CHAPTER: III

OBJECTIVE 1

To Study the Neuroprotective Efficacy and to Understand the Mechanisms of Neuroprotection Mediated by hDPSCs/hBM-MSCs and hDPSCs/hBM-MSCs Derived CM Against Kainic Acid Induced Hippocampal Neurodegeneration in an In Vitro Condition.

1. INTRODUCTION

Hippocampal formation plays a pivotal role in spatial learning and memory (1). Damage to the hippocampus leads to severe learning and memory deficits as observed in neurodegenerative diseases like AD (2). To date, there is no promising treatment available to prevent neuronal loss and to enhance functional recovery in many neurodegenerative diseases. Stem cell therapy was explored extensively in the past and the functional outcomes following such therapies were convincing in spite of various debates about the possible mechanisms by which functional recovery would have happened. Nevertheless, recent studies convincingly demonstrated that the cellular and functional recovery following cell therapy could be recapitulated with secretome per se thereby shifting the dogma from cell therapy to cell “based” therapy (3). Such studies demonstrated that CM possess therapeutic biomolecules that have the competence to stimulate host cell repair response, thereby circumventing the need for volumes of graft cell transplantation. Given the biological complexity of the CM, the current objective was designed to compare the neuroprotective potential of CM derived from hDPSCs/hBM-MSCs with direct hippocampal-hDPSCs/hBM-MSCs co-culture system and to explore the mechanisms of neuroprotection mediated by hDPSCs/hBM-MSCs and their CM in an in vitro model of hippocampal neurodegeneration.
2. MATERIALS AND METHODS

2.1 Human DPSCs and Human BM-MSCs Culture

Materials

- Mesenchymal stem cell (MSC) media
- 70% ethanol
- Plastic ware - Culture dishes and Centrifuge Tubes
- CO₂ Incubator - SalvisLab, Switzerland
- Light Microscope - Nikon Eclipse TE 200-s, Chiyoda-Ku, Japan
- Biosafety Cabinet Level 2 - Thermo Scientific, Waltham, Massachusetts, United States.

MSC Media Composition

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knock out-DMEM</td>
<td>Make up to 50 mL</td>
</tr>
<tr>
<td>10% FBS</td>
<td>5 mL</td>
</tr>
<tr>
<td>200 mM L-Glutamine</td>
<td>500 µL</td>
</tr>
<tr>
<td>100 µg/ml Penicillin-Streptomycin</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Methodology

Commercially available research grade hDPSCs and hBM-MSCs from healthy donors were obtained from Advanced Neuroscience Allies Pvt Ltd (ANSA), Bangalore, India. The Institutional Ethical Committee approval was obtained from Manipal Hospital (Protocol # MIRM/SCR/023) Bangalore, India. Human DPSCs and human BM-MSCs were cultured in Knock Out-Dulbecco’s modified eagle medium (KO-DMEM) (Invitrogen; Carlsbad, California, USA) supplemented with 10% (v/v) Fetal bovine serum (FBS) (Hi-media Laboratories; L.B.S Marg, Mumbai, India), 100 µg/ml penicillin, 100 µg/ml streptomycin (Invitrogen; Carlsbad, California, USA), and 2 mM L-Glutamine (Invitrogen; Carlsbad, California, USA) and maintained in a humid atmosphere at 37°C and 5% CO₂. After every 48 hours, the spent media was removed and replaced with fresh growth media. On attaining a confluence of 70-80% the cells were sub-cultured and the hDPSCs/hBM-MSCs from passage 4-5 were used to explore the neuroprotective potential.
2.2 Characterization of hDPSCs/hBM-MSCs

2.2.1 Expression of Cell-Surface Markers by RT-PCR

Conventional reverse transcriptase polymerase chain reaction (RT-PCR) was performed to evaluate the expression of mesenchymal stem cell-surface antigens in hDPSCs/hBM-MSCs (CD 90, CD 105, CD 44, CD 34 and CD 45). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Total RNA was isolated from hDPSCs/hBM-MSCs using RNAiso Plus reagent (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan). The amount of RNA was measured by Nano Drop 1000 (Thermo Scientific; Waltham, MA, USA). Single stranded cDNA (complimentary Deoxyribose Nucleic Acid) was synthesized using a Verso cDNA synthesis kit (Thermo Scientific; Waltham, MA, USA). For conventional RT-PCR the resultant cDNA was amplified with Emerald AmpGT PCR Master Mix (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan) for 35 cycles under the following conditions: 5 min of incubation at 95°C, 45 s of denaturation at 94°C, 45 s of annealing at the primer-specific temperature and 45 s of extension at 72°C followed by 10 min final extension step at 72°C. After electrophoresis, the PCR products were visualized using ethidium bromide staining.

2.2.2 Flow Cytometry for Cell-Surface Antigen Expression

Human DPSCs/BM-MSCs were characterized for the presence of mesenchymal surface antigens (CD 90, CD 105 and CD 44) and for the absence of hematopoietic markers (CD 45, CD 34) and HLA-DR (MHC class II cell surface receptor). Antibodies against CD 90 and CD 34 were conjugated with phycoerythrin (PE) while antibodies against CD 105, CD 45, HLA-DR and CD 44 were conjugated with fluorescein isothiocynate (FITC) (BD Biosciences; San Jose, CA, USA) were used. Cells at the passage 4-5 and 80% confluence were enzymatically detached by trypsinization (0.25% trypsin/EDTA; Sigma Aldrich; St. Louis, MO, USA) and fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. The cells were then centrifuged with phosphate buffered saline (PBS) at 675 x g for 5 minutes. Subsequently the cells were then incubated with the conjugated antibodies for one hour and flow cytometry analysis was performed using FACS Calibre flow analyser (Becton-Dickenson Biosciences, Franklin Lakes, NJ, USA) and Cell Quest Pro software (Becton-Dickinson).

2.2.3 In vitro Multi-lineage Differentiation of hDPSCs/hBM-MSCs

The tri-lineage potential of hDPSCs/hBM-MSCs was assessed as described below

**Adipogenic Differentiation:** Human DPSCs/BM-MSCs were plated in 6 well plates as two replicates for the control and two replicates for differentiation. The cells were allowed to reach 70% confluence. The control medium consisted of KO-DMEM (Invitrogen; Carlsbad,
California, USA) supplemented with 10% FBS (Hi-media Laboratories; L.B.S Marg, Mumbai, India), 100 µg/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Carlsbad, California, USA) and 2 mM L-Glutamine (Invitrogen; Carlsbad, California, USA). The adipogenic differentiation media consisted of StemPro differentiation basal medium and StemPro adipocyte supplement in the ratio 9:1 (Invitrogen; Carlsbad, California, USA). This media was used to induce differentiation for 21 days with media change on every 48 hours. After 21 days, adipogenic differentiation was assessed by Oil Red-O staining.

**Oil Red-O Staining for Adipogenic Cultures:** The Oil Red-O (Hi-media Laboratories; L.B.S Marg, Mumbai, India) stock solution was prepared by adding 300 mg of Oil Red-O stain powder in 100 ml of 99% isopropanol (Hi-media Laboratories; L.B.S Marg, Mumbai, India). The Oil Red-O working solution was prepared by mixing 3 parts of the Oil Red-O stock solution with 2 parts of distilled water and was allowed to settle at room temperature for 10 minutes. The culture medium was removed from the wells and the cells were washed once with sterile PBS. The cells were then fixed with 10% formalin (Sigma-Aldrich, St. Louis, Missouri, US) for 30-60 minutes at room temperature. The formalin was removed and the cells were washed once with sterile water. Two ml of 60% isopropanol was added to each well and incubated for 2-5 minutes. Isopropanol was aspirated after incubation and working solution of Oil Red-O was added along the side of each well and incubated for 5-10 minutes at room temperature. The cells were rinsed with tap water and viewed under a light microscope (Nikon Eclipse TE 200-s, Chiyoda, Japan) for the formation of oil droplets.

**Chondrocyte Differentiation:** Human DPSCs/hBM-MSCs were plated in 6 well plates as two replicates for the control and two replicates for differentiation. The cells were allowed to reach 70% confluence. The control medium consisted of KO-DMEM (Invitrogen; Carlsbad, California, USA) supplemented with 10% FBS (Hi-media Laboratories; L.B.S Marg, Mumbai, India), 100 µg/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Carlsbad, California, USA) and 2 mM L-Glutamine (Invitrogen; Carlsbad, California, USA). The chondrogenic differentiation media consisted of StemPro differentiation basal medium and StemPro chondrogenesis supplement in the ratio 9:1 (Invitrogen; Carlsbad, California, USA). This media was used to induce chondrogenic differentiation for 21 days with media change on every 48 hours. After 21 days the cells were stained with Alcian Blue which stains acidic polysaccharides such as glycosaminoglycan in cartilages, as a result blue staining was observed.
**Alcian Blue Staining for Chondrogenic Cultures:** The culture medium was removed from the control wells and induced wells and then the cells were washed once with sterile PBS. The cells were fixed in 4% PFA (Sigma-Aldrich, St. Louis, Missouri, US) solution for 30 minutes at room temperature. After fixation, the cells were rinsed twice with PBS. 1% Alcian blue (Sigma-Aldrich, St. Louis, Missouri, US) solution prepared in 0.1N HCl (Hi-media Laboratories; L.B.S Marg, Mumbai, India) was added to the cells and incubated for 30 minutes at room temperature. The cells were rinsed with 0.1N HCl and then neutralized with distilled water and observed under a light microscope (Nikon Eclipse TE 200-s, Chiyoda, Japan).

**Osteogenic Differentiation:** Human DPSCs/BM-MSCs were plated in 6 well plates, two replicates for the control and two replicates for differentiation. The cells were allowed to reach 70% confluence. The control medium consisted of KO-DMEM (Invitrogen; Carlsbad, California, USA) supplemented with 10% FBS (Hi-media Laboratories; L.B.S Marg, Mumbai, India), 100 µg/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Carlsbad, California, USA) and 2 mM L-Glutamine (Invitrogen; Carlsbad, California, USA). The osteogenic differentiation media consisted of StemPro differentiation basal medium and StemPro osteogenesis supplement in the ratio 9:1 (Invitrogen; Carlsbad, California, USA). This media was used to induce differentiation for 21 days with media change every 48 hours. After 21 days the cells were stained with Vonkossa.

**Vonkossa Staining for Osteogenic Cultures:** The culture medium was removed and the cells were fixed in 10% formalin (Sigma-Aldrich, St. Louis, Missouri, US) for 30 minutes at room temperature. After fixation, the formalin was removed and the cells were washed twice with distilled water. 1 ml of freshly prepared 5% silver nitrate solution (Hi-media Laboratories; L.B.S Marg, Mumbai, India) was added to the cells and kept under ultraviolet (UV) for 1 hour. Subsequently, silver nitrate was removed and 2.5% sodium thiosulfate solution (Hi-media Laboratories; L.B.S Marg, Mumbai, India) was added and incubated for 5 minutes. Later, the cells were washed with water and observed under a light microscope (Nikon Eclipse TE 200-s, Chiyoda, Japan) for calcium deposits.

**2.2.4 Expression of Neural Crest Markers in hDPSCs/hBM-MSCs by RT-PCR**

The expression of neural crest markers (p-75, Pax-6, Pax-3, Sox-9, Sox-2 and Twist) in hDPSCs as compared to hBM-MSCs was assessed by conventional RT-PCR. GAPDH was used as a housekeeping gene. Total RNA was isolated from hDPSCs/hBM-MSCs using RNAiso Plus reagent (Takara Bio Inc; Nōjihigashi, Kasatsu, Shiga, Japan). The amount of
RNA was measured by Nano Drop 1000 (Thermo Scientific; Waltham, MA, USA). Single stranded cDNA was synthesized using a Verso cDNA synthesis kit (Thermo Scientific; Waltham, MA, USA). For conventional RT-PCR the resultant cDNA was amplified with Emerald AmpGT PCR master mix (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan) for 35 cycles under the following conditions: 5 min of incubation at 95°C, 45 s of denaturation at 94°C, 45 s of annealing at the primer-specific temperature and 45 s of extension at 72°C followed by 10 min final extension step at 72°C. After electrophoresis, the PCR products were visualized using ethidium bromide staining.

2.3 Preparation of CM from hDPSCs and hBM-MSCs

Human DPSCs and hBM-MSCs at passage 4-5 were used for collection of CM. When the culture reached ~70% confluence, cultures were washed thrice with PBS and replaced with serum free KO-DMEM. After 24 hours, CM was collected, filtered through 0.2 µM sterile filter, (Millipore, Massachusetts, United States) centrifuged at 250 x g for 10 minutes, aliquoted under sterile conditions and stored at -80°C until further use (4,5).

2.4 Hippocampal Cell (H3) Culture

Materials

- Hippocampal cell line media
- 70% ethanol
- Plastic ware - culture dishes and centrifuge Tubes
- CO₂ Incubator - SalvisLab, Switzerland
- Light Microscope - Nikon Eclipse TE 200-s, Chiyoda-Ku, Japan
- Biosafety Cabinet Level 2 - Thermo Scientific, Waltham, Massachusetts, United States.

Hippocampal Cell Line Media Composition

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM High Glucose</td>
<td>Make up to 50 mL</td>
</tr>
<tr>
<td>10% FBS</td>
<td>5 mL</td>
</tr>
<tr>
<td>200 mM L-Glutamine</td>
<td>500 µL</td>
</tr>
<tr>
<td>100 µg/ml Penicillin-Streptomycin</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Methodology

The hippocampal cell line (H3) used in the present study was a generous gift from Prof. Mitradas Panikar, National Centre for Biological Sciences, Bangalore, India. The cell line was generated from postnatal day 1 Hippocampus of H2kb-ts A58 transgenic mice (6). The
hippocampal cells were maintained in Dulbecco’s modified eagle medium-high glucose (DMEM-HG) (Invitrogen; Carlsbad, California, USA) supplemented with 10% (v/v) FBS (Hi-media Laboratories; L.B.S Marg, Mumbai, India), 100 µg/ml penicillin, 100 µg/ml streptomycin (Invitrogen; Carlsbad, California, USA) and 2 mM L-Glutamine (Invitrogen; Carlsbad, California, USA) and differentiated using 1% FBS containing media (DMEM-HG, 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine) for 24 hours. The cells were maintained in a humid atmosphere at 37°C and 5% CO2.

2.5 Characterization of Hippocampal Cell Line

2.5.1 Immunocytochemistry

Materials

- 4% PFA
- Permeabilization buffer - 0.25% Triton-X 100 in PBS.
- Blocking buffer - 1% bovine serum albumin (BSA) + 0.1% Tween 20 in PBS.
- 1X PBS
- Primary Antibody Diluent - 1% BSA + 0.1% Tween 20 in PBS.
- Fluorescent Microscope - Nikon Eclipse 80i, Chiyoda-Ku, Japan.
- DABCO: 0.23g DABCO
  - 200 µl 1M Tris Hcl (pH 8)
  - 800 µl Distilled water
  - 9 ml Glycerol

Methodology

When the hippocampal cells attained 70 - 80% confluence, they were differentiated in 1% serum containing media. The differentiated cells were characterized for the expression of neural markers like β-III tubulin, nestin, neurofilament-M and glial markers like glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (IBA-1) and Rip. The characterization was done using immunofluorescence technique to localize the expression of the above-mentioned markers in the hippocampal cell line.

The hippocampal cells grown on the coverslips until they reached a confluence of 70% and fixed with 4% PFA (Sigma-Aldrich, St. Louis, Missouri, US) for 30 minutes at room temperature. Following that cells were washed with PBS for 3 times, 5 minutes each and the cells were incubated for 20-30 minutes with PBS containing 0.25% Triton-X (Sigma-Aldrich, St. Louis, Missouri, US). After the incubation, the cells were washed in PBS thrice for 5 minutes each and the cells were incubated in blocking buffer (1% BSA/10% goat serum in 0.1% Tween 20) for 45 mins at room temperature. Primary antibodies (Refer Table 1) at a
dilution of 1:200 in antibody diluent (1% BSA/10% goat serum in 0.1% Tween 20) were added to the cells and incubated for 24 hours at 4°C on rocker. The cells were then washed with PBS for 5 minutes each. Fluorophore conjugated secondary antibodies specific to primary antibody (Refer Table 1) at the dilution of 1:1000 in PBS were added to the cells and incubated for 2 hours at room temperature. The cells were then washed with PBS for 5 minutes each. Further, cells were incubated with 4’,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, California, USA) (1:10000 dilution) for 5 minutes and were washed with PBS once. The coverslips were then carefully removed and mounted on the slide using DABCO (Sigma-Aldrich, St. Louis, Missouri, US) and sealed with nail polish and observed under a fluorescent microscope.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primary Antibody</th>
<th>Blocking used</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-III Tubulin</td>
<td>Mouse monoclonal (MA1-110; Invitrogen,</td>
<td>10% Goat serum (ThermoFisher</td>
<td>Goat anti-mouse TR (SC-2781; Santa Cruz,</td>
</tr>
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<td>Carlsbad, California, USA)</td>
<td>Scientific, MA, USA)</td>
<td>Texaz, USA)</td>
</tr>
<tr>
<td>Nestin</td>
<td>Mouse monoclonal (MA1-110; Invitrogen,</td>
<td>10% Goat serum (ThermoFisher</td>
<td>Goat anti-mouse TR (SC-2781; Santa Cruz,</td>
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<tr>
<td></td>
<td>Carlsbad, California, USA)</td>
<td>Scientific, MA, USA)</td>
<td>Texaz, USA)</td>
</tr>
<tr>
<td>Neurofilament-M</td>
<td>Mouse Polyclonal (13-0500; Invitrogen,</td>
<td>10% Goat serum (ThermoFisher</td>
<td>Goat anti-mouse TR (SC-2781; Santa Cruz,</td>
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<td></td>
<td>Carlsbad, California, USA)</td>
<td>Scientific, MA, USA)</td>
<td>Texaz, USA)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Goat polyclonal (SC-6170; Santa Cruz,</td>
<td>1% BSA (Sigma-Aldrich, St.</td>
<td>Donkey anti-goat TR (SC-2783; Santa Cruz,</td>
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<td>Louis, Missouri, US)</td>
<td>Texaz, USA)</td>
</tr>
<tr>
<td>IBA-1</td>
<td>Goat polyclonal (SC-28530; Santa Cruz,</td>
<td>1% BSA (Sigma-Aldrich, St.</td>
<td>Donkey anti-goat TR (SC-2783; Santa Cruz,</td>
</tr>
<tr>
<td></td>
<td>Texaz, USA)</td>
<td>Louis, Missouri, US)</td>
<td>Texaz, USA)</td>
</tr>
<tr>
<td>Rip</td>
<td>Mouse Monoclonal (AB 531769; DSHB;</td>
<td>10% Goat serum (ThermoFisher</td>
<td>Goat anti-mouse TR (SC-2781; Santa Cruz,</td>
</tr>
<tr>
<td></td>
<td>Iowa city, Iowa)</td>
<td>Scientific, MA, USA)</td>
<td>Texaz, USA)</td>
</tr>
</tbody>
</table>

Table 1: Details of the Primary antibody, Blocking agent used and Secondary Antibody
2.5.2 Expression of Glutamate Receptor Subunits in Hippocampal Cell Line by RT-PCR

The expression of glutamate receptors such as Grin2a, Grin2b, Grin2c, Grin2d [corresponds to NMDA 2A-D subunits], Gria1, Gria2, Gria3, Gria4 [corresponds to AMPA1-4 subunits], Grik1, Grik2 [corresponding to kainate receptor sub-units KAR1 and KAR2] in hippocampal cell line were assessed by conventional RT-PCR. GAPDH was used as a housekeeping gene. Total RNA was isolated from hippocampal cells using RNAiso Plus reagent (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan). The amount of RNA was measured by Nano Drop 1000 (Thermo Scientific; Waltham, MA, USA). Single stranded cDNA was synthesized using a Verso cDNA synthesis kit (Thermo Scientific; Waltham, MA, USA). For conventional RT-PCR the resultant cDNA was amplified with Emerald AmpGT PCR Master Mix (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan) for 35 cycles under the following conditions: 5 minutes of incubation at 95°C, 45 s of denaturation at 94°C, 45 s of annealing at the primer-specific temperature and 45 s of extension at 72°C followed by 10 minutes final extension step at 72°C. After electrophoresis, the PCR products were visualized using ethidium bromide staining.

2.6 Dose Standardization of Kainic Acid

Kainic acid (Enzo Life Sciences; East Farmingdale, New York) was used as an excitotoxin in this study. Dose standardization was carried out by treating the hippocampal cells with KA at different concentrations. The percentage of hippocampal cell death (excitotoxicity) was assessed with KA at a concentration ranging from 100 µM-500 µM. Hippocampal cells were exposed to 100 µM-500 µM for 6 hours and the cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, 10 µl of MTT was added to each well and incubated at 37°C for 4 hours following which MTT solution was removed and the insoluble formazan crystals was dissolved in 100 µl of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a micro plate reader (Perkin Elmer multimode plate reader, St. Waltham, Massachusetts).

2.7 In Vitro Excitotoxicity Model of Neurodegeneration

Hippocampal cells were exposed to KA (300 µM) for 6 hours and then the KA containing culture medium was removed and replaced with fresh culture medium and cultured for 24 hours. Post 24 hours the cell viability, mode of cell death and expression of neuronal survival factors were assessed. The experimental design for an in vitro neurodegeneration is illustrated in the following diagram.
2.8 Hippocampal-Mesenchymal Stem Cell Co-Culture Approach to Assess Neuroprotection

In order to assess the neuroprotective potential of hDPSCs/BM-MSCs, hippocampal cells were pre-treated with 300 \( \mu \)M KA for 6 hours and then the KA containing culture medium was replaced with fresh hippocampal cell culture medium and directly co-cultured with mitomycin-C treated (10 \( \mu \)g/ml for 2 hours) hDPSCs/hBM-MSCs at a ratio of 1:1 for 18 hours and the neuroprotective effect and anti-apoptotic potential of hDPSCs/hBM-MSCs were assessed by MTT assay and caspase 3/7 activity assay respectively, the gene expression of various neuronal survival factors and the downstream activation of cell survival pathways such as PI3K pathway were assessed.

2.9 Assessment of the Neuroprotective Potential of CM Derived from hDPSCs/hBM-MSCs

To ascertain whether the paracrine factors released by hDPSCs/hBM-MSCs play an important role in protecting neurons against excitotoxicity, hippocampal cells were pre-treated with 300 \( \mu \)M of KA for 6 hours and then the KA containing culture medium was replaced with hDPSC-CM/hBM-MSC-CM and maintained for 18 hours. Following that, the neuroprotective effect and anti-apoptotic properties of CM derived from hDPSC/hBM-MSCs was assessed by MTT assay and caspase 3/7 activity assay respectively, the gene expression of various neuronal survival factors and the downstream activation of cell survival pathways such as PI3K pathway were assessed.
survival factors and the downstream activation of cell survival pathways such as PI3K pathway were assessed.

2.10 Cell Viability Assay

The hippocampal cell viability was assessed by MTT assay following various treatments. Briefly, hippocampal cells were plated in 96 well plates at a density of 6000 cells per well and incubated for 24 hours and then differentiated with 1% serum containing media followed by different treatment regimens. After the desired time point as mentioned in the section (2.7, 2.8 and 2.9), 10 µl of MTT (5 mg/ml) (Hi-Media, L.B.S Marg, Mumbai, India) was added to each well and incubated at 37°C for 4 hours, following that MTT solution was removed and the insoluble formazan crystals was dissolved in 100 µl of DMSO (Hi-Media, L.B.S Marg, Mumbai, India). For the spectrophotometric quantification, the optical density was read at 570 nm on an ELISA plate reader (Perkin Elmer multimode plate reader, St. Waltham, Massachusetts).

2.11 Caspase 3/7 Activity Assay

The caspase 3/7 activity was assessed using Caspase 3/7 Glo kit (Promega, Promega Corporation, Cat no: G8091, WI, USA). Towards this, hippocampal cells were plated in a 96 well plate at a density of $10^4$- $10^6$ cells/well. The Caspase- Glo 3/7 buffer and lyophilized Caspase-Glo 3/7 substrate were equilibrated to room temperature before use. The contents of the Caspase-Glo 3/7 buffer was transferred into the amber bottle containing Caspase-Glo 3/7 substrate. The contents were mixed by swirling until the substrate is thoroughly dissolved to form the Caspase-Glo 3/7 reagent. Before starting the assay, the Caspase-Glo 3/7 reagent was prepared and was allowed to equilibrate to room temperature. The 96-well plates containing cells was removed from the incubator and 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, negative control cells or treated cells in culture medium. The plate was covered with a
plate sealer or a lid. The contents of the wells was 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 Assessment of the Effect of hDPSCs/hBM-MSCs and its CM on Host’s Endogenous
Neuronal Survival Factor Expression

Neuronal pro-survival factors like brain derived neurotropic factor plays a significant role in
hippocampal neuronal survival and neuroprotection. We were interested in understanding the
effects of hDPSCs/hBM-MSCs and its CM treatments on the host’s endogenous neuronal pro-
survival factor expressions. Total RNA was isolated from hippocampal cells after the treatment
regimens using RNAiso Plus reagent (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan). The
amount of RNA was measured by Nano Drop 1000 (Thermo Scientific; Waltham, MA, USA).
Single stranded cDNA was synthesised using a Verso cDNA synthesis kit (Thermo Scientific;
Waltham, MA, USA). The expression of anti-apoptotic factor like Bcl-2 was measured by
quantitative Real-Time (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.
GAPDH was used for normalization of the data. We also assessed the endogenous BDNF
protein concentration using ELISA (mouse BDNF ELISA kit; GX-9030E1; Qiagen, Hilden,
Germany) which was performed as per the manufactures protocol. Furthermore, we carried out
experiments by neutralizing the BDNF in the treatment groups by adding anti-BDNF antibody
(1:100; ab203573; Abcam; Cambridge; UK) and assessed the alteration in neuroprotection by
MTT assay. Moreover, to reveal the downstream cell survival pathway through which
hDPSCs/hBM-MSCs and its CM protects neuron against KA mediated excitotoxicity, a PI3K
inhibitor (LY-294002 hydrochloride; Santa Cruz Biotechnology, USA) was added to the CM
at a concentration of 10 µM (7) and the cell viability was assessed by MTT assay.
To investigate whether hDPSCs/hBM-MSCs and their respective CM stimulates host’s
endogenous neuronal survival factors, hippocampal cells from different treatment groups were
analysed for an array of neuronal survival factors such as glial derived neurotrophic factor
(GDNF), ciliary neurotropic factor (CNTF), vascular endothelial growth factor (VEGF),
platelet derived growth factor (PDGF-A and PDGF-B), insulin-like growth factor-1 (IGF-1),
nerve growth factor (NGF), neurotrophin 3 (NT-3), basic fibroblast growth factor (bFGF) and erythropoietin (EPO) using qRT-PCR method. Total RNA was extracted from different treatment groups with RNAsiso plus reagent (Takara Bio Inc; Nojihigashi, Kasatsu, Shiga, Japan). The amount of RNA was measured by Nano Drop 1000 (Thermo Scientific; Waltham, MA, USA). Using total RNA, Single stranded cDNA was synthesized using a Verso cDNA synthesis kit (Thermo Scientific; Waltham, MA, USA) and Real-Time (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. GAPDH was used for normalization of the data.

2.13 Assessment of the Protein Expression of Pro-Cell Survival Factor Following hDPSCs/hBM-MSCs and Its CM Treatment

To estimate the protein expression of phosphorylated PI3Kinase (p-PI3K), a pro-cell survival factor following KA exposure, hDPSCs/hBM-MSCs co-culture and their CM treatment, hippocampal cells were scraped by adding a cold lysis buffer (0.1M Sodium Molybdate, 50mM Sodium Orthovanadate, 0.5M Sodium fluoride, 5M Sodium Chloride, 0.1% Triton X 100 and 1X Protease inhibitor). The cell 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 sodium dodecyl sulphate (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). Further, the proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and the blots were blocked with 5% BSA in 1X TBST (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 rabbit polyclonal anti-phosp-PI3 kinase (1:500; Santa Cruz Biotechnology, Inc, Texas, USA) at 4°C. Following several washes, the blots were incubated with HRP-conjugate 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).
2.14 Statistical Analysis

A minimum of three independent experiments were carried out with six replicates for MTT assay. For caspase-3/7 activity assay and western blotting three independent experiments with triplicates were carried out. All data are presented as mean ± SEM. For statistical comparisons between different treatment groups, one-way analysis of variance followed by Newman-Keuls multiple comparison test was used and p<0.05 was considered as significant.
3. RESULTS

3.1 Human Dental Pulp Stem Cells and Human Bone Marrow Mesenchymal Stem Cell Culture

Commercially available research grade hDPSCs and hBM-MSCs from healthy donors were obtained from ANSA, Bangalore. Human DPSCs and human BM-MSCs were cultured in KO-DMEM supplemented with 10% (v/v) FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-Glutamine and maintained in a humid atmosphere at 37°C and 5% CO₂. After every 48 hours, spent media was removed and replaced with fresh growth media. Cell cultures on attaining a confluency of 70-80% were sub-cultured and the hDPSCs/hBM-MSCs from passage number 4-5 were used to explore the neuroprotective potential. The morphology of the hDPSCs/hBM-MSCs are as shown in the Fig. 1A and 1B respectively.

![Figure 1: Bright field images of hDPSCs (A) and hBM-MSCs (B) at passage 4. Scale bar: 100 µm](image)

3.2 Characterization of hDPSCs and hBM-MSCs

3.2.1 Expression of Cell-Surface Markers by Reverse-Transcriptase Polymerase Chain Reaction

The expression of MSC cell surface marker genes were confirmed by RT-PCR. The expression patterns showed that hDPSCs/hBM-MSCs were positive for mesenchymal marker (CD 90, CD 105 and CD 44) and were negative for hematopoietic markers (CD 45 and CD 34) (Fig. 2A). Further expression of these markers were confirmed by flow cytometry.
3.2.2 Immunophenotypic Characterization

Flow cytometry analysis was carried out for a battery of MSC cell-surface markers. Figure 2B shows the immunophenotypic characterization of hDPSCs and hBM-MSCs respectively. Human DPSCs and hBM-MSCs were CD 90+, CD 105+ and CD 44+ which are common MSC markers. On the other hand, markers specific for hematopoietic cells, including CD 34, CD 45 and HLA-DR were not expressed.

3.2.3 In vitro Multi-lineage Differentiation of hDPSCs/hBM-MSCs

Differentiation into osteoblasts, adipocytes and chondrocytes are considered as an important characteristic feature of MSCs. Differentiation into adipocytes, chondrocytes and osteoblasts were verified by specific staining (Fig. 2C). Adipocytes were confirmed by the formation of fat droplets, which were detected by Oil Red-O staining (Fig. 2C). Furthermore, hDPSCs/hBM-MSCs differentiated into chondrocytes which were evidenced by extracellular matrix proteoglycan stained blue in colour with Alcian Blue stain (Fig. 2C). Also, hDPSCs/hBM-MSCs were able to form osteoblasts, which was confirmed by Vonkossa staining (Fig. 2C). These results confirmed that hDPSCs/hBM-MSCs are capable of differentiating into cells of multiple lineages.

3.2.4 Expression of Neural Crest Related Markers by hDPSCs/hBM-MSCs

We were interested to analyse the differences in the mRNA expressions of neural crest markers like p75, Pax 6, Pax 3, Sox 9, Sox 2 and Twist between hDPSCs and hBM-MSCs. Our results showed that hDPSCs expressed neural crest markers like p75, Pax 6, Pax 3, Sox 9, Sox 2 and Twist whereas hBM-MSCs expressed only Sox-9 and Twist highlighting the molecular differences between these two adult MSCs (Fig. 2D).

Figure 2A: Semi-quantitative PCR image showing cell surface gene expression of hDPSCs and hBM-MSCs. Note that hDPSCs and hBM-MSCs were positive for CD 90, CD 105 and CD 44 and negative for CD 34 and CD 45.
Figure 2B: Flow Cytometry analysis of cell-surface antigen expressions of hDPSCs and hBM-MSCs that were positive for CD 90, CD 105 and CD 44, which are markers for MSCs. In contrast, these cells were negative for markers of hematopoietic lineage, including CD 34, CD 45 and HLA-DR.

Figure 2C: Bright field images showing Adipogenic, Chondrogenic and Osteogenic Differentiation of hDPSCs and hBM-MSCs. Scale bar: 100 µm
Figure 2D: Semi-quantitative PCR gel image showing neural crest gene expression in hDPSCs and hBM-MSCs.

3.3 Hippocampal (H3) Cell Culture

The hippocampal cell line used in this study was generated from H-2Kb-ts A58 transgenic mice postnatal day 1 hippocampus. The hippocampal cells were cultured in DMEM-HG medium supplemented with 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-Glutamine and maintained at 37°C and 5% CO₂. Upon 70-80% confluence, hippocampal cells were grown in 1% serum containing media for differentiation. The morphology of the hippocampal cells grown in 1% serum is shown in the Fig. 3.

Figure 3: Bright field image of Hippocampal cells. Scale bar: 100 µm
3.4 Characterization of Hippocampal Cell Line

3.4.1 Hippocampal Cells Express Neuronal Markers

Upon differentiation with 1% serum, hippocampal cells exhibited clear neuronal morphology (Fig. 3A) and expressed neuronal markers β-III tubulin, Nestin and Neurofilament (Fig. 3a, 3b, 3c). Nonetheless, hippocampal cells did not express glial markers like GFAP, IBA-1 and Rip (Fig. 3d, 3e, 3f).

3.4.2 Glutamate Receptors Subunits Expression in Hippocampal cells by RT-PCR

Hippocampal cells upon differentiation with 1% serum demonstrated significantly higher levels of mRNA expressions for Grin2a (p<0.001) and Grin2b (p<0.001) [corresponds to NMDA 2A-B subunits], Gria1 (p<0.001), Gria3 (p<0.001), Gria4 (p<0.001) [corresponds to AMPA1, 3 and 4 subunits], Grik1, (p<0.001) Grik2 (p<0.001) [corresponding to kainic acid receptor sub-units KAR1 and KAR2] as compared to hippocampal cells grown in 10% serum. Whereas, mRNA expression of Grin2c (p<0.01) and Grin2d (p<0.001) which corresponds to NMDA-2C and NMDA-2D receptor subunits were downregulated in 1% serum treated hippocampal cells as compared to 10% serum treated hippocampal cells. Of note, the mRNA expression of Gria2 (corresponds to the AMPA-2 subunit) was not altered between 1% or 10% serum treated hippocampal cells.
Figure 3A: Immunofluorescent images showing hippocampal cells that are positive for β-III tubulin (a), Nestin (b), Neurofilament (NF-M) (c) and negative for GFAP (d), IBA-1 (e) and RIP (f). Scale bar: 100 µm
Figure 3B: Semi-quantitative PCR image showing the expressions of glutamate receptor subunits in hippocampal cells. Note a significant upregulation of mRNA expressions of glutamate ionotropic receptors in hippocampal neurons following 1% serum induced differentiation. * comparison between 10% serum vs 1% serum. **p<0.01; ***p<0.001.

3.5 Dosage Dependent Cytotoxic Effects of Kainic Acid

Hippocampal cells when exposed to toxic doses of KA (100 µM- 500 µM) for 6 hours degenerated in a dose dependent manner. We observed that 300 µM of KA induced ~40% cell death and therefore we chose this dose of KA for the rest of the study (Fig. 4).

Figure 4: Cell viability Assay: Bar diagram representing the percentage of viable hippocampal cells after KA exposure at different concentrations for 6 hours. Kainic acid (300 µM) exposure to hippocampal cells reduced the percentage of viable cells to ~40%. *comparison between NC vs treatment. *p<0.05; ***p<0.001.
3.6 Direct Co-Culture of hDPSCs/hBM-MSCs Protects Hippocampal Cells Against Excitotoxicity

To investigate the neuroprotective potential of hDPSCs/hBM-MSCs against KA induced excitotoxicity, MTT assay was carried out to assess the cell viability. Following 6 hours of KA treatment to hippocampal cells, there was a significant reduction in cell viability (40%; p<0.001; KA; Fig. 5). On the other hand, when KA treated hippocampal cells were directly co-cultured with mitomycin C treated hDPSCs/hBM-MSCs for 18 hours, there was a significant neuroprotection observed (99.15%; p<0.001; KA+DPSC; Fig. 5 and 97.47%; p<0.001; KA+BM-MSC; Fig. 5) as compared to KA group.

3.7 Human DPSCs/hBM-MSCs Derived CM Protects Hippocampal Cells Against Excitotoxicity

We further investigated whether direct cell (neuron)-to-cell (mesenchymal) contact is essential for the observed neuroprotection or it is due to the paracrine effects of MSCs. Towards this, the serum-free CM was obtained from hDPSCs/hBM-MSCs cultures and the neuroprotective efficacy of CM was elucidated in KA treated hippocampal cells. Like with cell-to-cell contact approach we found a profound neuroprotection when hDPSCs/hBM- MSCs CM per se was applied to KA treated hippocampal cells. As compared to KA group, KA+DPSC-CM group demonstrated significant neuroprotection with 107.26% (p<0.001; Fig. 5) viable cells and 102.48% viable cells were observed in KA+BM-MSC-CM group (p<0.001; Fig. 5). Thus suggesting that CM per se could protect the neurons against excitotoxicity in an in vitro condition.
Figure 5: Cell viability Assay: Bar diagram representing the percentage of viable cells in different treatment groups. Kainic acid exposure to hippocampal cells for 6 hours reduced the percentage of viable cells to ~60%. In contrast, co-culture with hDPSCs/hBM-MSCs significantly increased the cell viability to ~90% as compared to KA treatment group. Conditioned medium from hDPSCs/hBM-MSCs also significantly increased the hippocampal cell viability to ~90% as compared to KA treatment group. *comparison between NC vs treatment. +comparison between KA vs treatment. *p<0.05; **p<0.01; ***p<0.001; +++p<0.001.
3.8 Anti-Apoptotic Potential of hDPSCs/hBM-MSCs and Their CM

Next, we determined the anti-apoptotic potential of hDPSCs/hBM-MSCs and it’s CM in different groups by measuring the pro-apoptotic caspase 3/7 enzyme activity. Sham control groups treated with hDPSCs/hBM-MSCs and hDPSC-CM did not increase the caspase 3/7 activity significantly. However, treatment with CM derived from hBM-MSCs to sham control group (SH BM-MSC-CM; Fig. 6) resulted in increased caspase 3/7 activity (p<0.05; Fig. 6) as compared to NC group. Post KA treatment, caspase 3/7 activity was significantly increased (p<0.001; Fig. 6) in the KA group as compared to NC group. On the other hand, when KA treated hippocampal cells were co-cultured with hDPSCs/hBM-MSCs there was a significant decrease in caspase 3/7 activity (p<0.001; Fig. 6) compared to the KA group. There were no statistical differences in anti-apoptotic effects between hDPSCs and hBM-MSCs in neuronal~MSC co-culture approaches. The CM derived from hDPSCs/hBM-MSCs when treated to hippocampal cells after KA exposure significantly (p<0.001; Fig. 6) reduced the caspase 3/7 activity. When compared to co-culture and CM approach, CM from hDPSCs and hBM-MSCs showed significant anti-apoptotic effect than their respective co-culture groups (p<0.001; Fig. 6). Thus, suggesting that the CM derived from hDPSCs/hBM-MSCs possess better anti-apoptotic potential than co-culture approach.

![Figure 6: Caspase 3/7 Activity Assay](image)

**Figure 6: Caspase 3/7 Activity Assay:** Bar graph representing the luminescence (RLU) in different treatment groups. *comparison between NC vs treatment. +comparison between KA vs treatment. #comparison between KA+DPSC vs KA+DPSC-CM. $comparison between KA+BM-MSC vs KA+BM-MSC-CM. *p<0.05; ***p<0.001; +++p<0.001; ###p<0.001; $$$p<0.001. RLU: Relative Light Units**
3.9 Human DPSCs/hBM-MSCs and Its CM Treatment Increase Host’s Endogenous BDNF Expression

As brain derived neurotrophic factor is one of the essential growth factor for hippocampal neuronal survival (8,9), we estimated the mRNA and protein concentration of host’s endogenous BDNF by qRT-PCR and ELISA method. In KA treated group, there was a significant decrease in the mRNA (p<0.001; Fig. 7A) as well as protein concentration of BDNF (p<0.001; Fig. 7B) as compared to NC group. Interestingly, MSCs based therapeutic approaches, i.e. hippocampal-MSC co-culture and CM treatment resulted in significant (Fig. 7A and 7B) up-regulation of the host’s endogenous BDNF mRNA and protein level. Given these observations, we next determined to evaluate whether the increased BDNF level post-MSCs based treatment could be the cause for the neuroprotection observed. Towards this, BDNF neutralizing antibody was added to the experimental settings and the alteration in neuroprotection was evaluated by MTT assay. Our results revealed that neutralizing the BDNF in sham control groups and KA group significantly reduced the cell viability as compared to the NC group (p<0.001; Fig. 8). Similarly, neutralizing BDNF in the KA+treatment groups only partially abolished the neuroprotective potential of MSCs and their CM (Fig. 8), suggesting that there may be other trophic factors that might have acted in a synergistic manner to protect the neurons against excitotoxicity (Fig. 9 and 10).
Figure 7: (A) qPCR analysis of BDNF mRNA expression in different treatment groups expressed as fold change. *comparison between NC vs treatment. +comparison between KA vs treatment. $comparison between KA+DPSC-CM vs KA+BM-MSC-CM. #comparison between KA+DPSC vs KA+BM-MSC. ° comparison between KA+DPSC vs KA+DPSC-CM. **p<0.01; ***p<0.001; +++p<0.001; $$$p<0.001; ####p<0.001; ooo p<0.001.

Figure 7: (B) Secretion of BDNF (pg/ml) assessed by ELISA in different treatment groups. *comparison between NC vs treatment. +comparison between KA vs treatment. #comparison between KA+DPSC-CM vs KA+BM-MSC-CM. *comparison between KA+DPSC-CM vs KA+BM-MSC-CM. **p<0.01; ***p<0.001; +++p<0.001; #p<0.05.

Figure 8: Cell viability Assay: Bar diagram representing percentage of viable cells in different treatment groups with BDNF neutralizing antibody. *comparison between NC vs treatment. +comparison between KA vs treatment. ***p<0.001; +++p<0.001.
3.10 Human DPSCs/hBM-MSCs and Its CM Upregulates the Neuronal Survival Factors in Hippocampal Cells in an In Vitro Condition

In order to understand the effect of hDPSCs/hBM-MSCs co-culture and their respective CM treatment on neurotropic factors expression in hippocampal cells after KA exposure, qRT-PCR analysis was carried out for various nerve growth factors like GDNF, CNTF, VEGF, PDGF-A, PDGF-B, IGF-1, NGF, NT3, bFGF and EPO expression were analysed. Our results revealed that co-culture of hDPSCs/hBM-MSCs and their respective CM treatment showed a significant change in the mRNA expression of the growth factors in the hippocampal cells as explained below.

3.10.1 Kainic Acid Exposure to Hippocampal cells Reduced the mRNA Expression of Growth Factors

Exposure of KA to hippocampal cells induced a significant decrease in crucial growth factors like GDNF (p<0.001; Fig. 9A; KA), CNTF (p<0.001; Fig. 9B; KA), VEGF (p<0.001; Fig. 9C; KA), PDGF-A (p<0.001; Fig. 9D; KA), NGF (p<0.001; Fig. 9G; KA), NT3 (p<0.001; Fig. 9I; KA) and EPO (p<0.001; Fig. 9J; KA) as compared to NC group. No significant change in expression of PDGF-B (Fig. 9E; KA), IGF-1 (Fig. 9F; KA) and bFGF (Fig. 9H; KA) was observed following KA exposure as compared to NC group.

3.10.2 Co-Culture of hDPSCs/hBM-MSCs Alters the Growth Factor mRNA Expression in KA Treated Hippocampal Cells

Co-culturing hippocampal cells with hDPSCs after 6 hours of KA exposure significantly increased the mRNA expression of growth factors like GDNF (p<0.001; Fig. 9A; KA+DPSC), CNTF (p<0.001; Fig. 9B; KA+DPSC), VEGF (p<0.001; Fig. 9C; KA+DPSC), PDGF-A (p<0.001; Fig. 9D; KA+DPSC), PDGF-B (p<0.001; Fig. 9E; KA+DPSC), IGF-1 (p<0.001; Fig.9F; KA+DPSC), NGF (p<0.001; Fig. 9G; KA+DPSC) and EPO (p<0.001; Fig. 9J; KA+DPSC) as compared to KA group. Whereas, the co-culture approach significantly reduced the mRNA expression of NT3 (p<0.001; Fig. 9I; KA+DPSC) as compared to the KA group. No significant change was observed in the mRNA expression of bFGF (Fig. 9H; KA+DPSC) as compared to the KA group.

Likewise, we wanted to check the growth factor mRNA expression after co-culturing with hBM-MSCs post KA exposure for 6 hours. Our results revealed that there was a significant increase in the mRNA expression of growth factors like GDNF (p<0.001; Fig. 9A; KA+BM-
MSC), CNTF (p<0.001; Fig. 9B; KA+BM-MSC), VEGF (p<0.001; Fig. 9C; KA+BM-MSC), PDGF-A (p<0.001; Fig. 9D; KA+BM-MSC), IGF-1 (p<0.001; Fig. 9F; KA+BM-MSC), NGF (p<0.05; Fig. 9G; KA+BM-MSC) and bFGF (p<0.001; Fig. 9H; KA+BM-MSC) as compared to KA group. In contrast, co-culturing with hBM-MSCs decreased the mRNA expression of growth factors like PDGF-B (p<0.001; Fig. 9E; KA+BM-MSC) and NT3 (p<0.001; Fig. 9I; KA+BM-MSC) as compared to KA group. No significant change was observed in the mRNA expression of EPO (Fig. 9J; KA+BM-MSC) as compared to the KA group.

3.10.3 Conditioned Medium Derived from hDPSCs/hBM-MSCs Alters the Growth Factor mRNA expression in KA Treated Hippocampal Cells

Treating the hippocampal cells with hDPSC-CM after 6 hours of KA exposure significantly increased the mRNA expression of growth factors like GDNF (p<0.001; Fig. 9A; KA+DPSC-CM), CNTF (p<0.001; Fig. 9B; KA+DPSC-CM), VEGF (p<0.001; Fig. 9C; KA+DPSC-CM), PDGF-A (p<0.001; Fig. 9D; KA+DPSC-CM), PDGF-B (p<0.001; Fig. 9E; KA+DPSC-CM), IGF-1 (p<0.001; Fig. 9F; KA+DPSC-CM), NGF (p<0.001; Fig. 9G; KA+DPSC-CM), bFGF (p<0.001; Fig. 9H; KA+DPSC-CM) and EPO (p<0.001; Fig. 9J; KA+DPSC-CM) as compared to KA group. In contrast, treatment with hDPSC-CM significantly reduced the mRNA expression of NT3 (p<0.001; Fig. 9I; KA+DPSC-CM) as compared to KA group.

Similarly, when hippocampal cells were treated with hBM-MSC-CM after 6 hours of KA exposure there was a significant increase in the mRNA expression of growth factors like GDNF (p<0.001; Fig. 9A; KA+BM-MSC-CM), CNTF (p<0.001; Fig. 9B; KA+BM-MSC-CM), VEGF (p<0.001; Fig. 9C; KA+BM-MSC-CM), PDGF-A (p<0.05; Fig. 9D; KA+BM-MSC-CM), IGF-1 (p<0.001; Fig. 9F; KA+BM-MSC-CM) and NGF (p<0.001; Fig. 9G; KA+BM-MSC-CM) as compared to KA group. In contrast, there was significant reduction in the mRNA expression of bFGF (p<0.001; Fig. 9H; KA+BM-MSC-CM) and NT3 (p<0.001; Fig. 9I; KA+BM-MSC-CM). No significant change was observed in the mRNA expression of PDGF-B (Fig. 9E; KA+BM-MSC-CM) and EPO (p<0.001; Fig. 9J; KA+BM-MSC-CM) as compared to the KA group.
Figure 9: Bar diagram representing the expressions (fold change) of various nerve growth factors in different treatment groups. *comparison between NC vs treatment. +comparison between KA vs treatment. *p<0.05; **p<0.01; ***p<0.001; +++p<0.001.

Figure 10: Summary of the expression of neuronal survival factors in different groups

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- Indicates decreased expression of neuronal survival factors following KA treatment
- Indicates elevated expression of neuronal survival factors after treatment with hDPSCs/hBM-MSCs and their CM.
- Indicates decreased expression of neuronal survival factors after treatment with hDPSCs/hBM-MSCs and their CM.

* Comparison between NC versus treatment
+ Comparison between KA lesion versus treatment
3.11 Human DPSCs/hBM-MSCs and Its CM Activates PI3K Cell Survival Pathway

Nerve growth factors are known to activate cell survival pathways (10) and previous studies have highlighted the involvement of AKT/PI3K pathway in neuroprotection following cellular therapy (11,12). Therefore, we intend to study whether the neuroprotective effects of hDPSCs/hBM-MSCs and their respective CM is mediated through PI3K pathway. In order to address this, a PI3K inhibitor (PI3Ki) (LY-294002 hydrochloride; Santa Cruz Biotechnology, USA) at the concentration of 10 µM (7) was added to the experimental settings and the shift in the neuroprotective efficacy of hDPSCs/hBM-MSCs and its respective CM were assessed. As shown in Fig. 11A inhibiting PI3K significantly (p<0.001; Fig. 11A) reduced the cell viability in all the groups, suggesting its important role in neuronal survival. To substantiate this observation, we examined the p-PI3K in different treatment groups. Following KA treatment there was a significant reduction in the p-PI3K expression as compared to NC group (p<0.01; Fig. 11B and 11C). Co-culturing with hDPSCs/hBM-MSCs with KA treated hippocampal cells significantly increased the p-PI3K levels (p<0.001; Fig. 11B and 11C) and treatment with hDPSC-CM/hBM-MSC-CM significantly increased the p-PI3K expression as compared to KA group (p<0.001; Fig. 11B and 11C), suggesting that hDPSCs/hBM-MSCs and their CM treatment could possibly act through the PI3K pathway to protect neurons from KA induced excitotoxicity.
Figure 11: (A) Cell viability Assay: Bar diagram representing percentage of viable cells in different treatment groups with addition of PI3K inhibitor. (B) Representative western blot image showing protein expression of p-PI3K in different treatment groups. (C) Densitometric analysis of western blot for p-PI3K expression in different treatment groups expressed as fold change. *comparison between NC vs treatment. +comparison between KA vs treatment. **p<0.01; ***p<0.001; +++p<0.001.
4. DISCUSSION

This chapter focuses on comparing the neuroprotective and anti-apoptotic properties of neuron-MSC co-culture and neuron-MSC-CM treatment in an in vitro model of excitotoxicity. This chapter also compares the neuroprotective properties of neural crest derived DPSCs with commonly used BM-MSCs. Our results demonstrated that neuron-MSC co-culture and MSC-CM treatment significantly protected the hippocampal neurons against KA induced excitotoxicity through anti-apoptotic mechanisms.

Excitotoxicity is considered as one of the major causes for the loss of neurons in many neurodegenerative diseases like AD, TLE, Parkinson’s diseases and Amyotrophic lateral sclerosis (13). In objective 1, we used an in vitro model of hippocampal neurodegeneration by exposing hippocampal cells to a toxic dose of KA, a glutamate analogue. Conversely, hDPSCs/hBM-MSCs when co-cultured with hippocampal cells in a ratio of 1:1 rescued against excitotoxic cell death caused by KA. In line with our observation, reports have shown that co-culturing DPSCs with mesencephalic and hippocampal neurons significantly attenuated the neurotoxic effects of 6-OHDA (6-Hydroxydopamine) and amyloid beta peptide 1-42 induced toxicity (14). Similarly, MPP+/retonene treated mesencephalic neurons when co-cultured with DPSCs significantly diminished the neurotoxicity (15). Likewise, co-culturing DPSCs with the human neuroblastoma cell line, SH-SY5Y protect neurons against okasaic acid toxicity (16). Studies have shown that co-culturing mouse primary cortical neurons with BM-MSCs effectively protected the neurons against glutamate (NMDA) receptor induced excitotoxicity (17). As MSC-CM could play a bystander effect in protecting neurons against excitotoxicity, in the present study, we assessed the neuroprotective effect of the CM derived from hDPSCs/hBM-MSCs. Our results clearly demonstrated a significant neuroprotection with CM per se. In view of this, previous studies have shown that secretome derived from DPSCs when treated on amyloid beta (Aβ) peptide loaded human neuroblastoma cells reduced cytotoxicity through anti-apoptotic mechanism (18). Yet another study has demonstrated the neuritogenic potential of the secretome derived from DPSCs using human SH-SY5Y neuroblastoma cells (19). An interesting report by Mita et al. (2015) showed that the human exfoliated deciduous teeth (SHED) derived CM significantly protected the neurons, enhanced neuronal sprouting and decreased inflammation in a mouse model of Alzheimer disease (20). In cognate to CM derived from DPSCs, CM obtained from BM-MSCs also reduced staurosporine or amyloid beta peptide (Aβ) induced apoptosis in a concentration dependent manner in primary cortical neurons through activation of MAPK/Erk1/2 and Akt cell signalling pathways and by altering...
the NMDA receptor sub-unit expression (17,21). In line with this, Voulgari kokota et al. (2012) demonstrated that CM derived from BM-MSCs could modulate the neuronal excitability by altering the expression of NMDA receptor subunits (17).

Progressive neurodegeneration instigated by excessive activation of glutamate receptors that could lead to necrosis and apoptosis (22). Studies have shown that in in vitro conditions, excitotoxin KA induce cell death through apoptosis and necrosis (23). Recent evidence suggests that hDPSCs reduces apoptosis in okasaic acid mediated neuronal injury by re-establishing the changes in the chromatin morphology (16). Lin et al. (2015) demonstrated that co-culturing BM-MSCs with TNF-α primed PC-12 cells could significantly reduce apoptosis (24). Concurrent with previous reports, in the present study KA treatment to hippocampal neurons increased the activity of pro-apoptotic enzyme caspase-3/7. Of note, co-culturing hDPSCs/hBM-MSCs with KA treated hippocampal cells significantly reduced the caspase3/7 activity. Interestingly, CM derived from hDPSCs/hBM-MSCs could also significantly reduce the caspase 3/7 activity. The anti-apoptotic activity of DPSCs was recently reported wherein the CM derived from DPSCs could protect neurons against Aβ toxicity through anti-apoptotic mechanism (18). Likewise, studies have shown that CM derived from bone marrow stromal cells attenuates neuronal apoptosis induced by Aβ in a concentration dependent manner (25). Similarly, CM from human adipose-derived MSCs reduced apoptosis in cortical neurons pre-exposed to toxic doses of glutamate (26). In the current study, CM derived from hDPSCs/hBM-MSCs significantly diminished the caspase 3/7 activity signifying that the CM could be as effective as neurons-to-MSC co-culturing in reducing the caspase 3/7 activity.

The proposed mechanisms of neuroprotection following MSCs treatment could be differentiation of MSCs into neurons/glia (27,28), neuron-MSC fusion (29) and by secretome which could trigger the hosts’ endogenous cell survival system (30). In the current study, we have investigated the effects of hDPSCs/hBM-MSCs and their CM on host’s endogenous neurotropic factor expressions. Following KA treatment, the mRNA expression of neuronal survival factors like BDNF, GDNF, CNTF, VEGF, PDGF-A, NGF, NT3 and EPO in the hippocampal cells were significantly downregulated as compared to a normal control group. Interestingly, downregulation of neuronal survival factors following KA treatment was prevented when KA treated hippocampal cells were co-cultured with hDPSCs/hBM-MSCs or treated with CM derived from hDPSCs/hBM-MSCs.
Stimulation of cell survival pathways play a critical role in neuroprotection. Studies have shown BM-MSCs and its CM protected hippocampal neurons from Aβ-induced apoptosis by enhancing the Erk1/2 and Akt phosphorylation (25). Another study has shown that the human adipose-derived mesenchymal stem cells – conditioned media (AMSC-CM) protected PC-12 cells against glutamate toxicity through the PI3K/AKT pathway and MAPK/ErK pathways (11). In the present study, we investigated the activation of the PI3K cell survival pathway following neuron-MSC co-culture or MSC-CM treatment in an excitotoxicity condition. Treatment with KA decreased the expression of p-PI3K in hippocampal cells. Co-culture with hDPSCs/hBM-MSCs and their CM treatment post KA exposure increased the phosphorylation of PI3K. Thus protecting the neurons against KA induced excitotoxicity by activating the endogenous cell survival mechanisms.

In summary, we observed that the adult MSCs derived from two different tissue sources viz. dental pulp and bone marrow demonstrated comparable neuroprotection efficacy. Of note, the hDPSC-CM/hBM-MSC-CM treatment per se was sufficient enough to protect neurons from excitotoxicity suggesting the future possibilities of new therapeutic approaches using stem cell secretome to protect neurons in neurodegenerative diseases.
5. CONCLUSION

Stem cell-based therapy is emerging as a therapeutic strategy for treating many neurodegenerative diseases. As embryonic stem cells have numerous disadvantages, adult mesenchymal stem cells are a promising candidate for cell based therapeutic approaches. Bone marrow mesenchymal stem cells are one of the best-characterized MSCs that was initially considered as “master” MSCs for treating various diseases. However, due to its painful, invasive procedure that gives a fewer yield of MSCs along with the lower proliferation rate and differentiation capacity, there is a need to search for the alternate MSCs sources with easy isolation procedure as well as with comparable or even better neuroprotective potential than commonly used BM-MSCs. In the present study, we demonstrate that hDPSCs/hBM-MSCs and their CM could protect hippocampal cells against excitotoxicity through neurotrophic factors mediated PI3K cell survival pathway. Thus, our study illustrates the proof-of-principle on utilizing MSCs based “cell free” therapy for treating neurodegenerative diseases. Moreover, our results revealed that the anti-apoptotic potential of DPSCs derived CM was better than BM-MSCs derived CM. A study of this kind would help clinicians choose appropriate MSCs for deriving secretome for treating neurodegenerative diseases.