The Hematopoietic Growth Factor “Erythropoietin” Enhances the Therapeutic Effect of Mesenchymal Stem Cells in Alzheimer’s Disease

M.I. Khairallah, L. A. Kassem, N.A. Yassin, M.A. Gamal El Din, M. Zekri and M. Attia

Department of Physiology,
Department of Histology,
Faculty of Pharmacy and Biotechnology, German University in Cairo (GUC), Cairo, Egypt
Faculty of Medicine, Cairo University, Cairo, Egypt

Abstract: Alzheimer’s disease is a neurodegenerative disorder clinically characterized by cognitive dysfunction and by deposition of amyloid plaques, neurofibrillary tangles in the brain. The study investigated the therapeutic effect of combined mesenchymal stem cells and erythropoietin on Alzheimer’s disease. Five groups of mice were used: control group, Alzheimer’s disease was induced in four groups by a single intraperitoneal injection of 0.8 mg kg⁻¹ lipopolysaccharide and divided as follows: Alzheimer’s disease group, mesenchymal stem cells treated group by injecting mesenchymal stem cells into the tail vein (2×10⁶ cells), erythropoietin treated group (40 μg kg⁻¹ b.wt.) injected intraperitoneally 3 times/week for 5 weeks and mesenchymal stem cells and erythropoietin treated group. Locomotor activity and memory were tested using open field and Y-maze. Histological, histochemical, immunohistochemical studies, morphometric measurements were examined in brain sections of all groups. Choline transferase activity, brain derived neurotrophic factor expression and mitochondrial swellings were assessed in cerebral specimens. Lipopolysaccharide decreased locomotor activity, memory, choline transferase activity and brain derived neurotrophic factor. It increased mitochondrial swelling, apoptotic index and amyloid deposition. Combined mesenchymal stem cells and erythropoietin markedly improved all these parameters. This study proved the effective role of mesenchymal stem cells in relieving Alzheimer’s disease symptoms and manifestations, it highlighted the important role of erythropoietin in the treatment of Alzheimer’s disease.

Key words: Alzheimer’s disease, amyloid plaques, neurofibrillary tangles, lipopolysaccharide, mesenchymal stem cells, erythropoietin and brain derived neurotrophic factor

INTRODUCTION

Alzheimer’s Disease (AD) is a progressive, irreversible and age-related neurodegenerative disease, which leads to deterioration of mental functions including memory loss, behavioral and personality changes and eventual morbidity and death. The neuropathological hallmarks of AD are the excessive accumulation of amyloid plaques and Neurofibrillary Tangles (NFT) in brain tissue, along with neuronal loss, dystrophic neurites and gliosis (Pleckenstrie, 2010).

Many drugs have been approved for treating AD. The most commonly used are cholinesterase inhibitors which are approved for mild to moderate conditions and a glutamate N-methyl D-aspartate (NMDA) antagonist approved for moderate to severe cases of the disease (Salloway and Correia, 2009). The use of these drugs help to maintain neuronal functions through symptomatic therapy, however they do not impact the disease process significantly (Raina et al., 2008; Qaseem et al., 2008). Other disease modifying lines of treatment are also used, targeting amyloid plaques formation and amyloid peptides Aβ mediated cytotoxicity and neurofibrillary tangles generation (Kivelto and Solomon, 2008).

Current treatments for AD are unable to prevent, cure or halt the progress of the disease, but instead only provide some symptomatic relief. In order to reverse the progress of the disease and its symptoms, dead or dying nerve cells may need to be replaced (Miller et al., 2010).

Human Mesenchymal Stem Cells (MSCs) have emerged in recent years to hold enormous potential for cell replacement therapy for a wide variety of neurological disorders, including AD. MSC’s provide an alternative because they lack significant immunogenicity. This allows them to be transplanted without the need for immunosuppressive drugs. In addition, MSC’s are particularly useful because of the convenient isolation and their potential ability to differentiate into specific cell...
types as needed. Their use also poses no ethical controversy, therefore making them ideal to deal with illnesses that require cell replacement therapy such as AD (Sasaki et al., 2008; Tona et al., 2002).

Erythropoietin (EPO) was proven to be a candidate for investigation as a novel therapeutic agent in neurodegenerative diseases. Previously EPO has been used to treat anaemia, however, due to its ability to act directly through a non-haematopoietic EPO receptor system in the brain, its specific characteristics are being reviewed and its therapeutic potential is being studied (Brines and Cerami, 2005). By administering EPO systemically it has been shown to have neuroprotective effects in animal models with acute brain damage and/or chronic neurodegenerative conditions by directly crossing the blood brain barrier to damaged areas (Miskowiak et al., 2010). The aim of the present study is to examine the possible curative potential of using combined MSCs and EPO on an AD induced model and to investigate their effects on the memory and locomotor activity as well as on the underlying pathophysiological changes of the AD induced animal model. The main target is to find a source for replacing lost or damaged nerve cells.

The long-term objective of this research is to use genetically modified MSCs as an inexhaustible source for nerve cell repair. This could provide an alternative therapy for AD patients with whom available pharmacological treatments have not been successful.

MATERIALS AND METHODS

Animals: Sixty adult male mice weighing between 25-30 g were used in this study. Animals were purchased from the animal house of the National Research Center (NRC, Giza, Egypt). All experimental procedures were conducted according to NIH (National Institute of Health) guidelines for the treatment and care of laboratory animals published in the NIH publication 85-23 revised 1985.

Animals were housed in separate plastic cages (fifteen per cage) in the laboratory animal center of the German University in Cairo, under controlled temperature (22±2°C) and 50–55% relative humidity environment under a 12-h light/12-h dark cycle, for one week for stabilization. All animals had free access to food (standard diet) and water. All experiments were performed during the daylight hours.

Chemical: The following chemicals were purchased from the respective suppliers as below:

- Lipopolysaccharide (LPS) was used in this research to induce AD, it was purchased from (Sigma-Aldrich, USA) from LPS (Escherichia coli, serotype 0127:B8, Sigma, cat number: 63H-4010)
- EPO (Epoetin 4000 IU/1 mL of solution-SEDICO Pharmaceutical Co., 6 October City Cairo)
- MSCs obtained from tibial bone marrow of male mice, the marrow is flushed by Dulbecco’s modified Eagle’s medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine medium (GIBCO/BRL). Ficoll/Paque (Pharmacia) is used to isolate cells and 1% penicillin-streptomycin (GIBCO/BRL) for suspension
- FKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich)
- Avidin-Biotin immunoperoxidase polyclonal kit was provided by NEO Marker’s (Lab. Vision Corporation), for CD105 immunostaining of stem cells. The primary antibody (rabbit Ab) Supplier: Dako. Catalog Number: Z6311. Dilution: 1:1000 was done using IHC-Tek Antibody Diluent (Cat# IW-1000 or IW-1001), for S100B immunostaining

All other reagents and chemicals were of the highest quality commercially available.

Experimental protocol: Animals were equally divided into five groups, each containing 12 mice. The first group, served as control, receiving a daily dose of 1% tween 80, injected intraperitoneaally (i.p.) for 7 days.

AD was induced in the other 4 groups by injecting them with a single dose of lipopolysaccharide (LPS) 0.8 mg kg⁻¹ i.p. This was followed by a daily dose of 1% tween 80 for 7 days (Araki et al., 2001). One group was left without any treatment and served as the diseased group (group II). The other three groups were subjected to three different lines of treatment. Group III received a single MSCs injection into the tail vein at a concentration of 2×10⁶ cells suspended in 400 μL of Phosphate Buffered Saline (PBS) (Esneault et al., 2008). Group IV mice were treated by i.p., injection of EPO at a dose of 40 μg kg⁻¹ of body weight three times weekly for 5 consecutive weeks (Araki et al., 2001; Sheng et al., 2003). A combined therapy of both MSCs and EPO was given to the fifth group at same doses previously used (Araki et al., 2001; Sheng et al., 2003).

At the end of experimental protocol the following parameters were assessed.

Behavioral tests

The open field test: The open field test is a locomotor behavior assessment test (Kimani et al., 2008) which is used primarily to examine motor function by means of measuring spontaneous activity in an open field. The test was carried out in a big square metal box, each side of its base measures 80 cm in length and its height measures 40 cm (Cunha and Masur, 1978). The box walls were painted red in color while the floor was left white and it was divided into 16 equal squares.
Mice were placed individually into the central point of the open field and observed during a 3 min period, the floor and walls were cleaned after testing each mouse. The following parameters were recorded:

- **Ambulation frequency**: Represents the number of squares crossed by each mouse per minute. Entries were recorded manually, the total number during a 3 min period was used to compare between groups (El Sayed et al., 2009; Hrmkova et al., 2007)
- **Grooming frequency**: Is defined as the number of times of face washing, animal scratching itself with the hind leg, or licking of the fur and genitals while stationary per 3 min (El Sayed et al., 2009)
- **Rearing frequency**: Is the number of times the animal stood stretched on his hind limbs with or without fore limbs support during a 3 min period (Kwak et al., 2009)

**Spontaneous alternation Y-maze test**: Y-maze test is based on the innate curiosity of rodents to explore novel environments, where there is preference to alternate arms when exploring a new environment (Mandillo et al., 2008). It has been effectively used to assess exploratory behaviors, learning and memory function in rodents. It is used primarily to assess the working memory of mice by recording the percentage of spontaneous alternation behavior. In the present study, Y-maze apparatus consisted of three identical arms, connected via an equilateral triangular platform in the center. The arms dimensions were as follows: three arms each 325 mm in length, 85 mm in width and 150 mm in height, they were separated by angles of 120° (Arai et al., 2001; Mandillo et al., 2008). A mouse was placed in the center of the apparatus and was allowed to explore the maze for 8 min. An arm entry was counted when the mouse had all four paws inside an arm. Animals that made fewer than five entries were excluded from the analysis. The numbers of total arms entries, as well as the spontaneous alternation (number of triads) were recorded to calculate the percentage of spontaneous alternation.

A spontaneous alternation was defined as successive entries into all three arms consecutively, for example: ABC, BCA, CAB... etc., which are considered to be correct choices.

The percentage of spontaneous alternation for each mouse was determined by the following equation (Kim et al., 2007; Heo et al., 2009):

\[
\text{Alternation (\%) = } \frac{\text{No. of spontaneous alternations}}{\text{Total arm entries-2}} \times 100
\]

**Isolation of MSCs**: Bone marrow was harvested by flushing the tibiae and femurs of male mice with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine medium (GIBCO/BRL). Nucleated cells were isolated with a density gradient (Ficoll/Paque (Pharmacia)) and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO₂ for 12-14 days as primary culture or upon formation of large colonies. When large colonies developed (80-90% confluence), cultures were washed twice with Phosphate Buffer Saline (PBS) and cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation (at 2400 rpm for 20 min), cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask Falcon. The resulting cultures were referred to as first-passage cultures (Alhadlaq and Mao, 2004). Using the detection of CD 29, which are one of the surface markers of mice mesenchymal stem cells, MSCs were identified in culture (Rochefort et al., 2005; Zhu et al., 2010).

**Tracking of MSCs**: A red fluorochrome dye (PKH26) was used in groups III and V which were injected with MSCs to confirm homing of stem cells in the cerebral cortex of injected mice.

At the end of the experimental protocol of six weeks, behavioral tests were carried out on all studied groups to assess both locomotor activity and memory. Then the animals were anaesthetized with thiopental (60 mg kg⁻¹ intraperitoneal) and sacrificed by decapitation. The brains were isolated and divided into two hemispheres. One hemisphere was kept in 10% buffered formalin (for histological and morphometric studies), the other hemisphere was kept in Phosphate Buffered Saline (PBS) and stored at -80°C for further assessment of mitochondrial swelling, acetyl choline transferase enzyme activity and Brain Derived Neurotropic Factor (BDNF) gene expression.

**Histopathological studies**

**Histochemical study**: Cerebral specimens were stained with hematoxylin and eosin and were examined in all groups to demonstrate the dark blue nuclei. Congo red stain was used to demonstrate amyloid plaques. Finally, immunohistochemical staining was done using CD 105 immunostaining, acting as a marker for MSCs tracking and S100B immunostaining as marker for tau binding and amyloid plaques.

**Morphometric study**: All morphometric studies were done in the Histology department, Kasr Al Aini, Cairo University. The mean percentage area of dark nuclei, representing the apoptotic index was measured in
haematoxylin and eosin stained cerebral sections. The percentage area of amyloid plaques in Congo red stained cerebral sections and the percentage area of S100B immunoreactivity were recorded.

Biochemical and gene expression tests

**Determination of choline acetyltransferase:** Tissues were homogenized in 50 mM phosphate buffer, pH 7.4 and containing 0.5% TritonX-100, centrifuged at 5000×g for 10 min at 4°C. The supernatant was used as the source of enzyme. The assay was carried out in 50 mM phosphate buffer, pH 7.4, containing 0.2 M sodium chloride, 10 mM EDTA, 100 μM eserine, 5 mM choline chloride, 200 μM acetyl CoA (0.25 μCi [3H]acetyl CoA), in a final volume of 200 μL, for 30 min at 37°C. The reaction was stopped by adding an equal volume of 1.5% tetraphenyl boron in 3-heptanone, vortexed thoroughly and centrifuged at 5000×g for 5 min to separate the organic phase. The acetylcholine level was determined by counting the organic phase. Enzyme activity was expressed as pmol acetylcholine formed/min/mg protein (Fonnum, 1975).

**Detection of gene expression of brain derived neurotrophic factor (BDNF):** Total RNA was purified from mice brain tissue by (Qiagen, Valencia, CA) as recommended by the manufacturer. Extracted RNA was reverse transcribed into cDNA using RT-PCR kit (reverse transcription PCR) with M-MLV (Moloney-Murine Leukemia virus). BDNF gene expression was analyzed using reverse primer (UniGene Rn.25733) specific for 3¢BDNF coding reverse exons 5¢-CCAAAGAGAGGC CCAAATCCT-3¢ and Forward primer 5¢-CCCTAGCTGCCA GTTCCAAAG-3¢ specific non coding exons cDNA was amplified using real time PCR (qPCR system) (applied biosystem USA).

**Mitochondrial swelling assessment:** Mitochondria were isolated from brain tissue by mechanical lysis and differential centrifugation (Blattner et al., 2001). Tissues were washed briefly with cold PBS at 4°C and centrifuged at 450×g. The pellet was resuspended in cold isolation buffer (75 mmol L\(^{-1}\) sucrose, 20 mmol L\(^{-1}\) HEPES, 225 mmol L\(^{-1}\) mannitol, 0.5 mmol L\(^{-1}\) EDTA, pH 7.2) and the tissues were disrupted by homogenization. Nonlysed cells and nuclei were spun down by centrifugation at 750×g for 20 min. The supernatant was further spun at 10,000×g for 10 min twice. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer (140 mmol L\(^{-1}\) KCl, 10 mmol L\(^{-1}\) NaCl, 2 mmol L\(^{-1}\) MgCl\(_2\), 0.5 mmol L\(^{-1}\) KH\(_2 PO_4\), 20 mmol L\(^{-1}\) HEPES, 0.5 mmol L\(^{-1}\) EGTA; adjusted to pH 7.2 with KOH). Then mitochondrial swelling was measured spectrophotometrically. This method equates mitochondrial membrane permeability transition with high-amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm.

**Statistical analysis:** Statistical analysis was performed by one way Analysis of Variance (ANOVA) and multiple comparisons were done using post hoc test for quantitative variables. Data were recorded and analyzed using Graph-pad Prism version 5. They were expressed as Mean±standard Deviation (SD). A p value less than 0.05 was considered significant for all comparisons made (Altman, 2005).

**RESULTS**

**Effect of MSCs and EPO on LPS-induced AD locomotor and cognitive impairment in mice**

**The open field test:** As shown in Fig. 1a, the LPS induced AD group showed a significant decrease in ambulation frequency in affected mice when compared to the control group (group 1) (23±4.632 vs. 62.75±6.982). Treatment with either MSCs or EPO resulted in a significant increase in the ambulation frequency as compared to the untreated AD group; their mean values were (61.75±8.047, 52±7.447 vs. 23±4.632, respectively). Mice treated with the combined treatment of MSCs and EPO showed the best results with a mean value of (72.17±6.492) significantly higher than the control (62.75±6.982).

Grooming frequency was suppressed in the open field test for LPS induced AD group when compared to the control group (1.667±0.7785 vs. 4.083±1.165). Treatment of AD group of mice with MSCs alone or in combination with EPO resulted in a significant increase in grooming frequency as compared to AD group (4.417±1.24, 3.417±0.5962 vs. 1.667±0.7785). Whereas the group treated with EPO showed a slight increase in the grooming frequency but didn’t reach a significant value (p > 0.05) (Fig. 1b).

Rearing frequency was decreased in LPS induced AD group in the open field test when compared to the control group (group 1) (6.085±1.676 vs. 20.08±2.429). Treatment with MSCs and EPO each separately or both combined significantly increased the grooming frequency as compared to the AD group, the following mean values were recorded respectively (9.25±2.896, 8.75±2.563 and 14.25±2.832). However they were significantly different from the control group Fig. 1c.

All the above results of the open field tests showed improvement in all tested parameters, indicating almost complete recovery of locomotor activity.
Fig. 1(a-d): Effects of MSC and EPO on (a) Ambulation frequency, (b) Grooming frequency, (c) Rearing frequency, in 3 min while (d) Show percentage of spontaneous alteration in 8 min. The x axis represents the studied groups: control, AD: Alzheimer’s disease group, AD+MSCs: Alzheimer disease group treated with mesenchymal stem cells, AD+EPO: Alzheimer disease group treated with erythropoietin and AD+MSCs+EPO: Alzheimer disease group treated with mesenchymal stem cells and erythropoietin. Statistically significant change compared to its corresponding value in the control group (I) (p<0.05), AD group (II) (p<0.05), group (III) (p<0.05), group (IV) (p<0.05), respectively.

Y maze test: Testing memory of different groups of mice, by measuring the percentage of spontaneous alternation showed the following mean values: control group (27.16±3.214), AD group (16.99±3.214), MSCs treated group (23.22±3.349), EPO treated group (22.28±4.537) and the combined treated group (25.28±3.118) (Fig. 1d). All values of treated groups were significantly higher than the AD group (p<0.05). Meanwhile the combined therapy of both MSCs and EPO produced results that were not statistically significant from the control group, indicating almost a complete recovery of memory.

Effect of MSCs and EPO on LPS-induced AD histopathological and morphometric changes in the brain: With regards to the histopathology results, it is important to note that the control groups (I) of healthy brains show no areas of amyloid plaques or areas of dark nuclei (apoptotic index) and therefore the results of the treatment have been compared to the AD group (II). Cerebral sections of mice stained with hematoxylin and eosin, that had been administered LPS to induce AD showed multiple neurons exhibiting dark nuclei of pyramidal neurons surrounded by vacuolated cytoplasm. Blood vessels were dilated and contained spindle shaped cells (Fig. 2a). In LPS induced AD mice treated with MSCs or EPO (Fig. 2b, c) cerebral sections showed multiple neurons exhibiting dark nuclei but in lesser amounts when compared to the AD group. Treatment with combined stem cells and EPO, however, resulted in the appearance of occasional dark nuclei (Fig. 2d).

Examination of cerebral sections of control mice stained with Congo red revealed absence of deposited material or plaques. In LPS induced AD mice group sections showed multiple Congo red positive deposited material or plaques (Fig. 3a). In LPS induced AD mice group treated with EPO sections showed some positive Congo red plaques (Fig. 3c).
Fig. 2a: Cerebral cortex of AD stained with hematoxylin and eosin (x100), showing multiple neurons exhibiting many dark nuclei of pyramidal neurons surrounded by vacuolated cytoplasm. Blood vessels were dilated and contained spindle shaped cells.

Fig. 2b: Cerebral cortex of AD+MSCs stained with hematoxylin and eosin (x100) showing some neurons exhibiting dark nuclei.

These plaques became fewer in MSCs treated group (Fig. 3b) and occasional plaques could be observed in combined MSCs and EPO treated group (Fig. 3d).

In the present study, a high level of S100B immunoexpression was detected in the LPS induced AD group of mice (group II) (Fig. 4a). While in the MSCs or EPO treated AD groups, less S100B immuno-expression was found compared to the AD group.

Fig. 2c: Cerebral cortex of AD+EPO stained with hematoxylin and eosin (x100) showing multiple neurons exhibiting dark nuclei but less in comparison to the AD group. Blood vessels were dilated and congested.

Fig. 2d: Cerebral cortex of AD+MSCs+EPO stained with hematoxylin and eosin (x100), showing occasional dark nuclei (arrows).

The least S100B immuno-expression evident compared to the other experimental groups were shown in the group treated with combined MSCs and EPO (Fig. 4b).

Tracking of MSCs using CD105 immunostaining and PKH26 dye (a red flurochrome dye), was performed to groups III which were injected with MSCs: In LPS induced AD mice treated with stem cells (group III), cerebral sections showed multiple CD105 positive spindle...
Fig. 3a: Photomicrograph of a section in the cerebral cortex of AD stained with Congo red (x200), showing positive eosinophilic deposited material (P: plaques)

Fig. 3b: Photomicrograph of a section in the cerebral cortex of AD+MSCs stained with Congo red (x200) showing fewer Congo red positive eosinophilic deposited material (P: plaques) than those noted in AD cerebral cortex

Fig. 3c: Photomicrograph of a section in the cerebral cortex of AD+EPO stained with Congo red (x200), showing some Congo red positive eosinophilic deposited material (P: plaques)

Fig. 3d: Photomicrograph of a section in the cerebral cortex of AD+MSCs+EPO stained with Congo red (x200), showing occasional Congo red positive plaques (P)

shaped MSCs in the cerebral tissue (Fig. 5a). Cells were also tracked using fluorescent microscope in cerebral sections stained with PK26 dye (Fig. 5b). The same results were obtained when cerebral tissues of mice were treated with MSCs (group V).

Effect of MSCs and EPO on LPS-induced AD choline transferase activity, BDNF gene expression and mitochondrial swelling in the brain: The choline transferase enzyme activity for the AD group was significantly decreased when compared to the control group (642.4±123.6 vs. 1500±262.57 Pmol Ach/mg ptn/min). The LPS induced AD group treated with MSCs or EPO showed marked improvement when compared to AD group (1070±57.22, 1024±51.7 Pmol Ach/mg ptn/min vs. 642.4±123.6), respectively. While the group treated with the combined MSCs and EPO showed the most significant increase in choline transferase enzyme activity
Table 1: Comparison of choline transferase enzyme activity (Pmol Ach/mg pt/min), BDNF gene expression and mitochondrial swelling (OD/10^5 min) in all studied groups (groups I, II, III, IV and V)

<table>
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<tr>
<th>Parameters</th>
<th>Group (I)</th>
<th>Group (II)</th>
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<td></td>
<td>Control</td>
<td>AD</td>
<td>AD+MSCs</td>
<td>AD+EPO</td>
<td>AD+MSCs+EPO</td>
</tr>
<tr>
<td>Choline transferase enzyme activity (Pmol Ach/mg pt/min)</td>
<td>1500±62.57</td>
<td>642.4±123.6*</td>
<td>1070±57.22**</td>
<td>1024±51.7**</td>
<td>1166±47.3**</td>
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<td>BDNF gene expression</td>
<td>1.728±0.2697</td>
<td>0.284±0.1259**</td>
<td>0.679±0.07645**</td>
<td>0.4642±0.08575**</td>
<td>0.8692±0.06950**</td>
</tr>
<tr>
<td>Mitochondrial swelling (OD/10^5 min)</td>
<td>0.1237±0.05144</td>
<td>1.078±0.2180**</td>
<td>0.534±0.05752**</td>
<td>0.715±0.07**</td>
<td>0.3367±0.04716**</td>
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*<0.05 Statistically significant change compared to corresponding value in the control group I (p<0.05), AD group II (p<0.05), AD+MSCs group III (p<0.05), AD+EPO group IV (p<0.05), respectively. Values are Mean±SD

Fig. 4a: Photomicrograph of a section in the cerebral cortex of AD (group II) showing increased S100B immunoeexpression (S100B immunostaining, x200)

Fig. 4b: Photomicrograph of a section in the cerebral cortex of AD+MSCs+EPO (group V), showing very few S100B immunoeexpression (arrows) compared to Fig 4a (S100B immunostaining, x200) (1166±47.3 Pmol Ach/mg pt/min versus 642.4±123.6) when compared with the diseased group (Table 1).

Fig. 5a: Tracking of MSCs in brain tissues using CD105 immunostaining. Section in the cerebral, Cortex of group III (AD+MSCs), showing multiple CD 105 positive spindle cells (s) (x1000)

Fig. 5b: Labeled MSCs with PKH26 dye were detected using fluorescent microscope, in unstained brain tissues of group III that received MSCs, confirming that these cells were actually seeded into the brain tissues.

In the BDNF gene expression shown in Table 1, the AD group showed a significant decrease of 84% when
compared to the control group (0.2842±0.1259 vs. 1.728±0.2697). The BDNF gene expression for the treated groups with MSCs, EPO and both combined together when compared to the AD group showed a significant increase of 139, 63 and 206%, respectively in with the following mean values for, respectively (0.6792±0.07645, 0.4642±0.08575 and 0.8692±0.08959 vs. 0.2842±0.1259). The largest increase was observed in the group which received the combined treatment.

With regards to the mitochondrial swelling shown in Table 1, the AD group resulted in a very large increase in mitochondrial swelling when compared to the control group (1.078±0.2189 vs. 0.1237±0.05144OD/10^-2min). The swelling was reduced significantly in AD group treated with MSCs by 51% (0.53±0.05752OD/10^-2min), whereas the AD group treated with EPO showed less improvement (34%) with the mean value of (0.715±0.073OD/10^-2min). The LPS induced AD group that received the combined therapy of MSCs and EPO was the most successful and showed a significant reduction in mitochondrial swelling by 69% (0.3637±0.04716OD/10^-2min vs. 1.078±0.2189) when compared to AD group.

**DISCUSSION**

In this study we used an LPS-treated mice as a model of AD, based on previous study done by Viviani which proved that peripheral administration of LPS increases amyloid plaque accumulation and aggregation in brain tissues, which is considered a main hallmark of AD (Viviani et al., 2005).

The results of the present study are consistent with the belief that MSC-based therapies proved effective in ameliorating the effects of one of the most common neurodegenerative diseases, AD. In this context, we proved that EPO administration in combination with MSCs showed remarkable improvement of the AD condition and gave better results than the use of either MSCs or EPO alone. Our data suggests that this combined treatment caused full recovery of the memory deficit and ameliorate the AD pathology. It further enhances the differentiation of stem cells into cholinergic neurons as reflected by increased choline transferase activity. It also up regulates the BDNF expression in the affected brains which might contribute to the co-treatment of MSCs and EPO-mediated neuroprotection. This therapeutic approach could be used as an alternative way to improve the stem cell therapy.

In the present work, the spatial learning memory, a hippocampal-dependent form of memory, is highly impaired in the LPS-induced AD mice model. The current results demonstrated a significant reduction of the spontaneous percentage alternation in the Y maze of the AD group when compared with the control group which is consistent with previous reports (Corcoran et al., 2002; Lee et al., 2008). Furthermore, the motor activity was significantly suppressed in AD group indicating anxiety-induced locomotor activity. This is supported by previous findings of Swiergiel and Dunn (2007). Here we noticed almost complete improvement in the cognitive decline in the group of mice treated with the combined therapy of EPO and MSCs. Many authors showed that MSCs can give rise to neurons and glial-like cells in vitro by chemical or growth factor induction (Bossolasco et al., 2005; Lindvall and Kokaia, 2006; Levy et al., 2008). Munoz et al. (2005) proved that endogenous NSCs or progenitor cells are activated by chemokines secreted by MSCs (Munoz et al., 2005), the later acted directly on the NSCs to promote neurogenesis. However, it is also possible that factors secreted by the MSCs activated nearby astrocytes which in turn enhance neurogenesis, as they are located in close proximity to NSCs in the hippocampus (Nakayama et al., 2003; Song et al., 2002). Lledo et al. (2006) found a correlation between neurogenesis and cognitive function, indicating a link between these two processes (Lledo et al., 2006).

Considering the role of EPO as neuroprotective, Lee et al. (2012) investigated the therapeutic effects of EPO in animal models of AD, which is similar to this work, showing that the mice experienced an improvement in memory (Lee et al., 2012). It has been reported that EPO promotes brain repair by a number of mechanisms, including increased neurogenesis, modulation of inflammatory responses and increased recruitment of bone marrow-derived microglia into the brain (Viviani et al., 2005). In this light, it could suggest that EPO when added to MSCs could act in a synergetic way to potentiate neurogenesis in induced AD mice resulting in the rescue of the cognitive decline.

In the present study, cerebral sections of the AD induced mice showed multiple neurons exhibiting apoptotic features. Using Congo red stain, Congo red positive deposited material or plaques, blood vessels with deposited amyloid in their walls and increased area percentage of vascular angiopathy were markedly shown. Intraneuronal Aβ accumulation and fibrillization starts within cell bodies, neurites and synapses before plaque formation. Fibrillar Aβ aggregates were suggested to disrupt cytoarchitecture and pierce the cell membrane leading to degeneration of spines and neuritis. The current results are also in agreement with those of Lee et al. (2008) which demonstrated that systemic
inflammation induced by LPS caused beta amyloid deposition in the brain tissues through activation of β- and γ-secretases accompanied by inhibition of α-secretase, leading to elevated Aβ1–42 levels in both the cortex and hippocampus. Amyloid beta and proinflammatory cytokines activate microglia. Activated microglia, in turn, releases proinflammatory cytokines, such as IL-1-β, IL-6 and TNF-alpha. This co-elevated inflammation and amyloidogenesis results in apoptotic neuronal cell death and dysfunction (Lee et al., 2008).

The data from our study provides compelling evidence that the proposed combination therapy was the most successful line of therapy. There was maximum reduction in the percentage area of amyloid plaques and the percentage area of dark nuclei in the cerebral sections of the group of mice who received the combined therapy. This indicates that the use of both stem cells and EPO-based therapy appears likely to contribute to the improvement of the pathological picture of AD due to their neuroprotective and neuron replacement approaches.

As an important molecule in the pathogenesis of AD, Aβ has been linked to mitochondrial toxicity. Loss of mitochondrial volume homeostasis and accompanying mitochondrial swelling are the most striking signs of mitochondrial dysfunction and cell apoptosis (Du et al., 2011). In this study, the mitochondrial swelling observed in the LPS induced AD group was significantly reduced in the stem cell treated AD group compared to the EPO treated group and again the combination therapy showed the best results. It is interesting to note that the improvement in mitochondrial function goes in parallel with the reduction in apoptotic index and the Aβ confirming the link between them. Since mitochondrial damage is correlated to Aβ deposition in brain tissue, a reduction in Aβ deposition will spare mitochondrial damage. It has been reported that MSCs have a potential in reducing mitochondrial swelling and improving mitochondrial function indirectly through two mechanisms: by minimizing Aβ deposition and by their direct antioxidant effect. In a previous study done by Lee et al. (2012) it has been shown that bone marrow-derived MSCs reduce Aβ deposition and attenuated Aβ-induced apoptotic cell death when transplanted into induced AD mice brains. In addition, it diminished Aβ-induced oxidative stress (Lee et al., 2012).

The present study revealed a significant reduction in choline acetyltransferase (ChAT) enzyme activity in brain tissues of the LPS induced AD mice group compared to those of the control group which is in agreement with the work done by Liu et al. (2009). Degeneration of cholinergic neurons of the basal forebrain and neuronal cell loss were found predominantly in AD. In the advanced stages of late-onset AD, a severe loss of cortical cholinergic innervation has extensively been documented (Schliebs and Arendt, 2011). In the present study, the most significant ChAT activity was obtained with the combined treatment. This is consistent with the work done by Danielyan et al. (2009) who proved that MSCs exposed to EPO in vitro, showed a cholinergic neuron-like phenotype reflected by increased cellular levels of ChAT and ACh (Danielyan et al., 2009). They also showed that EPO enhanced the metabolism of Aβ protein in MSCs by increasing their neprilysin content. Their data indicated that cholinergic neuron-like differentiation of MSCs, their functionality and resistance to a neurotoxic environment are regulated and can be improved by EPO, highlighting its potential for optimizing cellular therapies of the CNS (Danielyan et al., 2009). Therefore, we cannot exclude a potential effect of the co-treatment MSCs and EPO on cholinergic neurons integrity and neurogenesis.

In the present study, BDNF gene expression was assessed in the brain tissues of all groups. The gene expression of the AD group showed a significant decrease of 84% compared to the control group. In agreement with our study, Schryndag et al. (2007) investigated changes in BDNF gene expression after the injection of a single dose of LPS into mice, where a marked decrease in BDNF was found after three days of injection when compared to their control group (Schryndag et al., 2007). Peng et al. (2009) stated that downregulation of BDNF in the cortex occurs early in the progression of AD (Peng et al., 2009). With respect to AD, BDNF has been shown to promote survival and differentiation of basal forebrain cholinergic neurons, as well as neurons in the hippocampus and cortex (Fahnstock et al., 2002). Since BDNF plays a critical role in neuronal survival, synaptic plasticity and memory, BDNF reduction may contribute to the synaptic and cellular loss and memory deficits characteristic of AD. It was also suggested that Aβ-associated neurotoxicity may be a consequence of BDNF deficiency (Schulte-Herbrüggen et al., 2008). The reduction of BDNF seen in AD could cripple the hippocampus in two ways: from a plasticity point of view, insufficient BDNF would weaken synaptic encoding strength or capacity and from a neurotrophic point of view, reduced BDNF makes hippocampal neurons more vulnerable to insult and degeneration (Lu, 2003).

Results of the present study demonstrated upregulation of BDNF in the AD group treated with MSCs which is consistent with the study of Wilkins et al. (2009). Furthermore, our study showed that treatment with EPO resulted in significant increase in BDNF expression when
compared to the AD group. The study of Viviani et al. (2005) supports our data (Viviani et al., 2005). Others proved that treatment with EPO in vivo significantly increased brain levels of BDNF and neurogenesis, suggesting that BDNF may stimulate neurogenesis. They suggested that EPO acts directly on cerebral endothelial cells that express EPO receptors and secrete BDNF which stimulates neurogenesis via a paracrine pathway. In addition, EPO also acts indirectly by inducing BDNF expression, which in turn augments the effect of EPO on neurogenesis (Wang et al., 2004). In the current study, BDNF gene expression obtained from mice receiving MSCs treatment alone was significantly higher than that in the group treated with EPO, highlighting the better efficacy of stem cells in secretion of BDNF which is essential for neuron outgrowth and synapse formation. The combined therapy of MSCs and EPO, on the other hand, proved to be most effective supporting the notion that EPO exerts a synergistic effect enhancing the differentiation of the MSCs and neurogenesis via increasing BDNF.

The S100B is a calcium-binding soluble protein released by glial cells. It is expressed in astrocytes and oligodendrocytes and used as a useful neurobiochemical marker of brain damage. It is associated with AD and may have a potential in predicting the efficiency of treatment and prognosis (Pena et al., 1995; Yordan et al., 2011). The results of this study showed that the S100 immunoexpression in all studied groups match the results of the other tested parameters in this work. As shown here, the AD group which received the combined treatment of EPO and MSCs was recorded to have the least S100 immunoexpression. This data provides strong evidence about the efficacy of the combined treatment to attenuate the brain damage in AD and to repopulate the affected neurons with new ones.

CONCLUSION

The current study demonstrated that using the combined therapy of EPO and MSCs on pathophysiologcal changes in AD offered the best results in all tested parameters. This could be due to the synergistic action of both EPO and MSCs against the pathophysiology of AD. Taken together, this data supports the potential use of such a combination as a promising new line of treatment for AD patients.

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REFERENCES


