Therapeutic effect of bone marrow mesenchymal stem cells on cold stress induced changes in the hippocampus of rats

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Abstract
The present study aims to evaluate the effect of bone marrow mesenchymal stem cells on cold stress induced neuronal changes in hippocampal CA1 region of Wistar rats. Bone marrow mesenchymal stem cells were isolated from a 6-week-old Wistar rat. Bone marrow from adult femora and tibia was collected and mesenchymal stem cells were cultured in minimal essential medium containing 10% heat-inactivated fetal bovine serum and were sub-cultured. Passage 3 cells were analyzed by flow cytometry for positive expression of CD44 and CD90 and negative expression of CD45. Once CD44 and CD90 positive expression was achieved, the cells were cultured again to 90% confluence for later experiments. Twenty-four rats aged 8 weeks old were randomly and evenly divided into normal control, cold water swim stress (cold stress), cold stress + PBS (intravenous infusion), and cold stress + bone marrow mesenchymal stem cells (1×10^6; intravenous infusion) groups. The total period of study was 60 days which included 1 month stress period followed by 1 month treatment. Behavioral functional test was performed during the entire study period. After treatment, rats were sacrificed for histological studies. Treatment with bone marrow mesenchymal stem cells significantly increased the number of neuronal cells in hippocampal CA1 region. Adult bone marrow mesenchymal stem cells injected by intravenous administration show potential therapeutic effects in cognitive decline associated with stress-related lesions.

Key Words: *nerve regeneration; bone marrow; mesenchymal stem cells; hippocampus; cold stress; intravenous; cognition; neuronal cells; CA1 region; neural regeneration*

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Introduction
Stress may be defined as the reaction of an organism to different stimuli. These stimuli are collectively called as stressors (Selye, 1936). Stress is the feeling we have when under pressure, while stressors are the things we respond to in our environment. Stress would manifest itself as physiological, biochemical and behavioral changes. Different types of stresses are used in previous studies on experiments with rats. One such stress is compulsive swimming in cold water, which produces output of norepinephrine in specific parts of the brain structures and also causes alteration in blood cortisol level (Medina et al., 1983). Inescapable stress is associated with impairments in learning and memory manifested by deficits in maze escape behaviors. These deficits appear to be related to alterations in brain structures involved in memory, including the amygdala and hippocampus (Squire and Zola-Morgan, 1991). The hippocampus sends outputs which have been implicated in the production and modulation of locomotor activity, rotational movement, and stereotyped behavior induced by the indirect dopamine agonists, methamphetamine and amphetamine (Pijnenburg et al., 1973). Repeated exposure to inescapable stress is associated with a neither eventual depletion of norepinephrine in the hypothalamus and hippocampus (Anisman and Zacharko, 1986; Weiss et al., 1981). The current drug therapies on stress management have dilemma including poor efficacy, withdrawal symptoms and side effects (Bachurin, 2003). Recently, much attention has been focused on the relevance of hippocampal neurogenesis to the pathophysiology and treatment of memory loss. Adult neural tissues have limited sources of stem cells, which makes neural regeneration in them less promising. Stem cell transplantation seems to be a promising strategy for treatment of several central nervous system degenerative diseases like Alzheimer's disease, Parkinson's disease (Wu et al., 2010). Bone marrow mesenchymal stem cells (BMSCs) are an example of self-renewing multipotential cells with the developmental capacity to give rise to certain cell types (McKay, 1997; Cameron and McKay, 1998). These cells seem to be able to differentiate into hepatocytes, osteocytes, cardiomyocytes, and neural cells *in vitro* (Brazelton et al., 2000; Mezey et al., 2000). A previous study has shown that the implanted mesenchymal stem cells (MSCs) at the site of injury are able to survive and integrate in the host brain (Woodbury et al., 2002). Lee et al. (2010) used

human umbilical cord blood MSCs in mice with Alzheimer's disease and demonstrated cognitive rescue with restoration of learning and memory functions. Regarding the fact that using autologous cell transplantation circumvents ethical and immunological problems, the present study was aimed to evaluate the therapeutic effects of MSCs on cold stress induced changes in the hippocampus and the cognitive function of Wistar rats.

Materials and Methods

Animals

Totally 25 male Wistar albino rats provided by the Saveetha University were used in this experiment. The study was conducted at BRULAC, Saveetha University, Tamil Nadu, India. The care and maintenance of the animals were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Three rats were housed in each polypropylene cage under standard laboratory conditions with food and water provided *ad libitum*. The experimental protocol was subjected to scrutiny by an institutional animal ethical committee for experimental clearance (IAEC).

Isolation and characterization of BMSCs

A 6-week-old Wistar rat weighing 80 g was used for isolation of BMSCs. The rat was anesthetized with 10 mg/kg xylozine and 80 mg/kg ketamine, cocktail. Following removal of femur and tibia, muscles and connective tissues were cleaned (Saraswathi and Saravanakumar, 2010). Both ends of the bones were cut and the marrow was slowly flushed out into an aseptic glass container with culture medium at one end of the bone. 20 mL of Dulbaco's minimal essential minimum medium (DMEM) (Gibco®, Grand Island, NY, USA) along with 10% fetal bovine serum (FBS) was added to a test tube. The medium was aspirated into a sterile 2 mL syringe and was forced into the medullary cavity of the long bones. The flushed medium was collected in a 15 mL Ependroff's tube. Bone marrow cells were thus suspended in DMEM containing 10% heat-inactivated FBS. Bone marrow was centrifuged at 1,500 r/min. After removal of supernatant solution, the adherent cells were pipetted into a T25 flask which was prefilled with 4 mL of DMEM and 10% FBS. The flasks were incubated at 5% CO_2 at 37°C. The cells were retrieved and passaged. From the first passage to the second passage, longer time duration was taken for the cells to adapt to *in vitro* condition. However, cells at passages 3–6 require relatively shorter time to adapt to new environment. Passage 6 cells were analyzed by flow cytometry (flowJo, India) for positive expression of CD44 and CD90 (specific markers for BMSCs) and negative expression of CD45. The viability of cells was determined by Trypan blue exclusion test as described by Tolnai (1975). Briefly, cells were incubated with Trypan blue dye for 1 minute. Blue CD44 and CD 90 positive cells and white CD 45 negative cells were counted in ten 20 × fields of view, and the percent of viable cells was calculated. Once the positivity was obtained, cells were cultured again to 90% confluence before they were used for the treatment.

Experimental groups

Another 24 male Wistar rats aged 8 weeks weighing 150–180 g were randomly divided into four groups (n = 6 per group): (1) normal control group: rats were kept under ideal laboratory conditions; cold water swim stress (cold stress) group: rats were forced to swim in the cold water maintained at 10 ± 2°C (measured by thermometer) till it started to sink, daily once in the morning for a period of 1 month; cold stress + PBS: rats underwent cold water swim stress for 1 month followed by PBS injection *via* the jugular vein; cold stress + BMSCs: rats received cold water swim stress for 1 month followed by BMSCs (1×10^6) injection *via* the jugular vein. All injections were performed under the guidance of veterinary surgeon.

Behavioral assessment

Behavioral functional test was performed in all rats during stress and treatment period by an investigator who was blinded to experimental groups. During the stress period, rats were assessed for any behavioral dysfunction occurrence. Rats were placed on the floor and various movements were analyzed and scored. Both horizontal (locomotion) and vertical activities (rearing) were monitored. The motor activity and anxiety were graded using Neurological Severity Score (NSS; 0–3 points) by Chen et al. (2001). 0, Normal walk; 1, inability to walk straight; 2, circling; 3, fall down.

Histological assessment

After treatment for 1 month, rats were anesthetized with ether and intracardiacally perfused with normal saline followed by 10% formal saline. Rat brains were dissected out and fixed in 10% chilled neutral formalin for 14 hours at 4°C. Paraffin embedded sections (Bregma –1.8 mm to –3.8 mm) of 10 μm thickness were cut on rotary microtome and stained with hematoxilin and eosin for demonstration of nerve cell bodies. Quantitative analysis of neuron cell bodies in the pyramidal cell layer of hippocampal CA1 region (Wang et al., 2005) was performed using a calibrated ocular micrometer. The cell count was corrected according to Abercrombie's formula (Abercrombie, 1946): $P = A \times M / (L + M)$ where P is the average number of nuclear points per section, A is the crude count of number of nuclei seen in the section, M is the thickness (in μm) of the section, and L is the average length (in μm) of the nuclei. Round, clear, middle-sized or large-sized neurons with distinct nuclei were counted. Darkly stained, shrunken cells and cells with fragmented nuclei were excluded from counting. Only adequately impregnated CA1 neurons were selected for study. The first six neurons encountered in each rat when moving from medial to lateral in the sections were selected for quantitative analysis. In the treatment groups, formalin-fixed, paraffin-embedded tissue samples were studied immunohistochemically for the presence of S-100 protein as described by Schmidt-Kastner and Szymas (1990).

Statistical analysis

Values are expressed as the mean ± SEM. One-way analysis of variance followed by Student-Newman-Keuls multiple

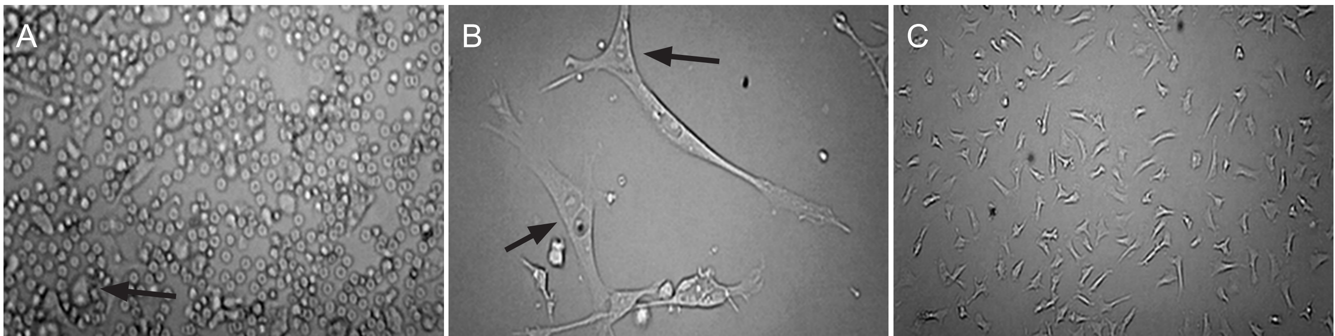

Figure 1 Identification and morphology of rat bone marrow mesenchymal stem cells.
(A; 40 ×) A mixed population of both hematopoietic and mesenchymal stem cells (MSCs) on the 3rd day after culture. Arrow indicates rat MSCs. (B; 200 ×) Change in morphology of MSCs on the 5th day after culture. Long arrow indicates triangular cells and short arrow indicates star-shaped cells. (C; 40 ×) Homogenous population of MSCs showing spindle-shaped morphology on the 14th day after culture.

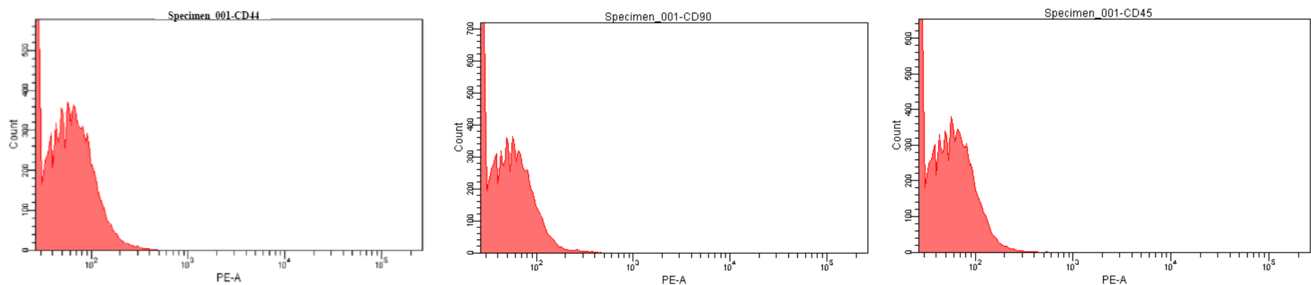

Figure 2 Phenotypic characteristic of rat bone marrow mesenchymal stem cells.
Flow cytometry analysis revealed that all passage 3 cells were positive for CD44, CD90, and negative for CD45.

comparisons test was used for comparison of the means. A level of $P < 0.05$ was taken as statistically significant. Statistical analysis was carried out using SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

Results

Culture observations

On the 3rd day of culture, we observed the mixed population of cells, and only a few MSCs were present. The MSCs showed a tendency to adhere to the tissue culture plastic and were triangular-shaped with eccentric nucleus. Other mixed cells (**Figure 1A**) were round. The ratio between MSCs and other mixed cell populations was small. On the 5th day of the culture, MSCs had started to grow with their long processes extended (**Figure 1B**) among the mixed cells. Compared to the 3rd day, there were more MSCs. On the 8th day, clusters of MSCs with long processes, one nucleus and one nucleolus were present. The MSCs had gradually changed from triangular to star-shaped then to spindle-shaped. During the subculture study, homogeneous adult MSCs were obtained. On the 14th day, multiple colonies of MSCs were present, which were thickly populated. There was a linear increase in the number of colonies and mesenhymal stem cells (**Figure 1C**). Viability of cells determined by Trypan blue dye reveals that about 99% of the cells were viable.

Flow cytometry analysis for BMSCs specific markers CD90 and CD44 expression

The cells at passage 3 were trypsinized, washed, and analyzed by flow cytometry after culture expansion. Flow cytometry analysis showed that 99.9% of cells were positive for CD90, 100% were positive for CD44, and 90.6% were negative for CD45 (**Figure 2**). From the 1st to 2nd passage, cells required longer time duration to adapt to *in vitro* condition. The time duration for environmental adaptation was shortened from the 3rd to 6th passage, indicating that the cells had adapted to the new environment.

Behavioral function

Rats subjected to cold stress exhibited unique spontaneous rotations and horizontal locomotion behaviors were increased. The rotations per minute were increased and the diameter of rotation was decreased as the stress was sustained for a long period of time. NSS score was 0 in the normal control group. In the cold stress and cold stress + PBS groups, the NSS score on average was increased. In the cold stress + BMSCs group, rotations per minute on the 10th day after intravenous injection of BMSCs were increased compared to the 3rd day, and the NSS score on the 10th day was 0, indicating that rats could walk normally.

Histological features of hippocampal CA1 region

Hematoxylin-eosin-stained sections of hippocampal CA1 region were observed under 40 × objective lens of a microscope. Neurons were counted under 400-fold magnification. The region for cell calculation was selected randomly from the serial sections in each group. The total number of cells in hippocampal CA1 region (**Figure 3**) was 63.5 ± 5.0/0.14

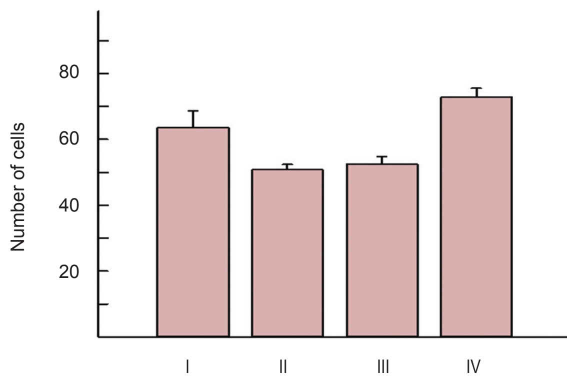

Figure 3 Total number of neuronal cells in hippocampal CA1 region sections (hematoxylin-eosin staining).

The values are the percentage of average number of cells per 0.14 mm^2. One-way analysis of variance showed that there was significant statistical difference between groups. The Student-Newman-Keuls test showed that the total number of neuronal cells was significantly higher in the IV group than in the other three groups ($P < 0.05$). I: Normal control group; II: cold stress group; III: cold stress + PBS group; IV: cold stress + bone marrow mesenchymal stem cells group.

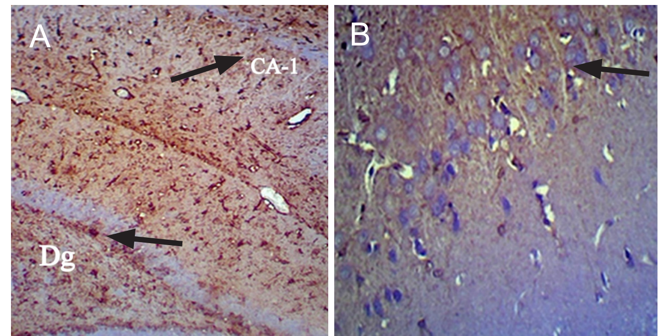

Figure 4 Immunohistochemical staining for S-100 protein in the hippocampal CA1 (CA-1) region of rats treated with intravenous injection of bone marrow mesenchymal stem cells after forced cold water swim stress.

(A) Arrows indicate the dentate gyrus (Dg) and CA-1 region of the hippocampus. (B) 200 × of CA-1 region. Arrow indicates glial cells with dendrites.

mm^2 in the normal control group and 50.83 ± 1.57/0.14 mm^2 in the cold stress group. The increased vascular space and darkly stained nucleus in the cold stress group indicate the presence of apoptotic cells. The total number of cells in hippocampal CA1 region was 52.5 ± 2.35/0.14 mm^2 in the cold stress + PBS group and 72.83 ± 2.70/0.14 mm^2 in the cold stress + BMSCs group. In the cold stress + BMSCs group, numerous cells with centrally placed nuclei with few darkly stained cells were seen. One-way analysis of variance results showed that there was significant statistical difference between cold stress + PBS and cold stress + BMSCs groups ($P < 0.01$). The Student-Newman-Keuls test for multiple comparisons showed that the total number of cells in the hippocampal CA1 region was significantly higher compared to other groups ($P < 0.05$). In the cold stress + BMSCs group, staining for S-100 protein revealed that numerous astrocytes appeared in the hippocampal CA1 region (**Figure 4**).

Discussion

Bone marrow is a rich source of MSCs, which plays a vital role in modern regenerative medicine. Prockop (1997) reported that MSCs could be isolated in a relatively high number from cultures of bone marrow by selecting the cells that are adhered to tissue culture plastic and proliferate rapidly. MSC population can be isolated by the methods similar to that used by Friedenstein et al. (1970), which utilized the physical property of plastic adherence. In the present study, BMSCs were isolated from a 6-week-old Wistar albino rat as described by Saraswathi and Saravanakumar (2010). There are many similar culture studies (Friedenstein et al., 1970; Ohazama et al., 2004; Kassis et al., 2006; Raimondo et al., 2006; Koc et al., 2008; Kumar et al., 2013). The mixed cell populations including a few MSCs with star-shaped appearance were seen on the 3rd day. The MSCs were distinct from other cells and can be differentiated clearly as described by Raimondo et al. (2006) who reported the presence of mesenchymal stem cells indicating the pseudopodia. Results from this study showed that when the culture medium was changed, MSCs were adherent to tissue culture plastic as described by Prockop (1997), Saraswathi and Saravanakumar (2010). On the 5th day, MSCs started to grow and their long processes were seen among the mixed cells. Kassis et al. (2006) reported that spindle-shaped MSCs can be isolated from mixed cell populations using fibrin microbeads, which have special advantage in isolating MSC from sources. On the 8th day, clusters of MSCs with nucleus, nucleolus and long processes were seen as studied by Kumar et al. (2013). When the culture medium was changed, MSCs were adherent to tissue culture plastic as described by Prockop (1997). Homogenous adult MSCs were obtained and colonies of adult MSCs were seen and were capable of quickly multiplying about 15 million cells per cm^3. Friedenstein et al. (1970) who cultured the bone marrow of guinea pig reported a linear increase in the number of colonies with an increase in number of explanted cells. Cells changed from star-shaped to spindle-shaped and grew as described by Saraswathi and Saravanakumar (2010). Flow cytometry analysis showed that 99.9% of cells were positive for CD90 and 100% of cells were positive for CD44, which are specific markers for BMSCs (Lowell and Mayadas, 2012). In the present behavioral study, a spontaneous rotation and an increased amount of horizontal locomotion were observed. Similarly, Mickley et al. (1989) described that radiation-induced damage to the hippocampal formation caused rats placed in bowls to spontaneously turn in and also exhibited other behavioral characteristics of hippocampal damage including spontaneous exploration of the arms of a maze, retarded acquisition of a passive avoidance task and increased amount of horizontal locomotion. Dopamine might have played a fundamental role in this damage. Another explanation could be the decreased blood flow to the hippocampus after stressful conditions. Hypoperfusion and reciprocal inhibition between frontal and limbic areas has been hypothesized in theories on the etiology of schizophrenia (Rabinowicz et al., 1997). In the present study, we showed that intravenous-

ly injected BMSCs reached hippocampal CA1 region and differentiated into neuronal cells. S100 staining showed the presence of numerous glial cells and these cells would have given rise to new neurons, which was already proved by Bettina Seri et al. (2001) who reported that astrocytes gave rise to new neurons in the adult mammalian hippocampus. Chu et al. (2003) intravenously injected human neural stem cells and they found that these cells migrated to the damaged hippocampus, proliferated and differentiated into mature neurons and astrocytes in the adult rat brain with transient forebrain ischemia. One possible reason is disruption of the blood-brain barrier, which was evident in the histology in the present study that vascular space was enlarged in the cold stress groups, and the disruption may inhibit the progression of selective entry of MSCs into the brain. Another possible mechanism is the natural migration of BMSCs from one hematopoietic microenvironment to the other and the genetical factor as described by Quesenberry et al. (1998). Intravenous injections have some limitations: (1) they might have diffused to the other systemic organs and (2) might be less effective after direct delivery to the brain region. But they are less invasive. A previous study has shown that direct transplantation of cells into the brain region resulted in tissue damage along the needle passages (Sinden et al., 1997).

Taken together, our results demonstrated that intravenously injected BMSCs gave rise to new neurons in hippocampal CA1 and improved cold stress-induced behavioral changes.

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Author contributions: *Saravana Kumar S was responsible for conception and design of this study and data collection. Vijayaraghavan R supervised this study, performed statistical analysis, analyzed data and drafted the manuscript. Saraswathi P supervised this study and provided technical assistance. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*

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