannot be ruled out that the mice might have had seizure during the post-surgery period. Given the hippocampal neurodegeneration and neuroinflammation following KA injection into the hippocampus, these mice demonstrated significant hippocampal dependent spatial learning and memory deficits as assessed by eight arm radial maze task. These observations are in line with previous reports that hippocampal neurodegeneration and neuroinflammation culminates in spatial learning and memory deficits (22,23). Mitigation of progressive hippocampal neurodegeneration and associated behavioural co-morbidities is a major challenge in AD and TLE. Several pharmacological agents are being tried in mitigating the neurodegeneration and preserving the higher order brain functions. However, such approaches only provided symptomatic relief and failed to arrest the disease progression. As nerve growth factors play a major role in neuronal survival, several clinical trials were been initiated to evaluate the therapeutic potential of growth factors in a spectrum of neurodegenerative diseases (24,25). However, the side effects of growth factor treatment outweighed the therapeutic outcomes (26). In this context, stem cell therapy is gaining significant attention as it could aid in regeneration of the nervous system or by restoring lost neurons/glia through exogenous cell grafting or by activating endogenous neural stem cell populations. Mesenchymal stem cells are more preferred for cell therapy as compared to embryonic/induced pluripotent stem cells because of its easy availability, immune modulatory properties with less ethical constraints. Bone marrow mesenchymal stem cells were used extensively in many disease conditions with encouraging functional outcomes (27). In the present study, transplantation of human BM-MSCs into the hippocampal damaged mice significantly prevented the KA induced hippocampal abnormalities such as neurodegeneration, neuroinflammation and improved hippocampal learning and memory functions. In line with this, studies have shown that BM-MSCs transplantation in 6-hydroxydopamine lesioned rats prevented dopaminergic neuron degeneration and reduced neuroinflammation (28). Similarly, intravitreal transplantation of BM-MSCs following optic nerve crush in adult rats promoted the regeneration of injured axons (29). Yi Xu et al. (2014), demonstrated that transplantation of human BM-MSCs through the femoral vein exhibited significant neuroprotection against cerebral ischemia via a mechanism associated with the ability to attenuate axonal injury which includes demyelination after cerebral ischemia, prevention of axonal degeneration, and promotion of axonal regeneration (30). Mahmood et al. (2005), reported that BM-MSCs administered either directly into the CNS or through systemic injection one day post traumatic
brain injury could efficiently induce neural stem cell proliferation and differentiation and aided in motor functional recovery. Of note, even delayed intervention with BM-MSCs two months post-traumatic brain injury resulted in enhanced hippocampal neurogenesis and significant motor function recovery in rats (31). Corroborating these reports with our observation in the current study that BM-MSCs transplantation into hippocampal damaged mice resulted in significant neurogenesis in the DG suggesting that the engrafted BM-MSCs might release various growth factors which in turn stimulate quiescent endogenous neural stem cells for proliferation and neural/glial differentiation. Thus, in the present study, we investigated whether CM derived from BM-MSCs per se could be able to recapitulate the direct intrahippocampal BM-MSCs transplantation in hippocampal damaged mice. Of note, BM-MSCs derived CM per se could prevent KA induced hippocampal neurodegeneration and improved spatial learning and memory function in KA treated mice. However, as compared to significant anti-inflammatory effect observed following BM-MSCs transplantation, BM-MSCs derived CM infusion into the hippocampus failed to exert anti-inflammatory effect. Interestingly, BM-MSCs derived CM infusion into the hippocampus induced significant upregulation of DG neurogenesis similar to direct BM-MSCs cell grafting into the hippocampus of KA treated mice. Our observation and the previous reports by other research groups (32–34) highlights the possible paracrine mediated mechanisms of neuroprotection following BM-MSCs transplantation in neurodegenerative diseases.

Though BM-MSCs mediated neuroprotection, neurorestoration (endogenous neurogenesis) and hippocampal functional recovery provided a proof-of-principle in using MSCs for treating neurodegenerative diseases, the donor availability, less proliferation index and most importantly the excruciating bone marrow extraction procedure limit the utilization of BM-MSCs for cell therapy. As necessity is the mother of invention, several research groups globally explored the possibilities of identifying MSCs that could be extracted/isolated much easily with less pain, possess better proliferation rate and have neuroprotective potential equal to or better than BM-MSCs. Such research highlights the importance of utilizing neural crest originated DPSCs for treating a spectrum of diseases/disorders associated with central nervous system. Dental pulp can be extracted from a third molar tooth which exhibits MSCs characteristic features with multipotent capacity (35). The population doubling index of DPSCs is far better than BM-MSCs and given their ectodermal origin, DPSCs possess an inherent differentiation propensity towards neural lineage when conducive milieu is provided (36,37). In the current study, we observed that DPSCs transplantation into the hippocampus of KA treated mice demonstrated equivalent neuroprotective and anti-inflammatory efficacy. In accordance with
our observations, previous studies have demonstrated that intracerebral transplantation of hDPSCs twenty four hours after focal cerebral ischemia resulted in neuroprotection and demonstrated significant improvement in forelimb sensorimotor function (38). Sakai et al. (2011), demonstrated that transplantation of hDPSCs or human exfoliated deciduous teeth (SHED) into completely transected adult rat spinal cord significantly attenuated neurodegeneration through anti-apoptotic mechanism (39). Likewise, transplantation of hDPSCs into sciatic-nerve resected areas in rats resulted in increased myelination and negligible percentage of transplanted hDPSCs could differentiate into neurons (40). The neurogenic potential of DPSCs in naïve mice has been demonstrated previously (41). However, whether DPSCs possess similar neurogenic properties in a neurodegenerative milieu has not been investigated. Our study is the first of its kind to demonstrate that hDPSCs transplantation into a degenerating milieu induced significant neurogenesis in the DG which was several fold higher than that of hBM-MSCs induced neurogenesis. As a consequent of enhanced hippocampal neurogenesis following hDPSCs transplantation, the hippocampal lesioned mice that received hDPSC cell grafting demonstrated early acquisition of spatial learning in eight arm radial maze task as compared to hBM-MSCs transplanted group. Recent studies demonstrated that DPSCs secrete greater titre of growth factors essential for neuronal survival as compared to BM-MSCs (42) and that cell therapy with DPSCs in animal models of spinal cord injury (39) or retinal degeneration (35,36) resulted in cellular and behavioural recoveries that was far superior to BM-MSCs. Sakai et al. (2011), demonstrated that transplantation of DPSCs/SHEDs into injured spinal cord of rats resulted in motor functional recovery with better hind limb coordination as compared to BM-MSCs treated spinal cord injured rats. Furthermore, microarray analysis between DPSCs and BM-MSCs demonstrated that DPSCs exhibit 2 fold higher gene expressions related to cell proliferation, extracellular matrix, embryonic development and tissue regeneration (39).

In order to assess the paracrine mediated neuroprotection, CM derived from hDPSCs was injected into the hippocampus one day following KA injection. Human DPSCs derived CM injection into hippocampus demonstrated equivalent neuroprotection, neurogenesis and hippocampal learning and memory performance comparable to hDPSCs transplantation again emphasising on the paracrine/trophic mechanisms of neuroprotection culminating in better behavioural recovery. Of note, the anti-inflammatory, hippocampal neurogenic and hippocampal functional recovery of DPSCs derived CM was better than that of BM-MSCs CM which support an earlier report by Tsuneyuki Mita et al. (2015), in which dental stem cell derived CM demonstrated better anti-inflammatory and memory improvement as compared to
BM-MSCs derived CM in an animal model of AD. They also demonstrated that dental stem cell derived CM treatment, but not with BM-MSCs derived CM treatment elevated anti-inflammatory cytokines and the anti-inflammatory markers like Ym-1, Arginase1 and Fizz1 (43) thus supporting our observation that the hDPSCs derived CM possess strong anti-neuroinflammatory properties than BM-MSCs derived CM. Besides, CM derived from DPSCs/SHEDs could promote neuritogenesis of transected spinal cord axons better than BM-MSCs derived CM by clearing axon growth inhibitors such as chondroitin sulfate proteoglycans and myelin-associated glycoprotein (39). Furthermore, DPSCs derived CM have shown to facilitate axon regeneration and guide regenerating axons to their appropriate target through chemoattractant factor stromal derived factor-alpha and its cognate receptor CXCR-4 (44). In the current study, we observed that the morphology of doublecortin positive neurons in the sub-granular zone were well matured with more dendritic branches ramified into granule cell layers as compared to BM-MSCs or its CM treatment group highlighting the superior characteristic features of DPSCs and its CM in activation of endogenous stem cell proliferation, neural differentiation and guidance to the appropriate target.

We evaluated the possible neuroprotective mechanisms of hDPSCs/hBM-MSCs transplantation or its CM treatment in hippocampal neurodegenerative conditions. As neurotrophic factors play a major role in neuronal survival (45), we explored whether hDPSCs/hBM-MSCs transplantation or their respective CM treatment could enhance the hosts’ endogenous neurotrophic factor expressions. Human DPSCs/hBM-MSCs and its CM treatment induced significant increase in the expressions of various neurotrophic factors such as BDNF, GDNF, CNTF, VEGF, NGF, and IGF-1, most of these factors being involved in neuronal survival (46). NT3 was upregulated with hDPSCs and its CM treatment, but not with BM-MSCs or its CM treatment. Significant increase in EPO was observed with hDPSCs/hBM-MSCs transplantation group and in hDPSCs derived CM treated mice, but not with hBM-MSCs derived CM treated mice. Thus, hDPSCs/hBM-MSCs or its CM influence hosts’ endogenous neurotrophic factor expressions in a distinct manner. Neurotrophic factors brings about its biological consequences through activation of cell survival pathways such as AKT/PI3K pathway which in turn increase the expression of anti-apoptotic factors like Bcl-2 (32,47). In the present study, as the downstream implications of enhanced neurotrophic factor expressions, we observed increased expressions of phosphorylated PI3K and Bcl-2 following hDPSCs/hBM-MSCs transplantation as well as its CM treatment. In contrast, pro-apoptotic markers like caspase 3/7 activity was significantly downregulated following hDPSCs/hBM-MSCs transplantation as well as its CM treatments. This is in line with previous studies that
cell therapy or secretome treatments lead to enhanced anti-apoptotic proteins and downregulation of pro-apoptotic proteins (47–51).
5. CONCLUSION

We evaluated and compared the neuroprotective, anti-inflammatory and neurogenic potential of MSCs derived from two different sources, i.e. dental pulp stem cells and bone-marrow-MSCs and their respective CM in hippocampus injured mice. Our data revealed that intrahippocampal injection of KA induced significant neurodegeneration and elevated neuroinflammation. Interestingly, intrahippocampal transplantation of hDPSCs/hBM-MSCs and injection of their respective CM significantly protected hippocampal neurons against KA induced excitotoxicity. Likewise, hDPSCs/hBM-MSCs and their CM were equally effective in reducing neuroinflammation following hippocampal injury. Human DPSCs/hBM-MSCs transplantation or their respective CM injection into the hippocampus induced neurogenesis to several folds as compared to NC or KA treatment group. Interestingly, the neurogenic potential of hDPSCs/hDPSC-CM was far greater than that of hBM-MSCs/hBM-MSC-CM suggesting that MSCs from ectodermal germ cell origin like DPSCs may be a more appropriate stem cell candidate for neural regeneration. Remarkably, intrahippocampal transplantation of hDPSCs/hBM-MSCs significantly attenuated the learning and memory deficits in hippocampus damaged mice. Likewise, intrahippocampal injection of CM derived from hBM-MSCs and hDPSCs also attenuated the learning and memory deficits, suggesting that the CM per se could be sufficient to recapitulate the therapeutic effects of stem cell transplantation. Our study is also the first of its kind to demonstrate that the CM derived from MSCs were equally potent like MSCs cell transplantation itself in protecting hippocampal neurons against excitotoxicity. Interestingly, there was a significant difference in anti-inflammatory and neurogenic potential of CM derived from hDPSCs and hBM-MSCs. The neurogenic and anti-inflammatory potential of the DPSCs CM was greater than the BM-MSCs derived CM, suggesting that DPSCs and its CM would be an ideal MSCs source for treating neurodegenerative diseases. Both hBM-MSCs/hDPSCs and their respective CM were effective in restoring the learning/memory functions. However, the behavioural improvement in hippocampal damaged mice were observed much earlier in DPSC/DPSC-CM treated mice when compared to BM-MSC/BM-MSC-CM treated mice. Furthermore, hDPSCs/hBM-MSCs cell transplantation approach or their CM treatments per se prevented neurodegeneration by downregulating the pro-apoptotic caspase-3/7 activity and up-regulating the expression of anti-apoptotic factors like Bcl-2 and phosphorylated PI3K.