Original Article

Impact of Intraventricular Human Adipose-Derived Stem Cells Transplantation with Pregnenolone Treatment on Remyelination of Corpus Callosum in A Rat Model of Multiple Sclerosis

Mohammad Mardani, Ph.D.[1]*, Raosul Ganji, Ph.D.[1], Nazem Ghasemi, Ph.D.[1], Mohammad Kazemi, Ph.D.[2], Shahnaz Razavi, Ph.D.[1]*

1. Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
2. Department of Genetics, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Corresponding Address: P.O.Box: 81746-73461, Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
Emails: mardani@med.mui.ac.ir, razavi@med.mui.ac.ir

Received: 11/August/2021, Accepted: 25/January/2022

Abstract

Objective: Multiple sclerosis (MS) is known as a nerve tissue disorder, which causes demyelination of central nervous system (CNS) fibers. Cell-based treatment is a novel strategy for the treatment of demyelinating diseases such as MS. Adipose-derived stem cells (ADSCs) have neuroprotective and neuroregenerative effects and pregnenolone as a neurosteroid has remarkable roles in neurogenesis. We intend to examine the impact of intraventricular transplantation of human ADSCs and systemic injection of pregnenolone on the remyelination of a rat model cuprizone-induced demyelination.

Materials and Methods: This experimental study was performed on 36 male Wistar rats that received a regular diet and a cuprizone diet for 3 weeks for M.S. induction. Through lipoaspirate surgery, human-ADSCs (hADSCs) were obtained from a patient. Six groups of rats (n=6): healthy, MS, sham, pregnenolone injection, ADSCs transplantation, and pregnenolone injection/ADSCs transplantation were included in this study. For assessment of remyelination, transmission electron microscopy (TEM), immunohistochemistry staining, real-time reverse transcription-polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) were performed.

Results: TEM outcomes revealed an increase in the thickness of the fibers myelin in the treatment groups (P<0.05). We also observed a significant upregulation of MBP, PDGFR-α, and MOG after treatment with hADSCs and pregnenolone compared to other study groups (P<0.001). These results were confirmed by immunostaining analysis. Moreover, there was no significant difference between the ADSCs/pregnenolone group and the control group regarding the level of MBP, A2B5, and MOG proteins in ELISA.

Conclusion: Our data implied that the remyelination and cell recovery were more improved by intraventricular ADSCs transplantation and pregnenolone injection after inducing a rat model of MS.

Keywords: Adipose-Derived Stem Cells, Intraventricular, Multiple Sclerosis, Pregnenolone

Cell Journal(Yakhteh), Vol 24, No 12, December 2022, Pages: 748-756

Citation: Mardani M, Ganji R, Ghasemi N, Kazemi M, Razavi Sh. Impact of intraventricular human adipose-derived stem cells transplantation with pregnenolone treatment on remyelination of corpus callosum in a rat model of multiple sclerosis. Cell J. 2022; 24(12): 748-756. doi: 10.22074/cellj.2022.8173.

Introduction

Multiple sclerosis (MS) is known as a nervous tissue disorder that causes demyelination of central nervous system (CNS) fibers. Degeneration of myelin sheath can create a complicated pattern of nervous system defects such as motor inability, visual problems, or mental disorders (1). The pathology of MS is described by lesions in CNS with perivascular inflammatory cell infiltration, demyelination, axonal destruction, neuronal decadence, and gliosis (2, 3).

MS is one of the most common neurological disorders in young adults worldwide. Overall, the prevalence of MS is 83 per 100000 individuals, and in women near two fold that of men (4, 5).

It is not yet clear what exactly causes MS, and there is currently no definitive cure for it; however, some treatments have been followed to reduce the number of *recurrence*s and slow the progression of the disease (6, 7). Cell-based treatment is a novel strategy for treating demyelinating diseases in CNS like MS (8).

In some studies, human embryonic stem cells, human bone marrow-derived mesenchymal stem cells (BM-MSCs), and human placental MSCs were used for transplantation in animal models of MS (9, 10). For example, a study on MSCs reported that they secrete various growth factors, especially facilitating oligodendrocyte differentiation (11).

hADSCs as a type of adult stem cells are able to differentiate into various cells and generate many identified neurotrophic factors, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) (12, 13). Some studies have demonstrated that hADS are able to exert their effects by crossing the blood-brain barrier (BBB) (8, 11).

Transplantation of hADSCs in an animal model of MS could induce the regeneration of myelin sheaths and improve the complications of MS (8).

Pregnenolone is a cholesterol-derived neurosteroid synthesized in the central and peripheral nervous system, mainly in glial and neuron cells that other neurosteroids such as progesterone, estrogen, and cortisol are derived from it (14). It is demonstrated that pregnenolone and its related derivatives such as pregnenolone sulfate, allopregnanolone, and dehydroepiandrosterone (DHEA) enhance memory and learning and, relieve depression, and improve the conceptual functions of the brain (15). Also, pregnenolone and other neurosteroid have neuroprotective roles in some neuroinflammatory diseases, including MS and Alzheimer's disease (AD), and can benefit totreatment some disorders like schizophrenia, depression, and autism (16-18).

ADSCs, with a high proliferation rate for a long time, secrete neurotrophic factors (19) and may improve the remyelination process owing to the concomitant use of cell and pregnenolone.

In our previous study, the human ADSCs and pregnenolone were used for transplantation in an animal model in which demyelination induced by cuprizone and their efficacy in improving the histological structure and cell regeneration in demyelinated nerve tissue were examined. However, we found that ADSCs intravenously injected into rat MS model, were able to pass the BBB, and transplanting cells could improve remyelination. In this study, we aimed to transplant hADSCs into the cerebral ventricles with pregnenolone treatment, and the results of this procedure were compared with systemic injection.

Materials and Methods

Experimental design

This research was approved by the Ethical Committee of Isfahan University of Medical Sciences (IR.MUI.REC.1395.1.041) and was conducted at the Central Laboratory of this university in 2018.

Thirty-six, eight-week-old male Wistar rats weighing 200 ± 20 g were purchased from the Pasteur Institute, Tehran, Iran. Rats were maintained under 12 hours light/dark cycle, at basic room temperature, and had access to common food and water. The animals were randomly divided into six groups (six rats in each group) as follows: control group without any intervention (C), MS control group (demyelination was induced using cuprizone) (cup), sham group (demyelinated rats received culture medium intraventricular), the fourth group (rats underwent demyelination and injection of pregnenolone intraperitoneally) (Preg), the fifth group (demyelinated rats received PKH26-labeled hADSCs intraventricular), and the sixth group (demyelinated rats received pregnenolone intraperitoneally and hADSCs intraventricular simultaneously (ADS/Preg).

Induction of demyelination model

The demyelination lesion was induced using cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc C9012) a copper-chelating agent. To obtain the desired cuprizone model, we gavaged the rats for three weeks with 0.6% cuprizone, dissolved in corn oil. Three weeks after cuprizone feeding, a histological examination of the brain was done to verify the demyelination. Treatments led to oligodendrocyte apoptosis and demyelination of fibers which was examined in the corpus callosum (20).

Culture and labeling of human adipose-derived stem cells

After obtaining informed consent confirmed by the Ethic Committee of Isfahan University of Medical Sciences, adipose tissue was obtained by needle biopsy or liposuction aspiration from healthy adult donors during liposuction surgery. The obtained tissue was washed with phosphate-buffered saline (PBS, Sigma-Aldrich, UK, 806552-500ML). For the digestion of fat tissue, the 0.075% collagenase type I (Sigma-Aldrich, UK, SCR103) was added to the sample and incubated for 30 minutes at 37˚C, with 5% CO_2. This enzyme was prepared in PBS containing 2% penicillin/streptomycin (P/S, Bioidia, BI1036, Iran). The collagenase activity was then neutralized by DMEM/F12 (Bioidia, BI1027, Iran) containing 10% fetal bovine serum (FBS, Gibco, 10270106, US).

Afterward, the centrifuge was done for 10 minutes at 1400 rpm; and then the cell pellet was re-suspended in DMEM/F12, 10% FBS, and 1% P/S and cultured at 37˚C in a 5% CO_2 incubator. The medium was twice replaced over one week. When approximately 80% cell confluence was achieved, the cells were passaged. Detachment of cells was performed by 0.25% trypsin and 0.02% Ethylenediaminetetraacetic acid (EDTA). In the present study, passages 2-4 h-ADSCs were used for transplantation (12).

Before transplantation, the cells were labeled by PKH-26 based on instructions [(2 μmol of PKH26 for 1×10^7 cells) (Sigma-Aldrich, MINI26)]. Briefly, PKH26 was added to 1×10^6 cells/ml. After 1-5 minutes the labeling was stopped using 1% bovine serum albumin (BSA, Sigma-Aldrich A2153-10G, UK). To determine the percentage of labeled cells, hADSCs were assessed by fluorescent microscopy (Olympus BX51, Japan) after staining. Also, the survival of labeled cells was evaluated based on the culture of a portion of these cells (19).

Stereotaxic surgery, cell microinjection, and pregnenolone injection

Under deep anesthesia with an intraperitoneal injection (IP) of 10% ketamine (100 mg/kg) and 2% xylazine (10 mg/kg), the rats were positioned in a stereotaxic apparatus (Stoelting, USA). The scalp was dissected and the skull was cleaned. The lateral ventricles coordinates based on the Paxinos and Watson atlas (21) were as follows:

AP: -0.84 mm posterior to bregma; ML: ± 1.6 mm from the sagittal suture; and DV: 3-4 mm ventral from the skull surface.

Following a week of final cuprizone feeding for cell microinjections, 30-gauge stainless steel injector needles, connected to a Hamilton syringe by polyethylene tubing (PE-20), were stereo tactically proceeded to reach the lateral ventricles; subsequently, the solution was injected in a total volume of 5 ml/side containing 1×10^6 cell, over a 60 second period; the solution was then left in place for an extra 60s to facilitate the diffusion.

Also, pregnenolone sulfate (Pregnenolone sulfate sodium salt, Sigma-Aldrich, P162) was injected IP daily (2.5 mg) and dissolved in DMSO over the three weeks of cuprizone feeding.

Electron microscopic study

Rats were transcardially perfused with 1.6% glutaraldehyde (Sigma-Aldrich G5882, UK) in PBS (0.12 M, pH=7.4). The corpus callosum was detached and fixed by 1% osmium tetroxide (Sigma-Aldrich, UK, 75632) and embedded in epoxy resin after dehydration in graded ethanol. After embedding in resin, ultrathin 70 nm sections were prepared and stained with uranyl acetate and lead citrate and observed via a transmission electron microscopy (TEM, LEO 906 Germany, 100 kV). Images were taken from the corpus callosum after removing it from the rat brain. Pictures of the fibers cut in cross-section were taken and myelin status was assessed using ten images per specimen (×3000) analyzed by Digimizer Image Analysis Software 5.3.5 (copyright © 2005-2019 MedCalc software). The percentage of myelinated axons, axon diameter, myelin thickness, and G-ratio were measured using 50 fibers per sample. The G-ratio was calculated by the axon diameter/entire fiber diameter ratio. hence, a completely demyelinated fiber has a G-ratio of 1, whereas, in the myelinated fibers, the G-ratio is <1 (22).

Immunohistochemistry technique

The immunohistochemistry technique was accomplished to evaluate the myelin repair process and the rate of oligodendrocyte cell reproduction. For fixation of animals' brains, transcardial perfusion was done by 4% paraformaldehyde (PFA, Sigma, Germany) in 0.1 M PBS, pH=7.4, and post-fixation in the same fixative was followed overnight after brain removal. Then 3 µm-thick sections were prepared from Paraffin-embedded samples. After deparaffinization, rehydration, and heat unmasking, the samples were blocked with 10% normal goat serum, and then the samples were exposed overnight at 4˚C to the following primary antibodies: Mouse monoclonal Anti-MBP (1:1000; Abcam, Cambridge, MA, USA), Goat polyclonal Anti-MOG (1:1000; Abcam, Cambridge, MA, USA), Mouse monoclonal Anti-A2B5 (1:1000; Abcam, Cambridge, MA, USA. A2B5 is a cell surface ganglioside epitope expressed in oligodendrocyte progenitor cells.

The next day, the sections were washed by PBS and exposed to Goat anti-mouse and Anti-Goat (FITC) (1:500; Abcam, Cambridge, MA, USA)-conjugated secondary antibodies at room temperature for 1 hour. For cell nucleus staining we used 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. Finally, the number of MBP, MOG, and A2B5 positive cells in each section was counted using a fluorescence microscope (Olympus, BX51, Japan) (13).

Real-time reverse transcription-polymerase chain reaction analysis

The real-time reverse transcription-polymerase chain reaction (RT-PCR) method was used to evaluate the expression level of myelin basic protein (*MBP*), myelin oligodendrocyte glycoprotein (*MOG*), and platelet-derived growth factor receptor α (*PDGFR-α*) genes in the corpus callosum. Because there was no A2B5 primer, the *PDGFR-α* gene was employed to detect oligodendrocyte precursor cells. Total RNA was extracted from corpus callosum samples in all studied animals by Total RNA Prep Kit as stated in the manufacturer's protocol (BIOFACT, Korea). Reverse transcription of The RNA was performed by BioFact™ 5X RT Pre-Mix cDNA Synthesis Kit (BIOFACT) and oligo dT primers. To ensure that there is no DNA, the obtained RNA was treated with an RNase-free DNase kit (Qiagen, Germany).

The real-time RT-PCR was carried out by BIOFACT™ 2X Real-Time PCR Master Mix (BIOFACT) using the specifically mentioned primers and performed on a Step One Plus™ system (Applied Biosystems, US). The expression level of *MBP*, *MOG*, and *PGFRα* genes was assessed by quantitative real-time RT-PCR and compared to *β-actin* as a housekeeping gene (13). The PCR amplification conditions consisted of 15 minutes at 95˚C followed by 40 cycles of denaturation step at 95˚C for 20 seconds and annealing and extension for 1 minute at 60˚C. Melting curve analysis was used to determine the melting temperature of specific amplification products and primer. The comparative expression level of intended genes was calculated using the $2^{-\Delta\Delta ct}$ method. The primers were designed with Gene Runner 4.0 and tested by BLAST [http://blast. ncbi.nlm.nih.gov/Blast.cgi] to determine attachment to rats 'genome. The designed primers are presented in Table 1.

Enzyme-linked immunosorbent assay

The ELISA method was performed to evaluate the amount of MBP, MOG, and A2B5 protein. The rat's brain was stored at -70˚C after removal. When performing the ELISA process, corpus callosum separated from the brains and tissues was homogenized by a homogenizer. The homogenates were rotated at 6000 rpm for 10 minutes, and the pellets were resuspended in water at a pH of 3.0 for 1 hour. Then centrifuge at 10,000 rpm was done for 1 hour, and supernatants were stored at -20˚C at a pH of 8.8 by Tris-HCL buffer.

To perform the ELISA test, 50 µL of the sample was coated in each well, and on the next day after washing, 200 µL of blocking agent (5% BSA in PBS) was added

and incubated at 4˚C overnight. Next, washing was repeated 4 times, and 100 µL primary antibody was added as follows: anti-myelin basic protein (MBP) FITC antibody, anti-A2B5 FITC antibody, and anti-myelin oligodendrocyte glycoprotein (MOG) FITC antibody. The samples were incubated at 4˚C overnight. Then, after washing again, the plates were treated with the conjugated secondary antibody (goat anti-mouse HRP) for one hour. Then treatment by 100 µL tetramethylbenzidine (TMB) was done for 15 minutes at room temperature. Eventually, the ELISA reader measured optical density (OD) values of each sample at 450 nm and they were converted into concentration in pg/ml (23).

Statistical analysis

The results of the evaluation techniques were determined as mean± standard error of the mean (S.E.M). Data analysis was performed using SPSS 24 (IBM, US) software and the one-way analysis of variance (ANOVA), followed by the LSD post-hoc test. $P<0.05$ was defined for statistical significance.

Results

Transmission electron microscopy study

Transmission electron microscopic (TEM) images were applied to characterize myelin morphometric parameters, in the experimental groups. These photographs were obtained from the coronal sections of corpus callosum fibers and assessed by Digimizer Image Analysis Software to measure the myelinated axons percentage, axon diameter, thickness of myelin, and G-ratio. Three weeks after cuprizone feeding began the myelin of corpus callosum fibers degenerated. Myelin and axon degradation and also vacuole formation is seen in cuprizone and sham groups. In the treatment groups, especially the ADS/Preg group, myelination had been done relatively well (Fig.1A).

The mean of myelinated axons of the treatment groups was significantly increased compared with the cuprizone groups ($P<0.05$). Evaluation of TEM images showed a significant increase in the mean thickness of myelinated axons in the corpus callosum in the treatment groups compared with cuprizone and sham groups ($P<0.05$), but no significant difference with the control group was observed. Furthermore, a significant increase was observed in the mean of the G-ratio in cuprizone, and sham groups compared with the control group ($P<0.05$). On the other hand, in the treatment groups, the mean of this ratio was significantly reduced ($P<0.05$, Fig.1B).

Table 1: The designed both rat and human primers sequences for each gene

Gene	Primer sequences (5′-3′)	Annealing temperature (˚C)	PCR size bp
ACTB-H	F: GTTGTCGACGACGAGCG	60	93
	R: GCACAGAGCCTCGCCTT		
Actb-R	F: AGGCCCCTCTGAACCCTAAG	60	118
	R: CCAGAGGCATACAGGGACAA		
MBP-H	F: GGCCCCGTGGATGGA	60	76
	R: GAGGCGCGAAAGGAGATG		
Mbp-R	F: CACAGAAGAGACCCTCACAGCGAC	60	136
	R: CCGCTAAAGAAGCGCCCGATGGA		
MOG-H	F: ACCAGGCACCTGAATATCGG	60	195
	R: CAGGGCTCACCCAGTAGAAAG		
Mog-R	F: GAGGTTCTCGGATGAAGGAG	60	110
	R: CAGGGTTGATCCAGTAGAAGG		
PDGFR-H	F: TTGAAGGCAGGCACATTTACA	60	119
	R: GCGACAAGGTATAATGGCAGAAT		
Pdgfrα -R	F: AATGAAGGTGGCTGTGAAGATGC	60	102
	R: AGATGCGGTCCCAAGTGAGTC		

PCR; Polymerase chain reaction.

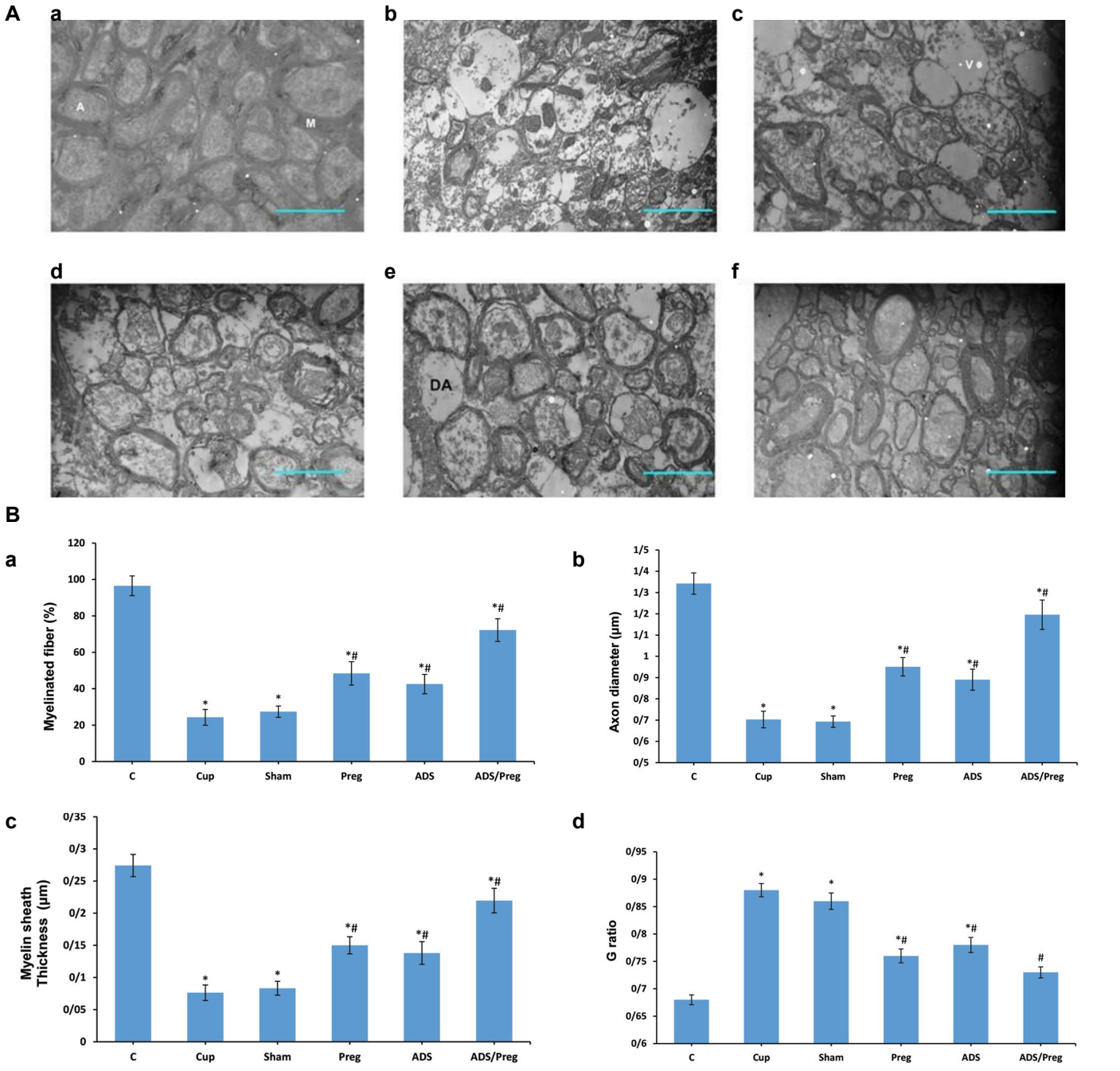

Fig.1: Transmission electron microscopic (TEM) study of the comparing groups. **A.** The TEM photos from the corpus callosum fibers of the rats' brains in the comparing groups**.** Demyelination of nerve fibers in the cuprizone and sham groups can be observed along with the destruction of demyelinated axons (DA) and vacuole formation (V). In ADS/Preg group, nerve fibers are properly remyelinated (TEM 2000x) (scale bar: 2 µm). **B.** Quantitative analysis of electron microscopy images**.** The mean percentage of myelinated fiber (a), axon diameter (b) and myelin thickness (c) in ADS/Preg group significantly increased compared with cuprizone and sham groups. Furthermore, the G-ratio (d) in the treatment groups, particularly ADS/Preg, was significantly reduced compared with cuprizone and sham groups. (mean ± SE) *; Significant difference with control group, #; Significant difference with cuprizone and sham group ($P<0.05$), C; Control group with any intervention, Cup; Cuprizone group, Sham; Sham group (received culture medium), Preg; Pregnenolone receiving group, ADS; hADSCs receiving group, and ADS/Preg; hADSCs and pregnenolone receiving group.

Real-time reverse transcription-polymerase chain reaction analysis

In this study, the source of ADSCs was from humans and examination was performed on rat corpus callosum to specify the level of myelination and oligodendrocyte retrieval; thus, rat and also human genes were analyzed. The results obtained from real-time RT-PCR showed that the injection of cuprizone significantly downregulated *Mbp*, *Mog*, and *PDGFR-α* genes in cuprizone and sham groups compared with the control group ($P<0.05$). However, *Mbp*, *Mog*, and *Pdgfr-α* expression significantly increased following treatment with hADSC and pregnenolone compared with cuprizone and sham groups. In the group simultaneously receiving hADSC and pregnenolone, the gene expression of *Mbp*, *Mog*, and *Pdgfr-α* increased even more than in healthy controls. Also, human *MBP, MOG* and *PDGFR-α* genes were significantly upregulated in ADS/Preg group in comparison to ADS group (Fig.2).

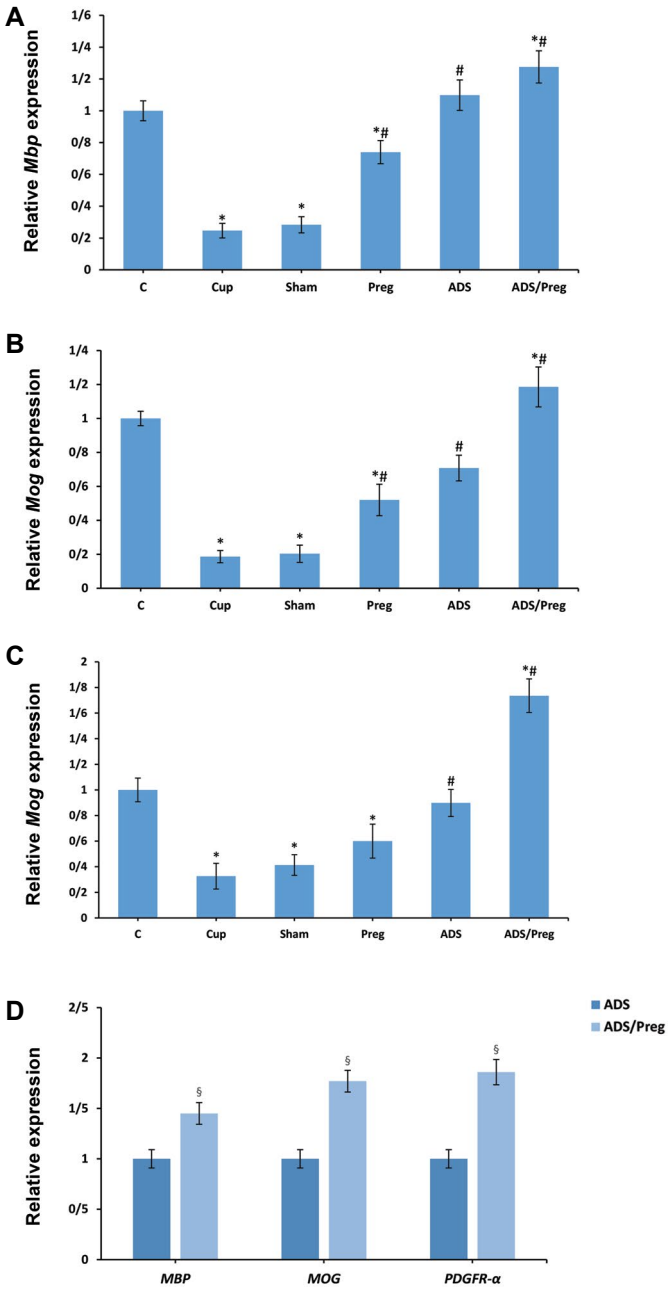

Fig.2: Comparison of the mean levels of *Mbp, Mog* and *Pdgfr-α* expression in corpus callusom of different groups using real time RT-PCR technique. **A.** The mean expression level of *Mbp* gene in ADS/Preg group significantly increased compared with other groups. **B.** The expression of *Mog* gene in ADS/Preg group significantly increased compared with other groups. **C.** The expression of *Pdgfr-α* gene in ADS/Preg group significantly increased compared with cuprizone, sham, and other treatment groups (mean ± SE). **D.** Human *MBP* and *MOG* and *PDGFR-α* genes were significantly upregulated in ADS/Preg group in comparison to ADS group. *; Significant difference with control group, #; Significant difference with cuprizone and sham group ((P<0.05), §: Significant difference between the ADS/Preg group and ADS and Preg groups, C; Control group with any intervention, Cup; Cuprizone group, Sham; Sham group (received culture medium), Preg; Pregnenolone receiving group, ADS; hADSCs receiving group, and ADS/Preg; hADSCs and pregnenolone receiving group.

Immunohistochemistry assay

After 3-4 cell passages of isolated hADSCs, the cells attached to the floor of the flasks had a homogenous morphology. The cells were then labeled by PKH26 and checked by fluorescent microscopy. The results showed that more than 85% of the isolated cells were labeled with PKH26.

Evaluation of the number of oligodendrocyte cells and remyelination in the corpus callosum was implemented by the IHC. Specific markers of immature oligodendrocyte (A2B5), mature oligodendrocyte (MOG), and myelination (MBP) were chosen for cell staining (Fig.3).

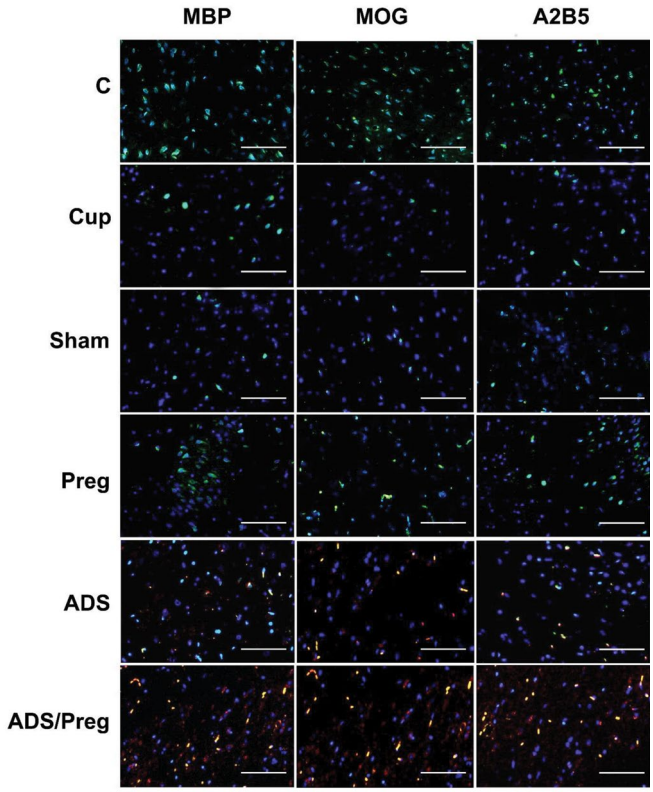

Fig.3: Immunohistochemistry images of paraffin section corpus callusom of studied groups for MBP, MOG and A2B5 four weeks after cell transplantation. The cells were depicted with a fluorescein isothiocyanate (FITC) (green), and 4' 6-diamidino-2-phenylindole (DAPI) (blue) for nuclear counterstaining. The PKH26 labeled cells (hADSCs) were red (scale bar: 50 µm). MBP; Myelin basic protein, MOG; Myelin oligodendrocyte glycoprotein, C; Control group with any intervention, Cup; Cuprizone group, Sham; Sham group (received culture medium), Preg; Pregnenolone receiving group, ADS; hADSCs receiving group, and ADS/Preg; hADSCs and pregnenolone receiving group.

Comparing the mean percentage of MBP, MOG, and A2B5 positive cells in different groups showed the mean percentage of MBP positive cells in all of the treated groups had a significant difference compared to the control group (P<0.05). Meanwhile, there was a significant increase in the Preg and ADS/Preg groups compared to the sham and cuprizone groups (P<0.05). Furthermore, the mean percentage of MOG-positive cells in treated groups showed a significant difference compared to the control group (P<0.05). However, the mean percentage of MOG-positive cells was significantly increased in the ADS and ADS/Preg groups compared to the sham and cuprizone groups (P<0.05). Similarly, the mean percentage of A2B5 positive cells showed a significant difference in all of the treated groups compared to the control group (P<0.05), but the ADS and ADS/Preg groups had a significant increase compared to sham and cuprizone groups (P<0.05, Fig.4).

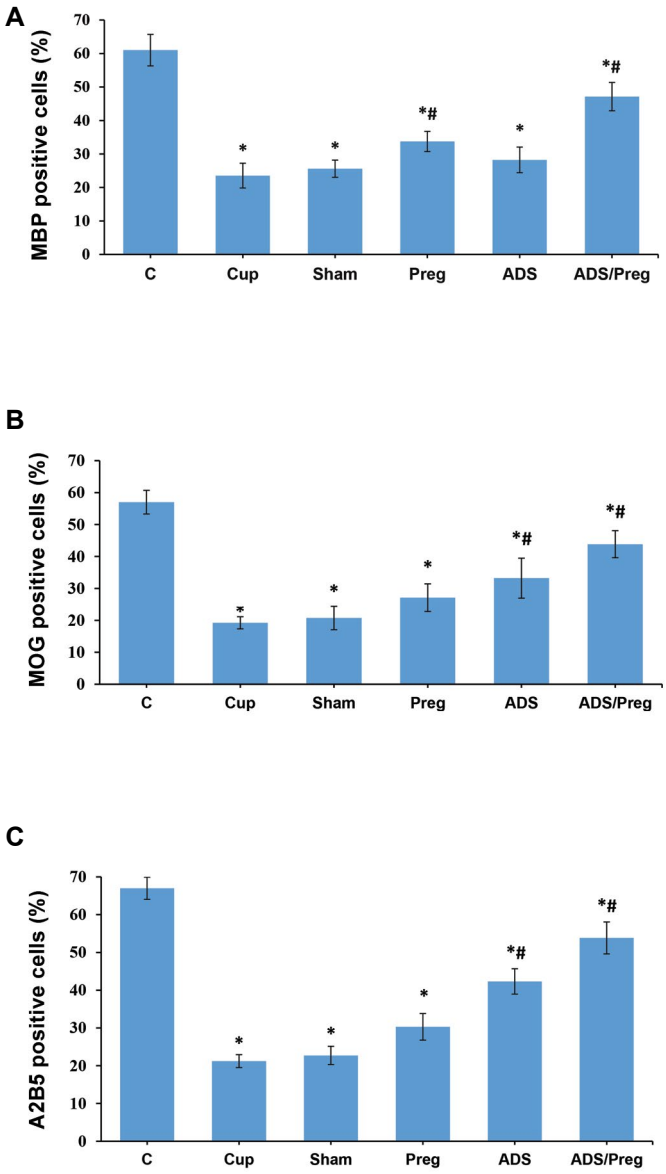

Fig.4: Comparison of the mean percentage of MBP, MOG and A2B5 positive cells in different groups. **A.** The mean percentage of myelin with MBP marker in all groups compared to the control group showed a significant difference (P<0.05). This percentage in the Preg and ADS/Preg treatment groups had a significant increase compared to the sham and cuprizone groups (P<0.05). **B.** The percentage of MOG positive cells in all groups compared to the control group showed a significant difference (P<0.05). This percentage had a significant increase in all treatment groups compared to sham and cuprizone groups (P<0.05). **C.** Similarly, the percentage of A2B5 positive cells in all groups compared to the control group showed a significant difference (P<0.05). This percentage in ADS and ADS/Preg treatment groups had a significant increase compared to sham and cuprizone groups. *; Significant difference with the control group, #; Significant difference with cuprizone and Sham groups (P<0.05), MBP; Myelin basic protein, MOG; Myelin oligodendrocyte glycoprotein, C; Control group with any intervention, Cup; Cuprizone group, Sham; Sham group (received culture medium), Preg; Pregnenolone receiving group, ADS; hADSCs receiving group, and ADS/Preg; hADSCs and pregnenolone receiving group.

Enzyme-linked immunosorbent assay

The protein level of MBP, MOG, and A2B5 significantly decreased in cuprizone, and sham groups compared to the control group (P<0.05), as presented in Figure 5. The protein levels of MBP, MOG, and A2B5 in the groups treated with hADSCs and pregnenolone were significantly higher than in the cuprizone and sham groups *(*P<0.05*)*. Treatment with hADSCs and pregnenolone in ADS/Preg increased the protein levels more than in other treatment groups, but no significant differences were observed (Fig.5).

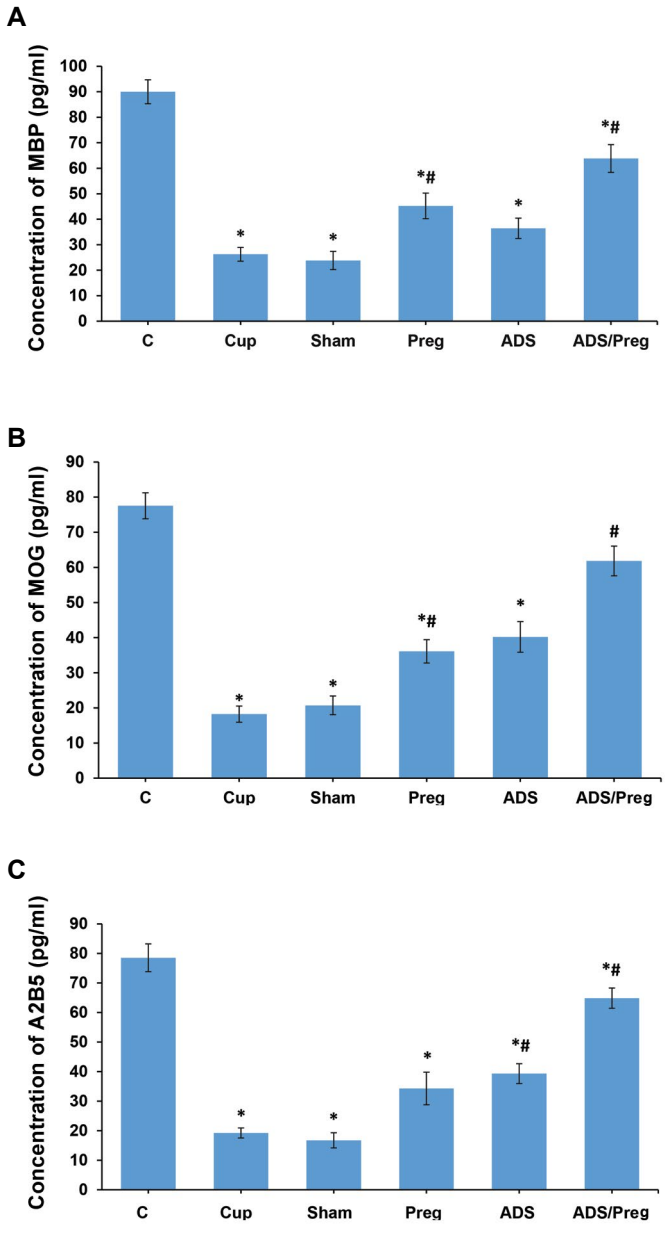

Fig.5: Comparison of the mean levels of MBP, MOG and A2B5 concentration in different groups using ELISA technique. The concentration of **A.** MBP, **B.** MOG and **C.** A2B5 proteins in groups reveals that the ADS/Preg and control group have no significant difference, but there is a significant increase in ADS/Preg group compared to the cuprizone and sham groups. *; Significant difference with control group, #; Significant difference with cuprizone and sham group (P<0.05), MBP; Myelin basic protein, MOG; Myelin oligodendrocyte glycoprotein, C; Control group with any intervention, Cup; Cuprizone group, Sham; Sham group (received culture medium), Preg; Pregnenolone receiving group, ADS; hADSCs receiving group, and ADS/Preg; hADSCs and pregnenolone receiving group.

Discussion

MS is a nerve tissue disorder specified by autoimmune and chronic inflammatory demyelination and degeneration of the CNS fibers (24).

Presently, some modulator drugs are used for treating MS, all of which influence the immune system (25), but there is no complete cure. Most medications have sedative and progress reduction aspects and remarkable drug efficacy is expected On the other hand, there are some side effects with them (26). Accordingly, it's essential to provide safer and complete treatments for this disorder.

Cell therapy is an interesting and effective strategy for treating neurodegenerative diseases; so far, different types of stem cells, for example, embryonic and MSCs, have been employed for this purpose. ADSCs have attracted the attention of many researchers because adipose tissues are abundant and have a large number of stem cells (27).

In this study, cuprizone was used orally to induce a model of MS in rats. Based on the obtained results, the MS model was induced effectively in the rats of the cuprizone-treated groups.

Cuprizone is a copper chelating agent that, when orally administrated, causes widespread demyelination in CNS fibers. After stopping cuprizone feeding, remyelination will resume, so this demyelination is dependent on the continuous feeding of cuprizone (20, 28). Corpus callosum is one of the main targets of cuprizone. Accumulation of microglia/macrophages in the corpus callosum is another effect of cuprizone (29).

Remyelination is the process of differentiation of oligodendrocytes from precursor cells, reconstruction of myelin sheaths on demyelinated fibers, prevention additional axonal damage, and re-establish saltatory conduction (30).

In our previous work, the human ADSCs and pregnenolone were used simultaneously through systemic injection in an animal model demyelination by cuprizone. We used behavior tests, histological examinations, real-time RT-PCR, and ELISA technique to evaluate functional status, remyelination progress, and myelin-producing cells recovery following 4 weeks. It was demonstrated that several ADSCs can migrate through blood circulation, cross the BBB and improve remyelination. Moreover, pregnenolone administration results in an increase in ADSCs differentiation into oligodendrocytes (31).

In the current study, to determine the effects of locally transplanted cells, near or at the site of injury, intraventricular transplantation of h-ADSCs and systemic injection of pregnenolone on remyelination in the corpus callosum of MS rat model induced by cuprizone.

Results of real-time RT-PCR and immunohistochemistry methods showed that transplantation of ADSCs into a model of MS leads to significant expression of some specific genes and markers of oligodendrocyte such as MOG, PDGFR-α, and A2B5 in ADS/Preg group than the other groups.

This process indicated an increase in the differentiation of oligodendrocytes from ADSCs, which was confirmed by increasing MBP expression by various methods. On the other hand, in this study, there was an increase in axon number and myelin thickness, which was confirmed by the TEM study. However, an increase in the amount and thickness of myelin suggests that differentiated oligodendrocytes may have the ability to regulate transcription of the major myelin genes that usually occur during active myelination (32). In addition, the increase in myelination by the ADS/Preg group may be due to the synergistic effect of the simultaneous use of ADSCs and pregnenolone.

Several studies have reported that the main mechanisms responsible for the therapeutic effects of stem cell transplantation are related to neurotrophic function and differentiation potential of stem cells in CNS (9, 10, 31).

The results of the current study were consistent with other studies. Aharonowiz et al. (33) found that human embryonic stem cell transplantation into the rat cerebral cortex had the potential to reduce the clinical signs in these models of MS and showed neuroprotective effects by suppressing the immune system. In addition, Burt et al. (34) showed that the use of hematopoietic stem cells incapable of producing myelin in patients with relapsing-remitting MS could reduce the disease progression and improve neurological symptoms.

In addition, the co-cell transplantation of hADSCs with neurotrophic factor secreting cells leads to more remyelination than transplantation of human ADSCs (19), and human ADSCs can differentiate into oligodendrocytes and enhance the remyelination process, possibly improving the motor functions (8).

We observed similar results given the fact that the number of positive cells for MBP, A2B5, and MOG significantly increased in the rats receiving ADSCs and pregnenolone as compared to other groups; this suggests that pregnenolone can have synergistic effects in the differentiation of ADSCs into oligodendrocyte.

Conclusion

Intraventricularly human ADSCs transplantation with pregnenolone injection can effectively improve remyelination of demyelinated nerve fibers in corpus callosum after inducing rat model of MS as compared to systemic transplantation of human ADSCs and pregnenolone treatment may be synergistic effects during differentiation of ADSCs into oligodendrocyte.

Acknowledgments

The author appreciates the Isfahan University of Medical Sciences for supporting this study (grant number: 195041). We would also like to thank the operating room of Alzahra Hospital for preparing the human adipose tissue. There is no conflict of interest in this study.

Authors' Contributions

M.M., R.G. Sh.R.; Contributed to conception and design. R.G.; N.Gh., M.K.; Contributed to all experimental work, data, and statistical analysis, and interpretation of data.

M.M., Sh.R.; Were responsible for overall supervision. R.G.; Drafted the manuscript, which was revised by Sh.R. All authors read and approved the final manuscript.

References

1. Ghasemi N, Razavi S, Nikzad E. Multiple sclerosis: pathogenesis, symptoms, diagnoses and cell-based therapy. Cell J. 2017; 19(1): 1-10.
2. Cohen JA, Imrey PB, Planchon SM, Bermel RA, Fisher E, Fox RJ, et al. Pilot trial of intravenous autologous culture-expanded mesenchymal stem cell transplantation in multiple sclerosis. Mult Scler. 2018; 24(4): 501-511.
3. Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci. 2008; 31: 247-269.
4. Kingwell E, Marriott JJ, Jetté N, Pringsheim T, Makhani N, Morrow SA, et al. Incidence and prevalence of multiple sclerosis in Europe: a systematic review. BMC Neurol. 2013; 13(1): 1-13.
5. Pugliatti M, Rosati G, Carton H, Riise T, Drulovic J, Vécsei L, et al. The epidemiology of multiple sclerosis in Europe. Eur J Neurol. 2006; 13(7): 700-722.
6. Boster A, Edan G, Frohman E, Javed A, Stuve O, Tselis A, et al. Intense immunosuppression in patients with rapidly worsening multiple sclerosis: treatment guidelines for the clinician. Lancet Neurol. 2008; 7(2): 173-183.
7. Stepien A, Dabrowska NL, Maciagowska M, Macoch RP, Zolocinska A, Mazur S, et al. Clinical application of autologous adipose stem cells in patients with multiple sclerosis: preliminary results. Mediators Inflamm. 2016; 2016: 5302120.
8. Ghasemi N, Razavi S, Mardani M, Esfandiari E, Salehi H, Esfahani SHZ. Transplantation of human adipose-derived stem cells enhances remyelination in lysolecithin-induced focal demyelination of rat spinal cord. Mol Biotechnol. 2014; 56(5): 470-478.
9. Bai L, Lennon DP, Eaton V, Maier K, Caplan AI, Miller SD, et al. Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. Glia. 2009; 57(11): 1192-1203.
10. Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells. 2010; 28(1): 152-163.
11. Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E, et al. Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells. 2009; 27(10): 2624-2635.
12. Razavi S, Mardani M, Kazemi M, Esfandiari E, Narimani M, Esmaeili A,et al. Effect of leukemia inhibitory factor on the myelinogenic ability of Schwann-like cells induced from human adipose-derived stem cells. Cell Mol Neurobiol. 2013 ; 33(2): 283-289.
13. Razavi S, Razavi MR, Kheirollahi-Kouhestani M, Mardani M, Mostafavi FS. Co-culture with neurotrophic factor secreting cells induced from adipose-derived stem cells: promotes neurogenic differentiation. Biochem Biophys Res Commun. 2013; 440(3): 381-387.
14. Agís-Balboa R C, Pinna G, Zhubi A, Maloku E, Veldic M, Costa E, et al. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. Proc Natl Acad Sci USA. 2006; 103(39): 14602-14607.
15. Vallée M, Mayo W, Le Moal M. Role of pregnenolone, dehydroepiandrosterone and their sulfate esters on learning and memory in cognitive aging. Brain Res Rev. 2001; 37(1-3): 301-312.
16. Daugherty DJ, Selvaraj V, Chechneva OV, Liu XB, Pleasure DE, Deng W. A TSPO ligand is protective in a mouse model of multiple sclerosis. EMBO Mol Med. 2013; 5(6): 891-903.
17. Naylor JC, Kilts JD, Hulette CM, Steffens DC, Blazer DG, Ervin JF, et al. Allopregnanolone levels are reduced in temporal cortex in patients with Alzheimer's disease compared to cognitively intact control subjects. Biochim Biophys Acta. 2010; 1801(8): 951-959.
18. Vallée M. Neurosteroids and potential therapeutics: focus on pregnenolone. J Steroid Biochem Mol Biol. 2016; 160: 78-87.
19. Razavi SR, Ghasemi N, Mardani M, Salehi H. Co-transplantation of human neurotrophic factor secreting cells and adipose-derived stem cells in rat model of multiple sclerosis. Cell J. 2018; 20(1): 46.
20. Torkildsen Ø, Brunborg L, Myhr K M, Bø L. The cuprizone model for demyelination. Acta Neurol Scand. 2008; 117: 72-76.
21. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. Oxford: Academic Press; 2007: 62.
22. Hedayatpour A, Ragerdi I, Pasbakhsh P, Kafami L, Atlasi N, Mahabadi VP, et al. Promotion of remyelination by adipose mesenchymal stem cell transplantation in a cuprizone model of multiple sclerosis. Cell J. 2013; 15(2): 142-151.
23. Guan D, Li P, Zhang Q, Zhang W, Zhang D, Jiang J. An ultra-sensitive monoclonal antibody-based competitive enzyme immunoassay for aflatoxin M1 in milk and infant milk products. Food Chem. 2011; 125(4): 1359-1364.
24. Milo R, Miller A. Revised diagnostic criteria of multiple sclerosis. Autoimmun Rev. 2014; 13(4-5): 518-524.
25. Baecher-Allan C, Kaskow BJ, Weiner HL. Multiple sclerosis: mechanisms and immunotherapy. Neuron. 2018; 97(4): 742-768.
26. Namjooyan F, Ghanavati R, Majdinasab N, Jokari S, Janbozorgi M. Uses of complementary and alternative medicine in multiple sclerosis. J Tradit Complement Med. 2014; 4(3): 145-152.
27. Sen A, Lea-Currie YR, Sujkowska D, Franklin DM, Wilkison WO, Halvorsen YDC, et al. Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. J Cell Biochem. 2001; 81(2): 312-319.
28. Skripuletz T, Gudi V, Hackstette D, Stangel M. De-and remyelination in the CNS white and grey matter induced by cuprizone: the old, the new, and the unexpected. Histol Histopathol. 2011; 26(12): 1585-1597.
29. Hiremath M, Saito Y, Knapp G, Ting JY, Suzuki K, Matsushima G. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. J Neuroimmunol. 1998; 92(1-2): 38-49.
30. Franklin RJ. Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci. 2008; 9(11): 839-855.
31. Ganji R, Razavi S, Ghasemi N, Mardani M. Improvement of remyelination in demyelinated corpus callosum using human adipose-derived stem cells (hADSCs) and pregnenolone in the cuprizone rat model of multiple sclerosis. J Mol Neurosci. 2020; 70(7): 1088-1099.
32. Ishii A, Fyffe-Maricich SL, Furusho M, Miller RH, Bansal R. ERK1/ERK2 MAPK signaling is required to increase myelin thickness independent of oligodendrocyte differentiation and initiation of myelination. J Neurosci. 2012; 32(26): 8855-8864.
33. Aharonowiz M, Einstein O, Fainstein N, Lassmann H, Reubinoff B, Ben-Hur T. Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis. PLoS One. 2008; 3(9): e3145.
34. Burt RK, Loh Y, Cohen B, Stefoski D, Balabanov R, Katsamakis G, et al. Autologous non-myeloablative haemopoietic stem cell transplantation in relapsing-remitting multiple sclerosis: a phase I/II study. Lancet Neurol. 2009; 8(3): 244-253.