17β-Estradiol Enhances the Efficacy of Adipose-Derived Mesenchymal Stem Cells on Remyelination in Mouse Model of Multiple Sclerosis

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Abstract- Previous studies have demonstrated the potential of monotherapy with either mesenchymal stem cells (MSCs) or estrogen in autoimmune and cuprizone models of multiple sclerosis (MS). The aim of this study was to examine the effects of co-administration of 17β-estradiol (E2) and adipose-derived mesenchymal stem cells (ADSCs) on remyelination of corpus callosum axons in a cuprizone model of MS. Forty eight male C57BL/6 mice were fed cuprizone (0.2%) for 6 weeks. At day 0 after cuprizone removal, animals were randomly divided into four groups. The E2 monotherapy, ADSCs monotherapy, E2/ADSCs combined therapy and vehicle control. Some mice of the same age were fed with their normal diet to serve as healthy control group. E2 pellets, designed to release 5.0 mg E2 over 10 days, were implanted subcutaneously. 10^6 PKH26 labeled ADSCs were transplanted into lateral tail. The extent of demyelination, remyelination, and cell type's composition of host brain were examined at 10 days post-transplantation in the body of the corpus callosum. Transplanted cells migrated to the corpus callosum injury. Histological examination revealed efficacy of intravenous ADSCs transplantation in remyelination of mouse cuprizone model of MS can be significantly enhanced by E2 administration. Flow cytometry showed that the mean percentages of expression of Iba-1, Olig2 and O4 were significantly increased in E2/ADSCs combined therapy in comparison with ADSCs monotherapy. In conclusion, the findings of this study revealed that E2 administration enhanced efficacy of intravenous ADSCs transplantation in remyelination of corpus callosum axons in mouse cuprizone model of MS.

Introduction

Stem cell transplantation has revolutionized the treatment of neurodegenerative disease such as multiple sclerosis (MS) (1). In recent years mesenchymal stem cells (MSCs) came into focus as a transplantable cell source to treat MS (2). Although bone marrow mesenchymal stem cells (BMSCs) are available from the bone marrow of adults (3), they are limited in number (4). Much attention has been paid recently to adipose-derived mesenchymal stem cells (ADSCs) because adipose tissue is an abundant, easily accessible, and appealing source of donor tissue for cell transplantation (5). Recently some reports have demonstrated that ADSCs express α4 integrin (6). A characteristic feature of α4 integrins is that their adhesive activity can be subjected to rapid modulation during the process of cell migration (6,7). Several studies have suggested that MSCs transplantation decrease the severity and progression of autoimmune demyelination (8,9). Constantine *et al.* have suggested that MSCs are able to secrete basic fibroblast growth factor (bFGF), brain-derived growth factor (BDNF) and platelet-derived growth factor (PDGF), all factors strongly supporting the process of oligodendrogenic differentiation (6).

In the other hand, Aghajanova *et al.* reported the presence of estrogen receptor on mesenchymal stem cells, suggesting that estrogen may modify the function of those cells (10). Previous studies showed that estrogen or estrogen-derivates are the protective factors

and it was postulated that the beneficial effects of estrogen in experimental models of MS may result from direct modulation of the adaptive immune system (11,12). Thus, to determine whether estrogen can increase the efficacy of ADSCs in the treatment of MS, we used the non-immune driven cuprizone intoxication model of MS. The aim of this study was to examine the effects of the co-administration of E2 pellet implantation and ADSCs transplantation on remyelination of corpus callosum axons in a cuprizone model of MS.

Materials and Methods

Isolation of adipose mesenchymal stem cells

Epididymal fat pads of 8-weeks male C57BL/6 mice was excised, placed on a sterile glass surface, and finely minced. The minced tissue was placed in a 50 ml conical tube (Greiner, Germany) containing 0.05% collagenase type 1 (Sigma) and 5% bovine serum albumin (Sigma). The tube was incubated at 37°C for 1 h. The tube contents, after filtering through a sterile 250 μm nylon mesh, were centrifuged at 250 g for 5 min. The cell pellet was re-suspended in ADSCs medium: Dulbecco's Modified Eagle's Media (DMEM; Gibco), 10% fetal bovine serum (Gibco), and 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma). Cell count was determined with a haemocytometer (4).

Characterization of isolated ADSCs by flow cytometry

Mice ADSCs within 3–5 passages after the initial plating of the primary culture were harvested by trypsinization, and then the 10^6 cells were fixed in neutralized 2% paraformaldehyde solution for 30 minutes. The fixed cells were washed twice with PBS and incubated with antibodies to the following antigens: CD31 (1:300), CD45 (1:300), CD73 (1:300) and CD90 (1:500) (Chemicon, CA) for 30 minutes. Primary antibodies were directly conjugated with Fluorescein isothiocyanate (FITC). The cells stained with FITC rat anti-mouse IgG served as controls. The specific fluorescence of 10,000 cells was analyzed on FACScalibur (Becton Dickinson, USA) using Cell Quest Pro software (4).

ADSCs multilineage differentiation potential

Cultured ADSCs of passage 3 were disseminated at a density of 10000 cells/cm^2 and maintained in growth medium for 3 days. Then medium was replaced by differentiation medium contained DMEM (Invitrogen), 10% FBS (Sigma), 2 mM L-glutamine, 100 U/ml

penicillin and 100 mg/ml streptomycin (Sigma) and additionally either 10 nM dexamethasone (Invitrogen), 50 mg/ml L-ascorbic acid (Gibco) and 10mM b glycerophosphate (Sigma) (osteogenic differentiation) or 10 nM dexamethasone (Sigma), 200 mg/ml indomethacin (Sigma), 5 mg/ml insulin (Gibco) and 0.5 mM IBMX (Sigma) (adipogenic differentiation). Media change was performed every 3-4 days. After 21 days, calcium deposition was determined by staining with von Kossa (Gibco) and intracellular droplets of adipocytes were visualized by oil red O staining (4).

Cuprizone mouse model and cell transplantation

A total of 48 male C57BL/6 mice (Pasteur Institute of Iran, Tehran) weighting 20 g at the start of the study were fed with 0.2% (w/w) cuprizone (Sigma) in ground breeder chow *ad libitum* for 6 weeks. This diet leads to selective oligodendrocyte death followed by demyelination of axons mainly in the corpus callosum (3). At day 0 after cuprizone removal, animals were randomly divided into four groups. The E2 monotherapy, ADSCs monotherapy, E2/ADSCs combined therapy and vehicle control, animals received 500 μl of DMEM with a placebo pellet implant. E2 pellets (Innovative Research), designed to release 5.0 mg E2 over 10 days, were implanted subcutaneously through a small incision made in the skin of the back (13). Quantities of 10^6 ADSCs were labeled with fluorescent dye PKH26, according to the manufacturer's instruction, in a volume of 500 μl of DMEM were transplanted into lateral tail vein using a 27-gauge needle, within more than 1 min. Some mice of the same age were fed with their normal diet to serve as healthy control group (n=12).

Single cell suspension of corpus callosum

The complete corpus callosum was microdissected from PBS-perfused mice 2 and 10 days after ADSCs transplantation and E2 implantation. The tissue was placed into a Petri dish containing 2 ml of digestion buffer, 1 mg/ml of collagenase D (Roche); 1 mg/ml of neutral protease (Worthington); DNase I (Qiagen), and diced into small pieces with a razor blade before incubation at 37°C for 30 min. Following the incubation PBS was added to stop the enzymatic digestion and cells washed through a 70 μm filter with FACS buffer and centrifuged at 2,000 rpm for 5 min at 4°C. The supernatant was aspirated and the pellet re-suspended in 8 ml of 40% Percoll (Sigma) and layered onto 3 ml of 70% Percoll. The gradient was centrifuged at 2,000 rpm for 25 min at room temperature without brakes. The

cells were collected at the interface of the 40 and 70% Percoll and washed with PBS by centrifugation. Cells were pooled and transferred to a separate 15 ml tube, washed twice with PBS, fixed in 4% formaldehyde (v/v in PBS) for 20 min on ice. The cells were then distributed between four microtubes per 15-ml tube and incubated with primary antibody at 4°C for 30 min. The secondary antibody was added to the samples and incubated for 20 min. The samples were then centrifuged at 3,000 rpm for 10 min. The pellets were washed twice in nuclear buffer. All of the samples were suspended in 500 µl of nuclear buffer and analyzed using a FACSCalibur Flow Cytometer (BD Biosciences). For the primary antibody, anti-GFAP antibody (1:500, Millipore) or anti-Iba-1 antibody (1:600, Millipore) or anti-Olig2 antibody (1:100, Millipore) or anti-O4 antibody (1:50, Millipore) was used. For the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Invitrogen, 1:100 dilution) was used (14).

Homing assay

The homing efficiency of transplanted ADSCs was assayed by labeling ADSCs with the red fluorescent dye PKH26 (Sigma). Animals were sacrificed 2 days after transplantation and single-cell suspensions obtained from corpus callosum was visualized by inverted fluorescence microscope (Olympus IX71). Nuclear staining was performed using DAPI to detect cells present in corpus callosum.

Preparation of brain tissue for histology

Ten days after ADSCs transplantation and E2 pellet implantation, mice (Healthy control and treated groups) were perfused with 4% paraformaldehyde (PFA, Fluka) for demyelination staining, Luxol fast blue, or with 4% glutaraldehyde (GLA, Fluka) for resin embedding (*n* = 3/group). Healthy control mice were also perfused with 4% PFA or 4% GLA.

Myelin staining

To stain for myelin content, tissue sections from mice were treated with Luxol fast blue (LFB, Sigma). Sections were stained overnight in LFP at 56°C and washed in 95% ethanol and distilled water to remove excess blue stain. The color was then differentiated (until white matter was easily distinguishable from gray matter) in lithium carbonate solution for 15 s, followed by distilled water and three washes of 80% alcohol. Slides were passed through fresh xylene twice, mounted with Entellan (Merck, Germany), and cover

slipped (3).

Electron microscopy examination

Mice were transcardially perfused with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS. Brains were removed, fixed with 1% osmium tetroxide and embedded in resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (LEO 906 Germany, 100 kV). Images were taken from four sections 50 µm apart for each animal at locations between −0.94 mm and −1.28 mm bregma with the midline corpus callosum at the epicenter. Photographs of the axons cut in cross section were taken and myelinated axons quantification was performed on four 3000× images per animal and analyzed as using Image tools software. For morphometric analysis, at least 100 axons were measured. We counted the total numbers of axons with and without myelin sheaths contained within all of the selected electron micrographs from each animal. We measured the axonal diameter as the shortest distance across the center of axons, avoiding the myelin sheath thickness. The axonal diameter plus the total myelin sheath thickness on both sides was defined as fiber diameter (15).

Statistics analysis

Statistical analysis was performed by one-way analysis of variance ANOVA. Data is reported as mean ± SEM. Each value represents the average of n=5 animals. Values of $P<0.05$ were considered statistically significant.

Results

Mouse ADSCs characterization

Flow cytometry analysis of mice ADSCs within 3–5 passages showed that ADSCs were CD73 (97%) and CD90 (96%) positive, but CD31, CD45 (hematopoietic marker) negative (Figure 1).

Differentiation of ADSCs into mesodermal lineages

ADSCs appeared as a monolayer of large, fibroblast like flattened cells (Figure 2A).We investigated whether ADSCs have multipotency including the ability to differentiate into osteoblasts and adipocytes. To test this, we first subjected the ADSCs to an osteoblast differentiation assay. After 21 days, the cells were stained with von Kossa, which stains calcium deposits.
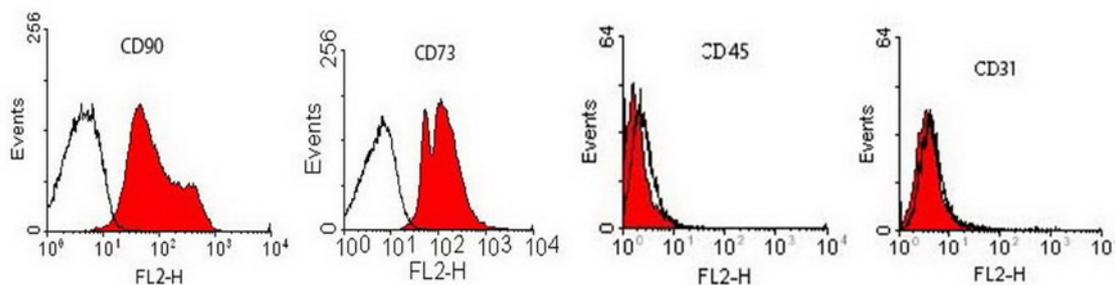
Figure 1. Flow cytometric analysis of C57BL/6 mice ADSCs showing that they do not express CD31, and CD45, but express CD73 and CD90. The white histograms show isotype-matched control staining.
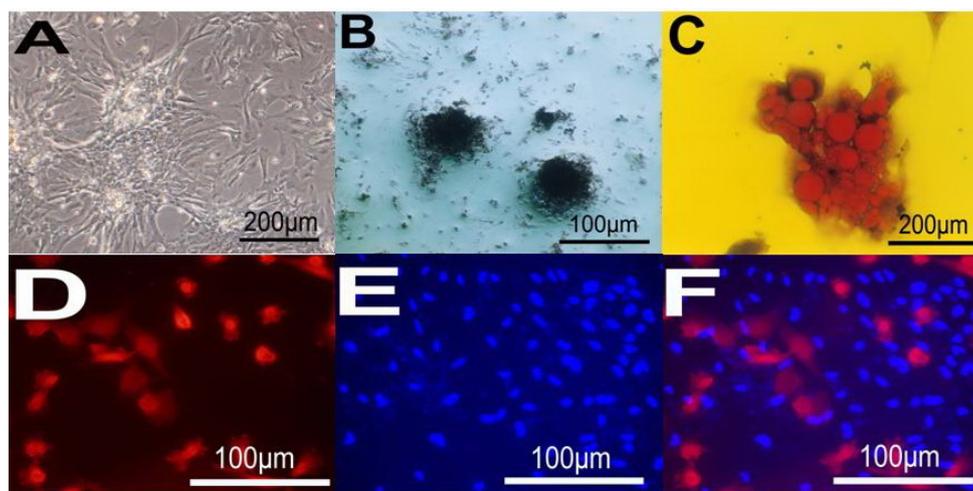

Figure 2. Undifferentiated ADSCs. Under inverted microscope, the cells display a flattened fibroblast-like morphology (A). Von Kossa staining of mineralized bone tissue (B) and Oil Red O positive intracellular lipid droplets (C) indicate that these cells can differentiate to osteoblasts and adipocytes respectively. The pooled corpus callosum single cell suspension from recipient mice 2 days after transplantation demonstrates PKH26-positive ADSCs (red colored), which are visualized among various cell types present in corpus callosum which their nuclei stained by DAPI (blue). ADSCs stained with PKH26 (D), nuclear staining with DAPI (E) and merge (F).

The ADSCs-derived cells stained positive for von Kossa (Figure 2B). These findings showed that the ADSCs-derived cells produce calcium in response to differentiation conditions. We next tested whether the ADSCs could be differentiated into adipocytes. For this, we cultured ADSCs in adipocyte differentiation media for 21 days and then stained the cultures for the presence of lipid droplets, a characteristic of mature adipocytes, using oil red O dye. The ADSCs-derived cells stained positive for oil red O (Figure 2C).

Homing properties of ADSCs

By fluorescent cell labeling, the transplanted ADSCs appeared to survive and exhibited homing specificity. Fluorescent PKH26 labeled ADSCs transplanted intravenously were localized almost exclusively in single cell suspension of injured corpus callosum at 2 days after transplantation (Figures 2D, E, and F).

Light microscope examination

Histological examination of tissue sections was performed at 10 days after treatment. LFB histologic stain was used to assess the extent of remyelination in corpus callosum. High intensity staining was evident in healthy control group (Figure 3*A*). In control vehicle group, partial remyelination was observed (Figure 3*B*). Conversely, the remyelinated area was markedly increased in mice that underwent ADSCs monotherapy (Figure 3*D)* or E2/ADSCs combined therapy (Figure 3E) and to a lesser extent in E2 monotherapy (Figure 3C).

Improved myelination in ADSCs transplantation

The transmission electron microscopic photographs were used for determined myelin morphometric parameters from corpus callosum in healthy control, vehicle control, E2, ADSCs monotherapy and ADSCs/E2 combined therapy groups (Figures 3F–J and 4). EM images were taken from sagittal ultrathin sections from corpus callosum and analyzed to quantify the percentage of myelinated axons, axonal diameter and myelin thickness (Figure 3).
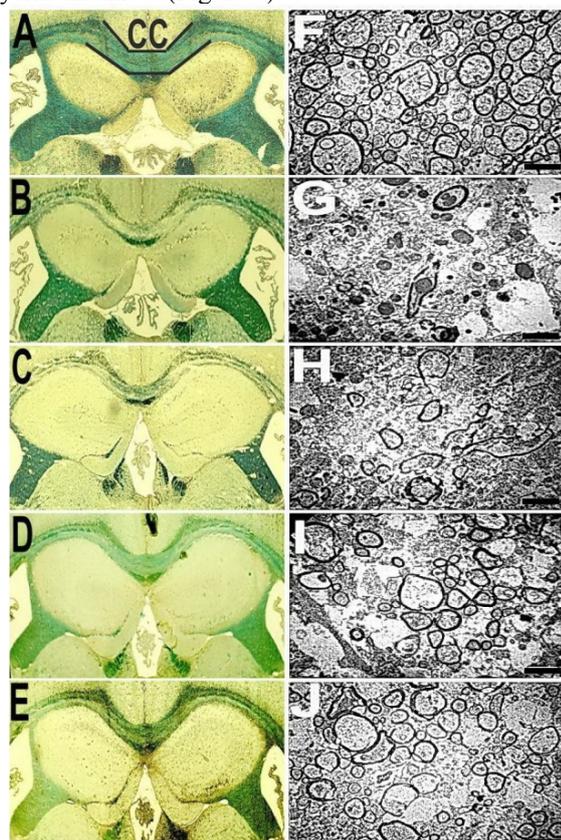

Figure 3. Light micrographs (left column) and transmission electron micrographs (right column) showing transplantation of ADSCs facilitates remyelination in the corpus callosum of mice after cuprizone-induced demyelination. The photomicrographs were taken from coronal (light micrographs) and sagittal sections (transmission electron micrographs) of corpus callosum of mice were killed 10 days after transplantation. A-E: Myelin content was evaluated by Luxol fast blue staining. Corpus callosum (CC) of a control health mouse (*A*, delineated by black lines), control vehicle (B), E2 monotherapy (C), ADSCs monotherapy (D) and E2/ADSCs combined therapy (E) groups. F–J: Electron micrographs show myelinated and unmyelinated axons at 10 days after treatment. Electron micrographs are at × 3000 magnification. (F) Control healthy, (G) control vehicle, (H) 17β-estradiol (E2) monotherapy, (I) ADSCs monotherapy and (J) E2/ADSCs. Scale bars, in F–J=1 μm.
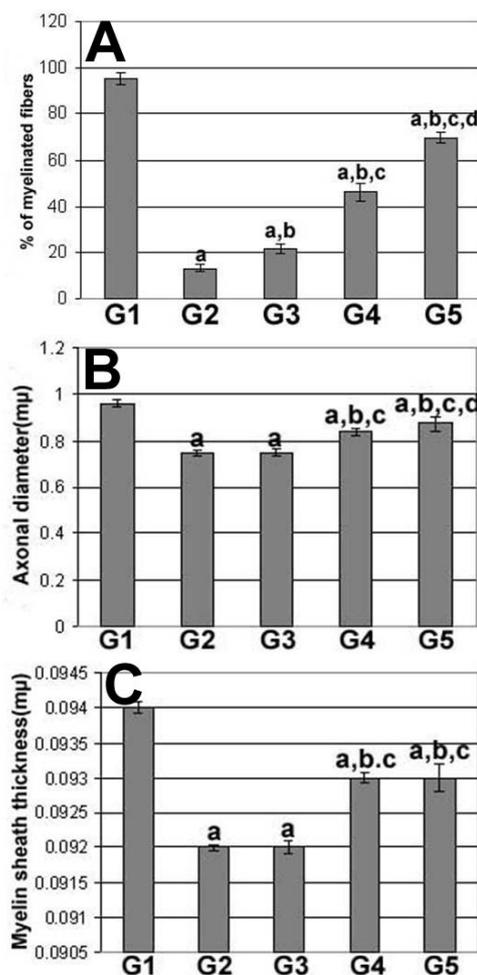

Figure 4. Percentage of myelinated axons in the corpus callosum (A), Mean of axon diameters (B) and mean of myelin sheath thickness (C). Quantitative analysis of the electron micrographs was carried out using the Image tools software. Results are the mean ± SEM from 4 different measurements for each experimental condition. * *P*<0.05. a: Significant difference with healthy control group, b: Significant difference with vehicle control group, c: Significant difference with E2 monotherapy group, d: Significant difference with ADSCs monotherapy group.G1; Healthy control, G2; vehicle control, G3; 17β-estradiol implanted, G4; ADSCs transplanted and G5; ADSCs transplanted with 17β-estradiol implanted.

The mean myelin fibers were significantly increased in E2/ADSCs combined therapy (*P*≤0.05; 69.5 ± 2.45) compared with in all the treated groups but reduced than healthy control group (*P*≤0.05; 95.3±2.47) (Figures 3, 4A). Figure 4B show that the mean axonal diameter was significantly increased in E2/ADSCs compound therapy (*P*≤0.05; 0.873±0.032) compared with vehicle control (*P*≤0.05; 0.746±0.012) and E2 monotherapy groups (*P*≤0.05; 0.75±0.017) but reduced than healthy control group (*P*≤0.05; 0.96±0.015). There was no significant

difference between ADSCs monotherapy ($P \leq 0.05$; 0.836±0.018) and E2/ADSCs combined therapy. The myelin sheath was significantly thicker in ADSCs monotherapy ($P \leq 0.001$; 0.093±0.0007 μm) and E2/ADSCs compound therapy ($P \leq 0.001$; 0.093±0.001 μm) compared with control vehicle ($P \leq 0.05$; 0.0.092±0.0005 μm) and E2 monotherapy groups ($P \leq 0.001$; 0.092±0.0009 μm), but thinner than healthy control group ($P \leq 0.001$; 0.0943±0.0008μm) (Figure 4C). There was no significant difference between ADSCs and E2/ADSCs combined therapy.

Determine the cellular composition

Effects of ADSCs transplantation, E2 implantation monotherapy and ADSCs/E2 compound therapy on neural cell composition of injured corpus callosum was evaluated by flow cytometry (Figure 5). The mean percentage of cell types in three independent samples per groups by flow cytometry is shown in table 1. According to table1, the mean percentages of expression of GFAP, an astrocyte marker, was significantly increased in vehicle control, E2 and ADSCs monotherapy groups compared with healthy control group ($P \leq 0.05$; 3.11±1.4) and this increase was significantly reversed in animals that received ADSCs/E2 combined therapy ($P \leq 0.05$; 4.06±1.52). The mean percentages of expression of Iba-1, a microglia marker, were significantly increased in all the treated groups compared with healthy control group.
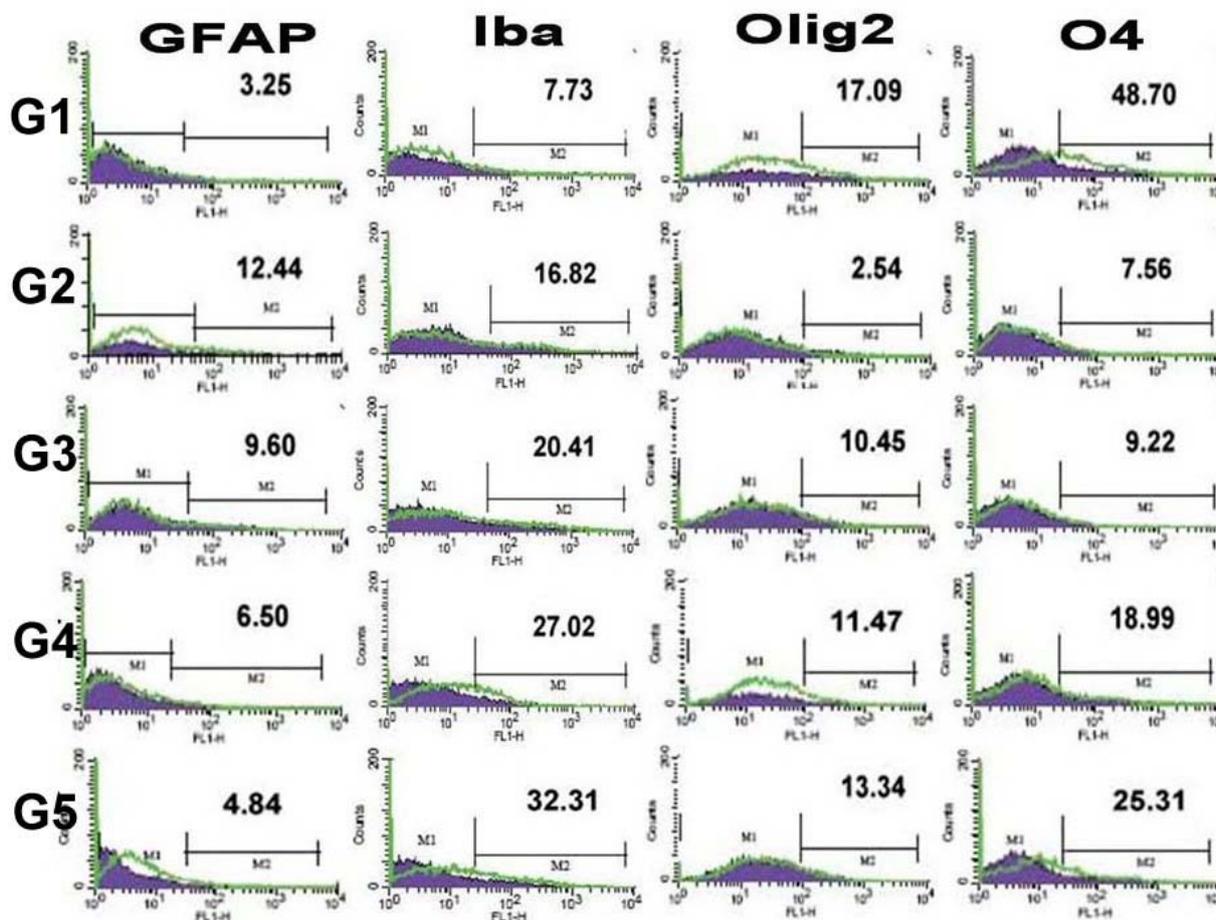

Figure 5. To analyze the changes in the cellular composition in the corpus callosum of cuprizone-induced demyelination mice treated with E2 monotherapy, ADSCs monotherapy and E2/ADSCs combined therapy. Mononuclear cells were isolated from corpus callosum and the frequencies of GFAP+ cells (astrocyte), Iba-1+cells (microglia), Olig2+cells (oligodendroglial progenitor cell) and O4+cells (oligodendrocyte) determined using flow cytometry 10 days after treatment. The respective isotype control is shown as violet. G1; Healthy control, G2; vehicle control, G3; E2 monotherapy, G4; ADSCs monotherapy and G5; E2/ADSCs combined therapy.

Table 1. The mean percentage of neural cell composition types in three independent samples per groups by flow cytometry.

Groups	GFAP	Iba-1	Olig2	O4
Healthy control	3.11±1.4	7.5±0.33	17.53±1.116	48.52±1.42
Vehicle control	12.82±1.27	16.96±1.78	2.78±0.51	7.7±1.37
E2 monotherapy	11.11±1.09	21.35±2.05	10.36±1.53	9.3±1.39
ADSCs monotherapy	6.42±0.84	24.21±4.37	12.59±1.72	18.55±1.55
E2/ ADSCs combined therapy	4.06±1.52	33.61±2.42	13.57±1.76	27.3±1.92

The mean percentages of expression of Olig2, an oligodendroglial progenitor cell marker, were significantly decreased in vehicle control group compared with healthy control group ($P \leq 0.05$; 17.53±1.116) and this decrease was significantly reversed in all the treated groups. There was significant difference between E2/ADSCs combined therapy ($P \leq 0.05$; 15.57±1.76) with ADSCs monotherapy ($P \leq 0.05$; 12.59±1.72). Likewise the mean percentages of expression of O4, an oligodendrocyte marker, were significantly increased in all the treated groups compared with vehicle control group. There was significant difference between E2/ADSCs compound therapy ($P \leq 0.05$; 27.3±1.92) with ADSCs monotherapy ($P \leq 0.05$; 18.55±1.55).

Discussion

A major finding of this study is that efficacy of intravenous ADSCs transplantation in remyelination of mouse cuprizone model of MS can be significantly enhanced by E2 administration. This result is consistent with several recent studies by others, which have also shown that ADSCs (6) and estrogen (11,12) monotherapy enhance remyelination of injured corpus callosum. Although the mechanisms responsible for the therapeutic effects of ADSCs on remyelination remain unclear, but two hypothesis need to be considered. ADSCs as MSCs may participate to remyelination by either differentiating into mature oligodendrocytes able to form new myelin or indirectly by promoting the survival and proliferation of endogenous precursor's cells (6). The possibility that MSCs benefit cerebral tissue by becoming brain cells is very unlikely (16). With intravenous injection numbering a few hundred thousand at most, there are very few cells present, even if they become brain cells, to replace a volume of tissue of more than a few cubic millimeters (16,17). Thus, replace damaged tissue as the mechanism by which ADSCs promote their beneficial effects is very unlikely (5). A far more reasonable explanation for the benefit is that MSCs induce cerebral tissue to activate endogenous restorative effects of the brain (3). MSCs may turn on reactions and interact with brain to activate restorative and possibly regenerative mechanisms (16). Constantine *et al.* (6) showed that ADSCs are able to secrete basic fibroblast growth factor (bFGF), brain-derived growth factor (BDNF) and platelet-derived growth factor (PDGF), all factors strongly supporting the process of oligodendrogenic differentiation. Another relevant finding of our study was that the remyelinated area was markedly increased in mice that received E2 implantation without ADSCs transplantation. Consistent with our findings, Acs *et al.* (18) also demonstrated that the application of estrogen in the male corpus callosum, using cuprizone as toxic agent, possesses the capacity of inhibiting mature oligodendrocyte damage and/or of positively stimulating remyelination. Previous studies showed that estrogen or estrogen-derivates are the protective factors and it was postulated that the beneficial effects of estrogen in experimental models of MS may result from direct modulation of the adaptive immune system (11,12). However, in cuprizone-induced demyelination, an invasion of T or B cells is not observed (18). Therefore, our study clearly shows that E2 treatment to be able enhances remyelination of injured corpus callosum also independent of a B and T cell-initiated autoimmune reaction. Some possible mechanisms may lead to the myelination demyelinated corpus callosum axons following E2 implantation with or without ADSCs transplantation: one of the possibilities is that estrogen enhances stem cell survival and mobilization (19). Recent study reported the presence of estrogen receptor on stem cells (10), suggesting that estrogen may modify the function of those cells. In this regard, Marin-Husstege *et al.* (20) reported that the addition of estradiol to the medium of cultured oligodendrocyte progenitor cells stimulated cell number and myelin formation. The therapeutic benefits of estrogen are not limited to the nervous system. For example, in heart disease, the E2 enhances the proliferation and migration of endothelial progenitor cells (EPCs) to the injured vessels, or ischemic myocardial tissues, which, through the process of

homing, help in repair and regeneration to compensate for the lost tissue (21).The other possibility is that estradiol may facilitate grafted cell migration by activating matrix metalloprotease-2 (MMP-2) in situ (22). Degradation of the ECM appears to be necessary for the grafted cells to integrate into a host tissue packed with cells (22). Because MMPs can degrade ECM molecules, their main function has been presumed to be remodeling of the extracellular matrix (ECM) (23). E2 regulates the expression of MMP-2 by activating estrogen receptors in a dose-dependent fashion in both in vivo and in vitro systems (24). Another relevant finding of our study was that ADSCs induced improvement was accompanied by changes in neural cell composition with increased microglia and decreased astrocytes in lesion areas. In similar experiments, Short *et al.* (14), Eglitis *et al.* (25) and Tambuyzer *et al.* (26) also reported that number of microglial cells increased in demyelination lesion after heterologus MSCs transplantation. Tambuyzer *et al.* (26) suggested that microglia is able to discriminate between self and non-self *in vivo*; although a clear mechanism for the latter still remains to be elucidated. A potential mechanism for clearing allografts in the CNS by microglia might be found in their production of TNF-α and/or NO on strong activation (26,27). It was recently demonstrated that interleukin-17 producing (ThIL-17) T cells mediate inflammatory pathology in certain autoimmune diseases including MS (28). Previous studies suggest that astrocytes can stimulate IL-17 production (29) and treatment with MSCs significantly down regulates IL-17 levels and results in a reduction in astrogliosis (30). In conclusion, the findings of this study revealed that that E2 administration enhanced efficacy of intravenous ADSCs transplantation in remyelination corpus callosum axons of mouse cuprizone model of MS.

Acknowledgment

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