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## COMPARATIVE STUDY ON EFFECT OF OMEGA-3 FATTY ACID AND BONE MARROW MESENCHYMAL STEM CELLS ON COLD STRESS INDUCED NEURONAL CHANGES IN THE HIPPOCAMPUS OF RATS.

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**ABSTRACT:** The present study aimed to compare the effect of Omega3 fatty acids (OFA) and bone marrow mesenchymal stem cells (BMSC's) on stress induced neuronal degeneration in CA1 hippocampus region of rats. For isolation and culture of BMSCs, a three week old Wistar rat was sacrificed, femora and tibiae were collected then mesenchymal cells cultured. The 6<sup>th</sup> passage cells are analyzed by flow cytometry for the expression of CD44, CD90 for positivity and CD45 for negative expression. In this experiment, thirty six rats aged 8 weeks old were divided into six Groups (n=6). Group I was control, Group II was given forced cold water swim stress, Group III: cold stress followed by saline Group IV and V, cold stress followed by oral gavages of omega-3 fatty acids 60mg/Kg for 2 weeks (short-term model) and 4 weeks (long term model) respectively and Group VI was cold stress followed by intravenous injection of  $1 \times 10^6$  BMSCs. Behavioural function test performed using Y-maze. After treatment, rats were sacrificed for Cortisol assay and histological studies. Results indicate treatment with BMSC's shows significantly increased neuronal cell and mean spontaneous alteration behavior compared to OFA. BMSC's shows better therapeutic effect than OFA in cognitive decline.

**INTRODUCTION:** Stress is the nonspecific response of the body to demand. A stressor is agent that produces stress at anytime <sup>17</sup>. Diverse types of stresses are used in previous studies on experiments with rats. Obsessive swimming in cold water is one such stress, which produces output of norepinephrine in definite parts of the brain structures and also causes alteration in blood cortisol level <sup>11</sup>.

Unescapable 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 amygdala and hippocampus <sup>19</sup>. The Y-maze can be used as a measure of short term memory, general locomotor activity and stereotypic behavior <sup>13</sup>.

Repeated exposure to inescapable stress is associated with a neither ultimate reduction of norepinephrine in the hypothalamus and hippocampus <sup>18</sup>. The current drug therapies on stress management have dilemma including poor efficiency, withdrawal symptom and adverse effects <sup>16</sup>. Recently, much awareness has been created on the relevance of hippocampal

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neurogenesis to the pathophysiology and treatment of memory loss. In the nervous system, especially brain contains higher concentration of PUFA. Omega-3 fatty acid are critical for the physiologic function of neuronal cell membrane. The important role of these fatty acids in central nervous system function, and the potential EFAs have in the treatment of various neuropsychiatric disorders. Omega-3 fatty acid supplementation decreases anxiety in the animal models of drug addiction<sup>7</sup>.

In humans, PUFAs have positive effects on the pathophysiology of a wide range of stress-related disorders<sup>9</sup>. 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<sup>23</sup>. 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<sup>10,4,1</sup>.

These cells seem to be able to differentiate into hepatocytes, osteocytes, cardiomyocytes, and neural cells in vitro<sup>3,12</sup>. Lee et al.<sup>8</sup> used human umbilical cord blood MSCs in mice with Alzheimer's disease and demonstrated cognitive rescue with restoration of learning and memory functions. The present study was aimed to compare the effects of omega-3 and MSCs fatty acid on cold stress induced neuronal changes in the hippocampus of rats.

## MATERIALS AND METHODS:

### Animals:

Totally 37 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 water and food *ad libitum*. The experimental protocol was subjected to scrutiny by an institutional animal ethical committee for

experimental clearance (IAEC) with approval number SU/BRULAC/016/2014.

### Isolation and characterization of BMSCs:

A 6-Week 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 tissue were cleaned. Both ends of the bone were cut and the marrow was slowly flushed out into an asptic glass container with culture medium at one end of the bone. 20 mL of Dulbaco's minimal essential medium (DMEM) along with 10% fetal bovine serum (FBS) was added to a test tube. The medium was aspirated into a sterile 2mL 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 pipette into a T25 flask which was filled with 4 mL of DMEM and 10% FBS. The flasks were incubated at 5% CO<sub>2</sub> 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 for positive expression of CD44 and CD90 and negative expression of CD45. The viability of cells was determined by Trypan blue exclusion test as described by Tolani<sup>20</sup>. Briefly, cells were incubated with Trypan blue dye for 1 minute. Blue CD44 and CD90 positive cells and white CD 45 negative cells were counted in ten 20 X 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 treatment.

### 2.3 Experimental group:

Thirty six rats aged 8 weeks old were divided into six Groups (n=6). Group I was control, Group II was given forced cold water swim stress, Group III: cold stress followed by saline Group IV and V, cold stress followed by oral gavages of omega-3

fatty acids 60 mg/Kg (Docosahexaenoic acid(DHA) and Eicosapentaenoic acid(EPA)) for 2 weeks (short-term model) and 4 weeks (long term model) respectively and Group VI was cold stress followed by intravenous injection of  $1 \times 10^6$  BMSCs. The total study was 60 days which included 1 month stress period followed by treatment.

#### **Behavioural assessment:**

Behavioural study was assessed by spontaneous alteration using Y-maze. The Y-maze can be used as a measure of short term memory, general locomotor activity and stereotypic behavior. Therefore, spontaneous alteration was assessed using a Y-maze composed of equally spaced arms ( $120^\circ$ , 41cm long and 15 cm high). The floor of each arm is made of Perspex and is 5cm wide. Each mouse was placed in one of the arm compartments and was allowed to move freely until its tail completely enters another arm. The sequence of arm entries is manually recorded, the arms being labelled A, B or C. An alteration is defined as entry into all three arms consecutively, for instance if the animal makes following arm entries; CAB,ABC,BCA,ACB,BAC.

The number of maximum spontaneous alteration is then the total number of arms entered minus two, and the percentage alteration is calculated as  $\{(actual\ alternations / maximum\ alternations) \times 100\}$ . For each animals the Y-maze was carried out for 5 minutes. The apparatus was cleaned with 5% alcohol and allowed to dry between sessions.

#### **Biochemical assay:**

Under aseptic condition, 5 ml of animal's blood was collected from the internal jugular vein using a 21 gauge 5 ml syringe. The blood was then transferred to a sterile plain vacationer tubes. The blood was allowed to stand for 30 minutes to enable clot formation. Then the blood was centrifuged at 3000 rpm in a centrifuge machine and 1 ml of serum was extracted. The serum was analyzed for corticosterone hormone. Cortisol assay was done using ADVIA Centaur System. The ADVIA Centaur cortisol assay is a competitive immunoassay using direct Chemiluminescence technology.

#### **Histological assessment:**

The animals were anaesthetized with ether and intra-cardiac perfusion of normal saline followed by 10% formal saline was done. After euthanized, the brain was rapidly removed by inserting the tip of the curved scissors into the foramen magnum and a single lateral cut was made into the skull extending forward on the left and right side. With a bone cutter the dorsal portion of cranium was peeled off and by means of blunt forceps brain was dropped onto the ice cold glass plate leaving the olfactory bulbs behind. The whole process of removing brain takes less than 2 minutes. Brain were dissected out from each animal and were fixed in 10% chilled neutral formalin for 14 hours at  $4^\circ\text{C}$ . Dissection was made on ice-cold glass plate. Further histological processing was done. Paraffin embedded sections (Bregma -1.8mm to -3.8mm) of  $10\ \mu\text{m}$  thickness were cut on rotary microtome and stained with hematoxylin and eosin for demonstration.

#### **RESULTS:**

##### **Spontaneous alteration:**

The spontaneous alteration as shown in **Fig.1** was  $67.33 \pm 2.67\%$  in control group and  $47.50 \pm 2.50\%$  in cold stress and  $47.50 \pm 2.50\%$  cold stress induced followed by saline injection respectively,  $54.83 \pm 5.17\%$  and  $62.17 \pm 7.83\%$  in cold stress induced followed by short term and long term treatment with and omega 3 fatty acids and  $77.17 \pm 2.83\%$  in cold stress induced followed by treatment with BMSCs.

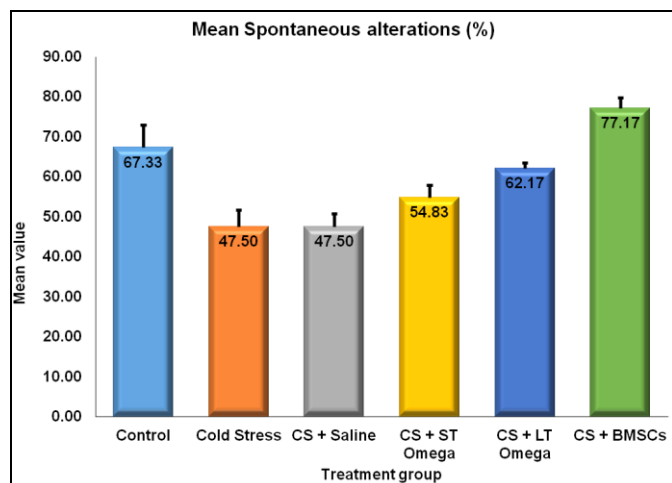
##### **Cortisol assay:**

The cortisol assay as shown in **Fig. 2** shows  $1.07 \pm 0.43$  in control group,  $1.74 \pm 0.26$  in cold stress group,  $1.71 \pm 0.29$  in cold stress followed by saline injection,  $1.52 \pm 0.48$  and  $0.93 \pm 0.07$  in cold stress followed by oral gavages of omega-3 fatty acid for short term and long term respectively and  $0.96 \pm 0.04$  in cold stress followed by treatment with BMSCs.

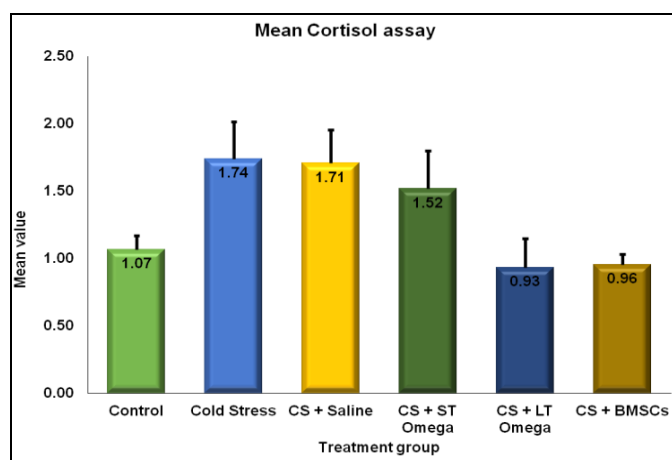
##### **Histological assessments:**

As shown in **Fig. 3** the total number of cells in control group was  $63.50 \pm 6.50$ , cold stress was  $50.67 \pm 9.33$ , cold stress followed by saline injection was  $49.17 \pm 0.83$ ,  $55.83 \pm 4.17$  and  $59.50 \pm 0.50$  in cold stress followed by short and long term

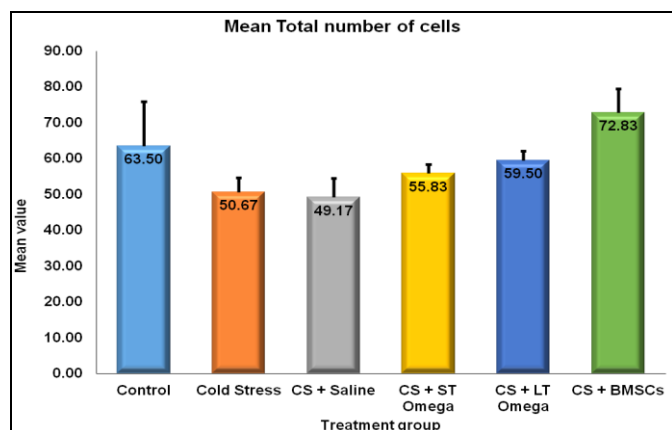
treatment with omega-3 fatty acids respectively and  $72.83 \pm 7.17$  in cold stress followed by treatment with BMSCs.



**FIG.1: BEHAVIOUR STUDY – MEAN SPONTANEOUS ALTERATIONS**



**FIG. 2: CORTISOL ASSAY**



**FIG. 3: TOTAL NUMBER OF CELLS**

**DISCUSSION:** The parts of brain structures are involved in learning and memory. Considering the role of memory, it is of interest that brain structures involved in memory are primarily involved in the

neurobiological response to unavoidable stress. In the present study cold stress was given to rats which cause neuronal loss in the hippocampal region<sup>16, 1, 18</sup>. Cold water swim stress induces major neuronal loss in hippocampus region of rats<sup>22</sup>. Behaviour was assessed by spontaneous alteration using Y-maze<sup>13</sup>.

Present study shows that Mean Spontaneous alteration(%) of cold stress followed by treatment with BMSCs was statistically significant when compared to the other groups, the cold stress followed by long term omega-3 fatty acid treated group was significant when compared with cold stress followed by short term omega-3 fatty acid treated group, the cold stress followed by short term omega-3 fatty acid treatment had higher percentage when compared to the cold stress followed by saline treatment group and cold stress group. The omega 3 fatty acid supplementation in low dose for long term cause anti-stress effect by decreasing anxiety<sup>15</sup>. Dietary intake of DHA is required for normal neural development and brain. Various experimental studies suggest that DHA decreases particularly in region of hippocampus which are crucial for higher brain functions such as memory formation and cognition<sup>6</sup>.

Comparable results have been reported using stress paradigms and corticosterone administration<sup>14, 21</sup>. Mean Cortisol assay was statistically significant in cold stress followed by long term omega-3 fatty acid treated group than the other groups. Followed by which there was significant result in cold stress followed by bone marrow mesenchymal stem cell treated group, cold stress followed by short term omega-3 fatty acid treated group, cold stress followed by saline infusion and cold stress group respectively in this study which showed increase in corticosteroid level in the stressed rats. Histological study reveals an increase in the total number of cells in hippocampus region of treatment group. Total number of neuronal cells in BMSCs treated group was significantly higher than other group, followed by significant increase in total number of cells in long term fatty acid treated group, short term fatty acid treated group, cold stress group and cold stress followed by intravenous injection respectively which shows when BMSCs when used

for treatment of stress yields a significant result<sup>2</sup>. When human neural stem cells are injected intravenously they found that these cells migrate to damaged hippocampus, proliferated and differentiated into mature neurons in the adult rat brain with transient forebrain ischemia<sup>5</sup>.

**CONCLUSION:** Taken together, our study demonstrated BMSC's had better therapeutic effect than OFA( short term or long term) followed by in cognitive decline associated with stress.

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