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Effect of mesenchymal stem cells on glial cells population in cuprizone induced demyelination model

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ABSTRACT

Mesenchymal stem cells (MSCs) have a notable potential to modulate immune responses and protect the central nervous system (CNS), mostly by secreting factors that affect inflammation. MSCs have the ability to improve several autoimmune diseases in animal models including multiple sclerosis (MS). MS is a disease of the CNS among adult humans and it is characterized by demyelination, neuroinflammation and gliosis. In this study, we first induced chronic demyelination by cuprizone, followed by intraventricular injection of MSC. Our results showed that MSC significantly decreased microgliosis and astrocytosis by secreting cytokines that have neuroprotective activity including TGF-β and CX3CL1. Also, downregulation of IL-1β and TNF-α as inflammatory chemokines was seen along with decreased astrocytes and microglia activation. Finally, these results showed that trophic factors secreted by MSC can increase oligodendrocyte population and remyelination rate by reducing pro-inflammatory factors.

These findings demonstrate that MSC could decrease inflammation, gliosis and demyelination with neuroprotective and immunomodulating properties in chronic cuprizone demyelination model. Therefore MSC transplantation can be considered as a suitable approach for enhancing myelination and reducing inflammation in diseases such as MS.

1. Introduction

Mesenchymal stem cells have properties such as self-renewal and differentiation to other linages and they could be used as a therapeutic source for several diseases. MSCs are multipotent and heterogeneous cells that have various roles including secretion of trophic factors, promotion of angiogenesis, reducing inflammation and increasing repair and regeneration (Singer and Caplan, 2011).

MSCs are able to improve numerous animal models of autoimmune diseases like MS (Bert et al., 2011; Bai et al., 2012). MS is a neurodegenerative autoimmune disease that have pathological symptoms such as inflammation, demyelination and axonal damage, astrocytosis and microglial activation (Höftberger and Lassmann, 2017). Oligodendrocytes, microglia and astrocytes have supportive effects on neurons and play an important role in repair and degenerative processes (Tejedor, 2015). During demyelination, oligodendrocytes that generate myelin become damaged and myelin gets destroyed (Rivera and Aigner, 2012). In reaction to demyelination events, oligodendrocyte precursor cells (OPCs) proliferate, differentiate and migrate to damaged sites in order to remyelinate, but this procedure is normally faulty (Dulamea, 2017). Remyelination, as a repair mechanism, happens naturally after demyelination. Remyelination process employs mature oligodendrocytes which originate from OPC to create new myelin sheaths around the axons (Plemel et al., 2017; Kastriti and Adameyko, 2017). Myelin sheaths are distinctive membranes with high lipid content and also have few proteins (Salzer and Zalc, 2016) that surround the axons and protect them by releasing trophic factors and also increase signal transduction velocity (Jana and Pahan, 2017). In inflammatory situation of MS, neurological disability occurs following demyelination (Lassmann, 2018) and axonal injury (Ineichen et al., 2017). At the same time, microglial activation and astrocytosis have been observed in these areas (Tejedor, 2015). Microglia are one of the immune cells of the nervous system in physiological situations and it have beneficial effects; But microglial activation have also been observed during pathological

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conditions (Gudi et al., 2014). Activated microglia have several phenotypes that depends on signals in their surrounding microenvironment. Besides toxic elements, such as reactive oxygen species, TNF-α and nitric oxide secreted by activated microglia contribute to inflammatory process (Lepka et al., 2017).

Astrocyte is a type of glial cells in the nervous system that support neurons and other cells. They have beneficial and detrimental roles during repair; they are also able to preserve the extracellular ion balance. Astrocytes have vital role during demyelination process and they form a glial scar when demyelination is completed (Brosnan and Raine, 2013). Astrocytes are main source of chemokines, such as TNF-α and IL-1β which are involved in the pathogenesis of MS (Sénécal et al., 2016).

Bone marrow mesenchymal stem cells (BMSCs) transplantation can be a suitable solution for these problems. MSCs are multipotent cells that have immunomodulating and regenerative effects (Cristofanilli et al., 2011). Furthermore, homing phenomenon which is stem cells ability to migrate to a damaged area is considered as one of the main areas of stem cell research (Yun et al., 2018).

It has been well established that MSCs inhibit the release of pro-inflammatory chemokines including TNF-α, IFNγ and IL-1β (Duffy et al., 2011). In order to assess the neuroprotective capacity of MSCs in chemically-induced demyelination model without any intervention by T cells, cuprizone model have been employed. The studies have already showed that MSC can modulate the inflammatory environment in the damaged sites and enhance repair by producing anti-inflammatory cytokines and trophic factors (Correale and Farez, 2015).

In present study, we evaluate the effects of intraventricular MSC transplantation in a cuprizone-induced demyelination model in corpus callosum of mice. We also assess the immunomodulatory and neuroregenerative effects of MSCs on remyelination and glial cells population of corpus callosum.

2. Materials and methods

2.1. Animals

Eight week old male C57BL/6 mice (n = 30) were used as experimental animals in this study. The animals were maintained in a controlled condition and supplied with standard rodent chow and water. C57BL/6 mice were breed in a pathogen free environment. All experiments were performed in accordance with Iran University of Medical Science guideline of animal care in research.

2.2. Experimental design

Mice were divided into 3 groups (n = 10) referred to as control (group that were fed by normal chow without any treatment), cuprizone (CPZ) (group that had a diet mixed with cuprizone) and BMSCs (CPZ + MSC) (group that received intraventricular injection of MSC after cuprizone digestion). We did this study according to Chart 1.

2.3. Cuprizone induced demyelination model

In order to induce chronic demyelination model, we used two months old male mice (19–21 g). They were fed by a standard rodent chow mixed with 0.2% cuprizone powder (bis-cyclohexanone oxaldihydrazone, Sigma–Aldrich, St.Louis, MO, USA) for 12 weeks (Kipp et al., 2011).

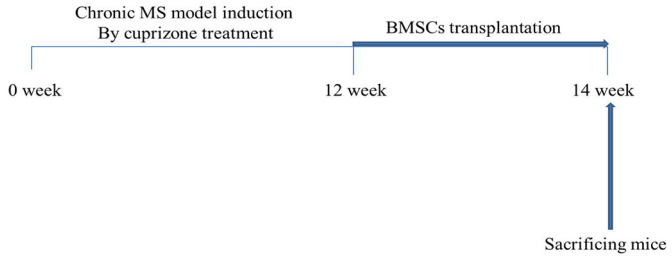

Chart 1. Experimental design in this study.

2.4. Isolation and culture of MSCs

The adult C57BL/6 mice were sacrificed by cervical dislocation and soft tissues were removed from their tibiae and femurs. The tip of the bones were cut by a rongeur, and contents of bone canal was transferred to a plate by flushing Dulbecco's Modified Eagle's Medium (Gibco, Washington, USA) by a syringe needle from opposite end of the bone. MSCs were cultured into 25 cm^2 flask in DMEM containing 15% fetal bovine serum (Biochrom, Germany), 100 u/ml streptomycin and penicillin (Sigma-Aldrich, St. Louis, MO, USA). Cells were reserved at 37 °C and 5% CO_2. After 1 week, when cells proliferated and reached confluence, the cells were treated with 0.025% Trypsin containing 0.02% EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 2 min at room temperature. The cells which were picking up were cultured in a 75 cm^2 flask. After 3 weeks, when the culture approximately reached to 80% confluence, they were used for next experiments (Nadri et al., 2002).

2.5. Phenotypic analysis by flowcytometry assay

After 3 passages, cultured MSCs were trypsinized and then washed with PBS (Sigma-Aldrich, St. Louis, MO, USA). After that, cells were centrifuged and platelet formed. We added PBS and mixed them. We put 5×10^5 cells in each tube and used for flow cytometric analysis. Antibody against CD34 (abcam, UK) and CD45 (abcam, UK) were used as negative control group and antibody against CD105 (abcam, UK) and CD44 (abcam, UK) as positive control group were added to tubes and incubated. Their histograms were drawn according to 2×10^4 cells/sample. The flow cytometric software analyzed the obtained data (Baksh et al., 2007).

2.6. Surgical procedures

In order to trace MSCs, they were labelled with 1, 1′-Dioctadecyl 3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate (DiI) (life technologies, California, USA) and then they were transplanted (Gutiérrez-Fernández et al., 2015).

10 mice were randomly chosen for MSCs transplantation. We anesthesized the animals by intraperitoneal injection of 10 ml xylazine and 100 ml ketamine per kg body weight. The mice were placed and fixed in a stereotaxic apparatus (Stoelting, Illinois, USA) for cell injection. Coordinates from bregma were as follows: −0.5 mm anteroposterior, +1.1 mm mediolateral and 2 mm dorsoventral from the surface of the brain. Approximately 3×10^5 cells with 2 µl culture medium were injected into right lateral ventricle using a 5 µl Hamilton syringe (Hamilton, Nevada, USA) during 4 min. After the injections, the incisions were sutured and animals were maintained in warm cages until full recovery.

2.7. Sacrifice and tissue preparation procedure

14 weeks after study beginning, mice were deeply anesthetized with ketamine/xylazine (10/1). Then all of the animals were transcardially perfused with saline. For histological studies, animals were perfused with 2% paraformaldehyde; (PFA) (Sigma-Aldrich, St. Louis, MO, USA) and for electron microscopy studies; they were perfused with 2.5% glutaraldehyde and 4% PFA. For gene expression level analysis, mice were perfused with saline and then their brains were rapidly detached, and their corpus callosums were dissected. Tissues were immediately transferred to liquid nitrogen tanks and then freezed and preserved at −80 °C.

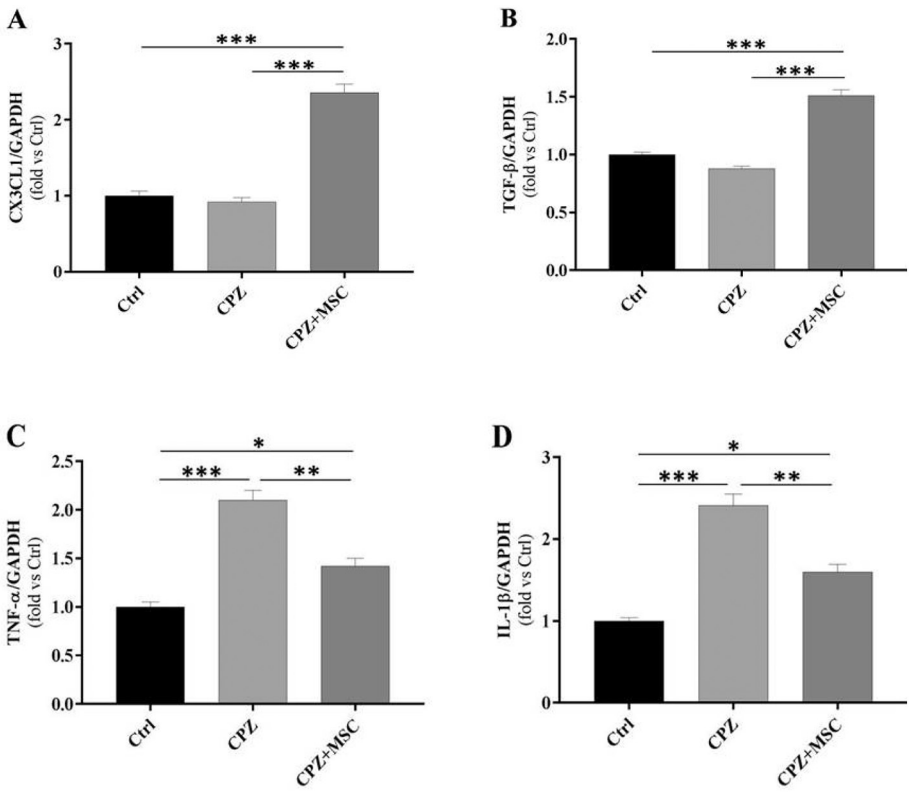

Fig. 1. The effect of MSC transplantation on trophic factors and pro-inflammatory genes assessed by qRT-PCR in cuprizone induced demyelination model. (A, B) QRT-PCR using primers for CX3CL1 and TGF-β as MSC trophic factors. Results showed that the expression of TGF-β and CX3CL1 mRNA were low in control and untreated cuprizone mice, while in MSC group their expression increased. (C, D) QRT-PCR analysis for specific primers of TNF-α and IL-1β as pro-inflammatory factors. MSC decreased TNF-α and IL-1β after CPZ treatment. GAPDH was used as an internal control for normalization. Values are expressed as the mean ± SEM. ***p < .001, **p < .01 and *p < .05.

2.8. Real time-PCR

The mRNA of tissues was isolated using the Trizol protocol (Invitrogen Life Technologies, Spain). For qRT-PCR, cDNA was synthetized from extracted mRNA and amplified by SYBR Green Master mix (Applied Biosystems Foster City, CA, USA). The samples were analyzed using the RT-PCR system (Applied Biosystems, Foster City, CA, USA). Quantification was done using the ΔCT method relative to GAPDH gene and normalized with control conditions. In this study, we used the following primer sequences for RT–PCR: GFAP, F: GTGGAGA GGGACAACTTTGC and R: TTCATCTGCCTCCTGTCTATACG; Iba-1, F: CTGCCAGCCTAAGACAACCA and R: GGATCATCGAGGAATTGCTTG TTG; Olig2, F: GAACCCCGAAAGGTGTGGAT and R: TTCCGAATGTGA ATTAGATTTGAGG; TNF-α, F: TGTAGCCCACGTCGTAGCAA and R: AGGTACAACCCATCGGCTGG; IL-1β, F: TGTGCAAGTGTCTGAAGCAGC and R: TGGAAGCAGCCCTTCATCTT; CX3CL1, F: CCGCGTTCTTCCATT TGTGT and R: CCACTGGGATTCGTGAGGTC; TGF-β, F: GGTTCATGTC ATGGATGGTGC and R: TGACGTCACTGGAGTTGTACGG; GAPDH, F: CAAGCTCATTTCCTGGTATGACAA and R: GGGATAGGGCCTCTCTT GCT.

2.9. Immunohistochemistry

After brain fixation with 4% PFA, the tissues were dehydrated and then embedded in paraffin. Coronal sections of brain were prepared by a microtome rotatory apparatus (Microm HM335E, Walldorf, Germany). For antigen retrieval, the sections were immersed into citrate buffer and then permeabilized by 20% tween for 30 min. Subsequently, sections were blocked by 0.1% BSA in 0.1% Triton to prevent binding of non-specific markers (Ghareghani et al., 2017). Then slides were covered with primary antibodies anti-GFAP (1:250) (Sigma) for astrocytes, anti-Iba-1 (1:250) (Wako chemicals, Virginia, USA) for microglia and anti-Olig2 (1:100) (Gene Tex, California, USA) for oligodendrocytes and were incubated at 4 °C overnight. We added secondary antibody (Alexa Fluor 488, 568 at 1:500) and DAPI (1:1000) for nucleus staining at room temperature for 60 min. Slices were observed with a Fluorescent microscope (Olympus BX51TRF, Japan) equipped with a digital camera (Olympus, Tokyo, Japan).

2.10. Luxol fast blue (LFB) staining

To assess the demyelination processes, we prepared 5 µm paraffin sections of the brain and located them on gelatin coated slides. The tissue processing was performed and then the tissues were incubated in a LFB solution (0.01%) overnight at 60 °C (myelinated fibers appear blue and demyelinated regions are white) (Dhakshinamoorthy et al., 2017). The images of stained sections were observed under the light microscope (Olympus, Japan) and captured by camera. Finally, the percentage of myelinated zone was calculated by dividing corpus callosum size in experimental groups to the same area in control group for each section. We used 10 sections per mouse.

2.11. Transmission electron microscopy (TEM)

Perfusion procedure was done using a 2% PFA and 2.5% glutaraldehyde as fixative solutions. The brains were dissected and corpus callosum tissue was immersed in 2% PFA and 2.5% glutaraldehyde and fixed by 1% osmic acid and then washed three times with PBS. Next dehydration procedure was done and samples were embedded in resin and ultrathin sections were prepared using an ultramicrotome. The sections were observed by CM120 Electron microscope. Approximately we chose 10 fields of view per each section and photographed the sections via TEM (LEO 906; Carl-Zeiss, Germany) at 80 kv (Chen et al., 2014). The G-ratio of the neuronal myelinated fibers in corpus callosum was quantified using ImageJ software (Cruz-Martinez et al., 2017).

2.12. Statistical analysis

SPSS software (Statistical Package for the Social Sciences, version 24, USA) was used to analyze the results. Data was shown as mean

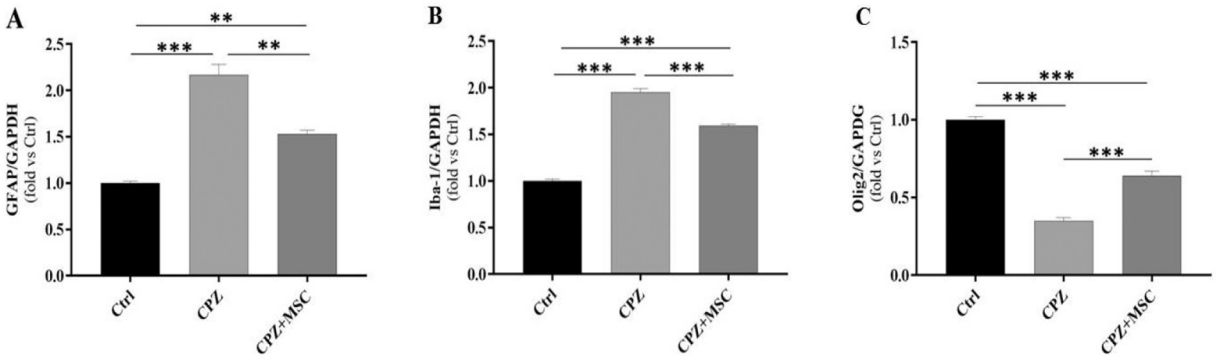

Fig. 2. The effect of MSC trophic factors on glial genes assessed by qRT-PCR in cuprizone induced demyelination model. QRT-PCR using specific primer for (A) GFAP, (B) Iba-1 and (C) Olig2 as astrocyte, microglia and oligodendrocyte markers, respectively. Results showed an upregulation in the expressions of GFAP and Iba-1 with a decrease in Olig2 gene in untreated CPZ mice, while treatment with MSC caused a significant reduction in the rate of GFAP and Iba-1 with a rise in Olig2 expression. GAPDH was used as an internal control for normalization. Values are expressed as the mean ± SEM. ***p < .001 and **p < .01.

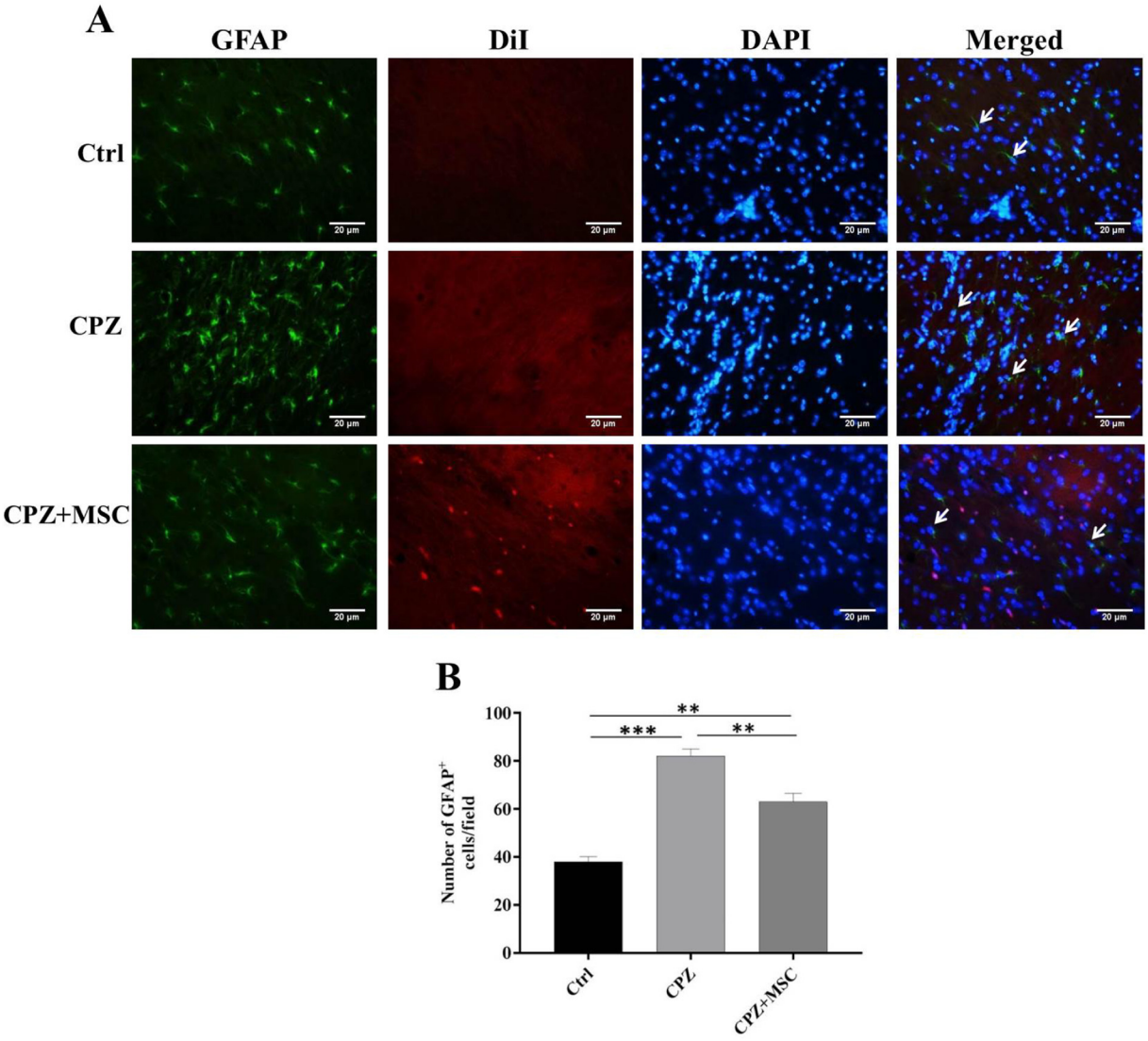

Fig. 3. Immunofluorescence evaluation of astrocyte associated marker GFAP. (A) Representative images are corpus callosum sections (40× field) of astrocytes (GFAP, green), MSC (DiI, red) and nuclei (DAPI, blue). Arrows indicate GFAP+ cells overlap with nuclei. (B) Quantification of immunofluorescence data showed that the changes in the expression of GFAP between study groups were significant. Results showed an upregulation in the expression of GFAP in untreated CPZ mice, while treatment with MSC caused a significant reduction in the GFAP expression. Values are expressed as the mean ± SEM. ***p < .001 and **p ≤ .01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

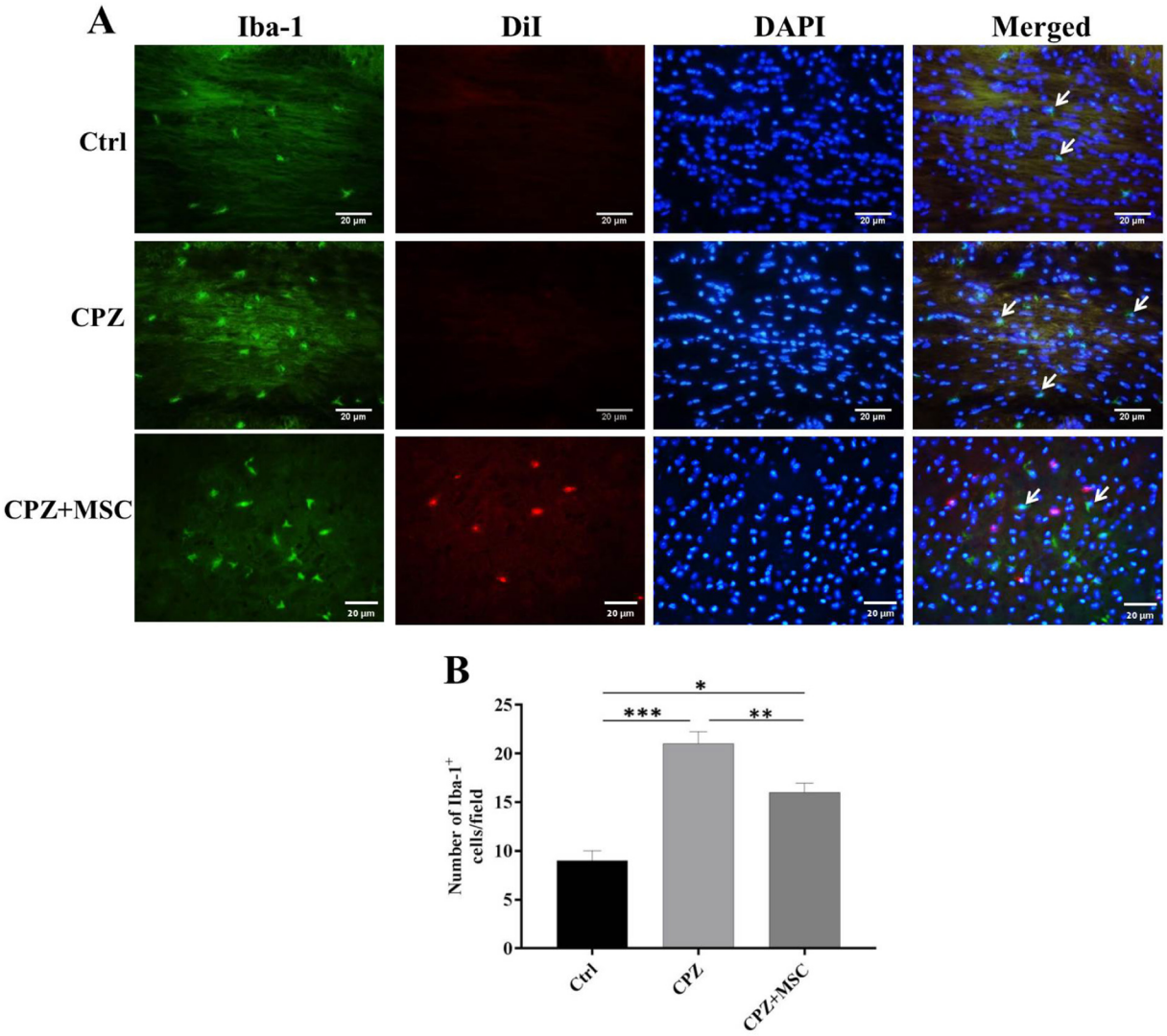

Fig. 4. Immunofluorescence assessment of microglia associated marker Iba-1. (A) Representative images are corpus callosum sections (40× field) of microglia (Iba-1, green), MSC (DiI, red) and nuclei (DAPI, blue). There is a rise inexpression of Iba-1 in the CPZ group, and MSC transplant decreased the Iba-1 protein expression. Arrows indicate Iba-1 + cells overlap with nuclei. (B) Quantification of immunofluorescence data showed that the changes in the expression of Iba-1 between groups were significant. Values are expressed as the mean ± SEM. ***p < .001, **p < .01 and *p < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values ± standard error of the mean (mean ± SEM). In order to compare the results in all of the groups, we used a one-way analysis of variance (ANOVA) following Tukey post-test with Graph Pad Prism (Version 7, USA). Significance is showed by ∗p < .05, ∗∗p < .01 and ∗∗∗p < .001.

3. Results

3.1. QRT-PCR results

The genes of trophic factors secreted by MSC and pro-inflammatory factors were evaluated at the transcriptional level by qRT-PCR. Our results showed that trophic factors such as TGF-β and CX3CL1 in the CPZ and control groups had a low level of expression. While the gene expression level was significantly increased in the cell transplantation group (CPZ + MSC) compared to the CPZ group (p ≤ .001) (Fig. 1-A, B).

Our results also indicate that the expression level of TNF-α and IL-1β significantly increased in the CPZ group (p ≤ .001 vs. control). After transplanting MSCs to animals that received cuprizone for 12 weeks, this increase was reduced prominently (p ≤ .01 vs. CPZ) (Fig. 1-C, D). These observations demonstrate that stem cells increase secretion of trophic factors such as TGF-β and CX3CL1 and also inhibit inflammation via reducing pro-inflammatory factors such as TNF-α and IL-1β.

Additionally the specific gene of astrocytes (GFAP), microglia (Iba-1) and oligodendrocyte (Olig2) within corpus callosum were evaluated using qRT-PCR. The obtained data by qRT-PCR showed that induction of chronic demyelination by CPZ increased the expression level of GFAP and Iba-1 genes (p < .001) compared to control group, which indicates that the number of astrocytes and microglia in the corpus callosum region have increased. The results showed that in the group treated with MSCs, there was a significant decrease in the expression level of GFAP (p ≤ .01) and Iba-1 genes (p ≤ .001) compared to CPZ group. Also, the mRNA levels showed that oligodendrocytes rate decreased in CPZ group (p ≤ .001 vs. control) which confirms that demyelination occurred in this model. Also, results of qRT-PCR showed that after 2 weeks of MSC transplantation, Olig2 gene level significantly increased in MSC group (p ≤ .001 vs. CPZ) (Fig. 2).

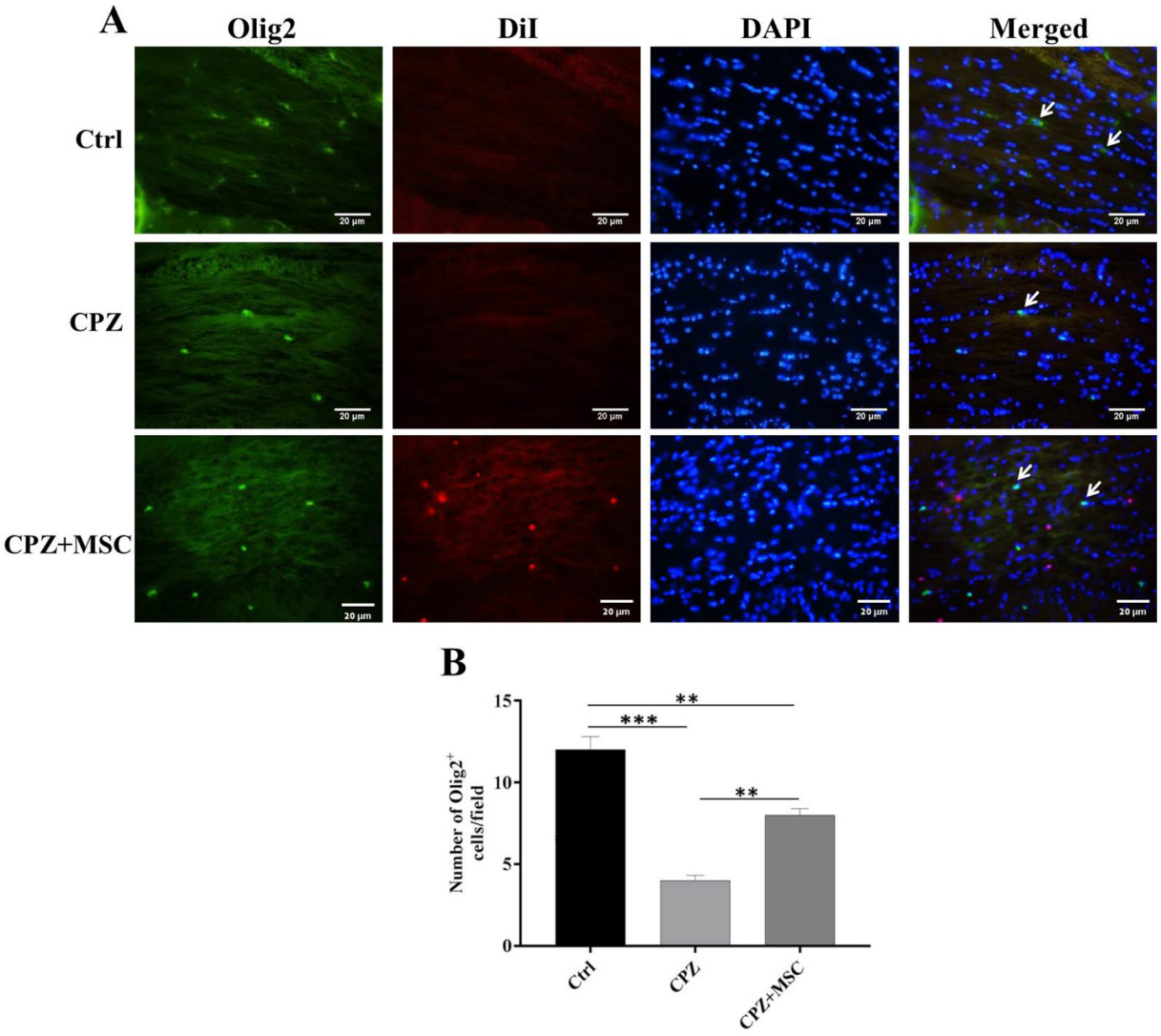

Fig. 5. Immunofluorescence assessment of Olig2 as oligodendrocytes marker. (A) The images show coronal sections (40× field) of corpus callosum oligodendrocyte (Olig2, green), MSC (DiI, red) and nuclei (DAPI, blue). Arrows indicate Olig2+ cells overlap with nuclei. (B) Quantification of the immunofluorescence data revealed that the reduction of Olig2 in the CPZ group and the rise of this marker in the CPZ + MSC group were significant. Values are expressed as the mean ± SEM. ***$p < .001$, **$p < .01$ and *$p < .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Immunofluorescence results

Astrocyte population was investigated at the translational level by immunofluorescence of GFAP. The images showed that astrogliosis occurred in demyelination model induced by cuprizone in the corpus callosum. The quantitative analysis of data confirmed that the rise in the expression of GFAP in the CPZ group was significant ($p \leq .001$ vs. control), and number of GFAP positive cells significantly ($p \leq .01$ vs. CPZ) decreased in MSC transplanted group. These results indicate that astrogliosis occurred in the CPZ group, and MSC could inhibit this process. Results of the qRT-PCR and immunofluorescence studies clearly indicate that neurotrophic factors secreted by MSC could reduce astrogliosis (Fig. 3).

The numbers of microglia inside corpus callosum in the studied groups were evaluated using immunofluorescence assessment of Iba-1. Immunofluorescence images showed signs of microgliosis in corpus callosum of CPZ group. The quantitative analysis of data showed that the rise in the expression of Iba-1 in the CPZ group was significant ($p \leq .001$ vs. control), and there was also a significant decrease in the number of Iba-1 positive cells in MSC transplant group compared to CPZ group ($p \leq .01$) (Fig. 4). These results indicate that microgliosis occurred in the CPZ group, and mesenchymal stem cell therapy could inhibit this process. Taken together, qRT-PCR and immunofluorescence results demonstrate that stem cell treatment have the potential to decrease microglial cells in CPZ receiving mice.

Olig2 protein expression was also assessed by immunofluorescence technique in order to evaluate MSC effects on oligodendrocyte population within the corpus callosum. A reduction in the number of oligodendrocytes was observed in the CPZ group which is a sign of demyelination in the corpus callosum. On the other hand, expression of Olig2 increased in CPZ group after MSC treatment. Quantification of the immunofluorescence data showed that the expression of the Olig2 significantly decreased ($p < .001$) in CPZ group compared to control group and the number of Olig2 positive cells were greater in CPZ + MSC group compared to CPZ group ($p < .01$) (Fig. 5). These results indicate that remyelination in MSC transplanted group was considerably higher than the untreated CPZ group.

3.3. LFB results

In this study, LFB staining was performed for assessment of the myelination level in the corpus callosum in 3 experimental groups.

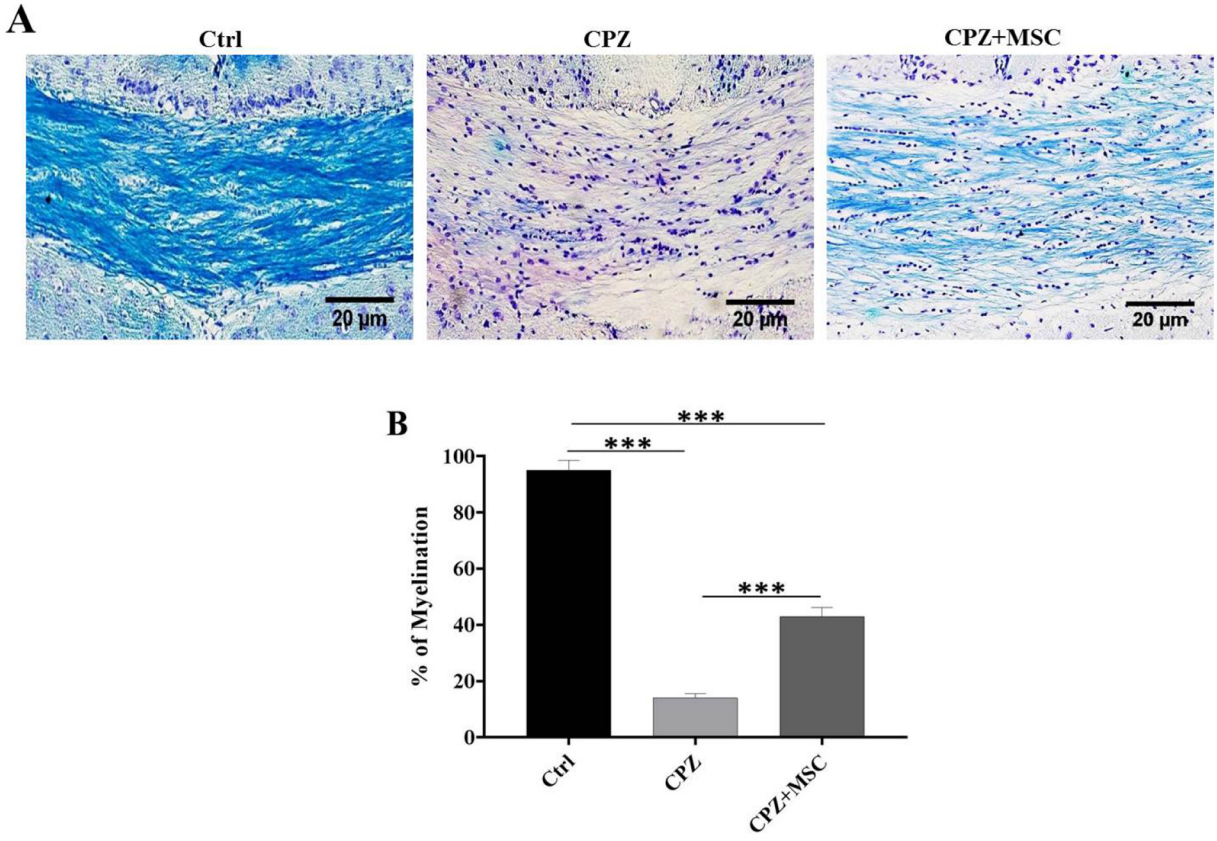

Fig. 6. Effect of MSC transplantation on myelination in corpus callosum. LFB staining showed an increase in the demyelinated area in corpus callosum in CPZ group which improved after stem cell therapy. Quantitative analysis of LFB showed that the difference between myelination rate was significant between experimental groups. Values are expressed as the mean ± SEM. ***p < .001.

Administration of cuprizone for 12 weeks induced chronic demyelination. Two weeks after MSC transplantation, the rate of myelination was increased in mice (Fig. 6-A). Quantification of the LFB staining showed that CPZ treatment increased demyelination in corpus callosum ($p < .001$ vs. control) and MSC injection increased myelinated area ($p < .001$ vs. CPZ) (Fig. 6-B). These results showed that factors secreted by stem cells could induce remyelination.

3.4. TEM findings

In order to further assess the ultrastructure of axons and myelin sheath, they were evaluated with TEM. The electron microscope images showed that the number of demyelinated fibers increased in CPZ group and also some gaps developed between the layers of myelin sheaths. On the other hand, in the CPZ + MSC group there was a noticeable decrease in the number of demyelinated fibers, confirming the results of LFB (Fig. 7-A). The quantification of TEM data was performed in order to assess the changes in the myelin sheath and axonal diameter. Quantification analysis by G-ratio showed that the myelin thickness significantly decreased in CPZ group ($p \leq .001$) compared to control group and increased ($p \leq .05$) after MSC treatment compared to cuprizone treatment alone (Fig. 7-B). These findings together with the LFB staining results indicate that MSC transplantation could be an effective approach to enhance remyelination and axonal repair.

4. Discussion

In the present study, we demonstrated that intraventricular transplanted MSCs can modulate glial cells activity and induce remyelination in cuprizone model. The damaging effects of astrocytes and microglia along with demyelination due to oligodendrocytes loss have been observed in neurodegenerative diseases such as MS (Domingues et al., 2016). Current therapeutics in MS which include immunomodulatory drugs, only reduce CNS inflammation and don't have any effects on remyelination process (Plemel et al., 2017). Therefore, novel approaches such as cell therapy are preferred to enhance remyelination and neuroregeneration (Rivera and Aigner, 2012). In order to assess the therapeutic effects of MSCs, we used the cuprizone model of demyelination and oligodendrocyte toxicity. Experimental models are a valuable tool to evaluate the cellular and molecular mechanisms involved in demyelination and remyelination processes. In the demyelination process induced by cuprizone the blood brain barrier remains intact, oligodendrocytes toxicity increases, myelin is lost and astrogliosis could be detected with microglial activation in damaged regions without any influence on the immune system (Skripuletz et al., 2011). MSCs have neurotrophic and immunomodulatory effects on glial cells that indirectly mediate neuroprotection (Uccelli et al., 2011).

Activated astrocytes exist in the margins of demyelinating regions and extend into adjacent areas and exacerbate injuries (Brosnan and Raine, 2013; Ponath et al., 2016). In this study, astrogliosis was seen in corpus callosum of mice that received cuprizone for 12 weeks. Esser et al. (2018) showed that reactive astrogliosis significantly increased in the hippocampus, corpus callosum and cortex of cuprizone mice (Esser et al., 2018). In active lesions, astrocytes have a hypertrophic morphology, with enlargement of the cell body and decreased process density and also form glial scars (Orthmann-Murphy et al., 2008). Our results showed that the number of astrocytes decreased in MSC transplanted mice. MSC transplantation also reduced inflammation. The decreased number of astrocytes was accompanied by a reduction in pro-inflammatory factors including IL-1β and TNF-α, which might be the result of the activity of MSCs trophic factors. Similarly, Park et al. (2015) indicated that LPS increased VEGF-A in astrocytes and IL-1β in

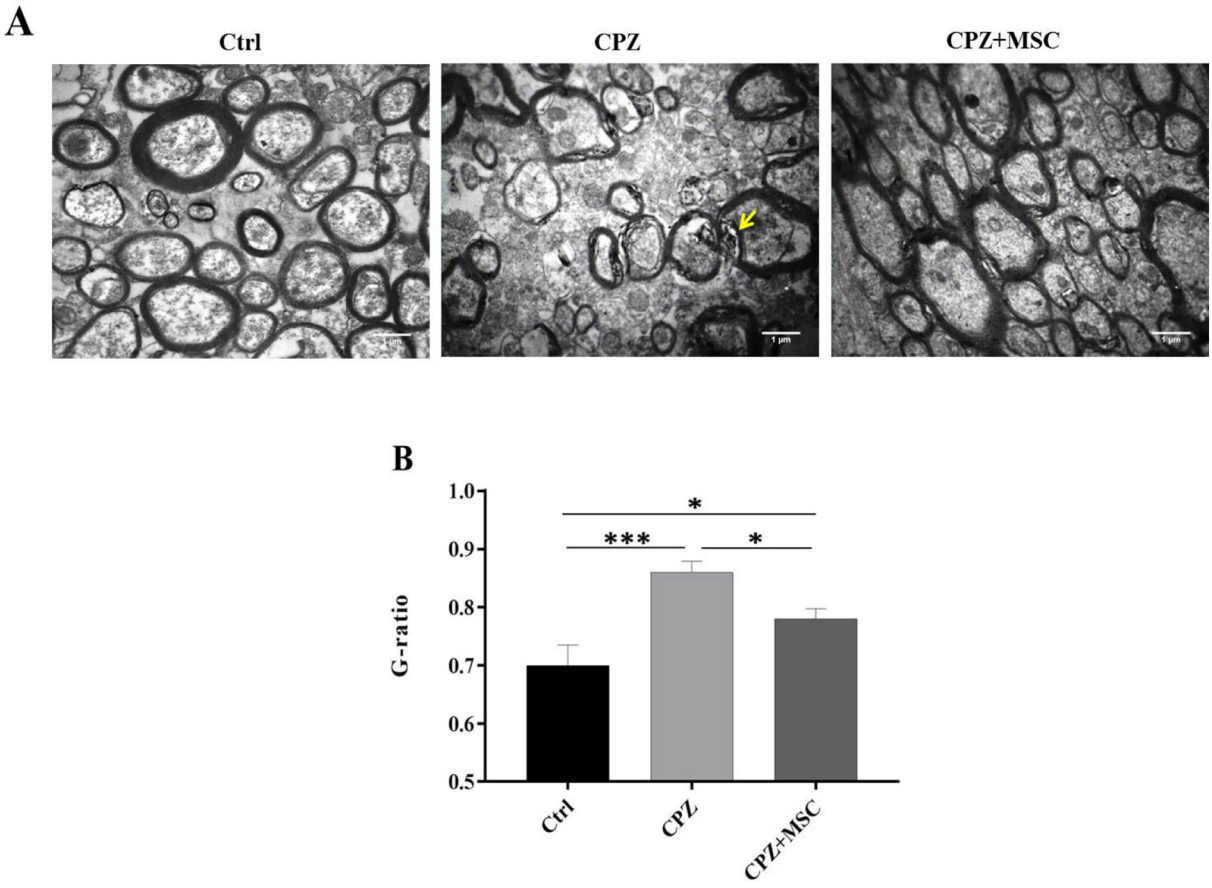

Fig. 7. Mesenchymal stem cells effect on ultrastructures of myelin sheath in cuprizone demyelination model. (A) The disruptions and gaps between layers of the myelin membranes were seen in the CPZ group. The yellow arrow indicates destroyed myelin sheath. (B) The graph depicts the G-ratio. Quantification of the TEM data showed that MSC could repair damaged myelin by cuprizone. Values are expressed as the mean ± SEM. ***p < .001 and *p < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microglia and MSC transplantation considerably reduced IL-1β and VEGF-A through an increase in IL-10 expression (Park et al., 2015).

Microglia which are the resident macrophages of the nervous system, become active in response to brain damage. They also change their phenotype and morphology in diseases and help to preserve brains homeostasis (Saijo and Glass, 2011). Various cytokines and inflammatory molecules regulate microglial phenotype and could shift microglia toward a detrimental or beneficial phenotype (Kettenmann et al., 2011). Interestingly, we identified a significant upregulation of IL-1β and TNF-α as cytokines that are usually involved in the inflammatory response in cuprizone mice that was according to other study (Tahmasebi et al., 2019). Meanwhile in MSC transplanted group, the rise of trophic factors such as TGF-β and CX3CL1 could suppress pro-inflammatory cytokines. In parallel to this result, Kettenmann and colleagues induced microglial activation by LPS and then added exogenous CX3CL1 to microglial culture. They showed that the CX3CL1 secreted by MSC can switch microglia from M1 phenotype that secrete proinflammatory molecules to M2 phenotype that produce anti-inflammatory cytokines (Kettenmann et al., 2011). Giunti et al. (2012) reported that MSCs decrease the expression of iNOS, TNF-α and oxidative stress factors, which are induced by LPS activated microglia, and thus prevent the production of proinflammatory molecules during chronic brain inflammation (Giunti et al., 2012).

Ion channels become disrupted in demyelinated axons, which leads to accumulation of ions, enlargement of the axolema, axonal damage and neurodegeneration (Dutta and Trapp, 2007). Remyelination could not occur in demyelination disorders such as MS and therapeutic approaches for remyelination enhancement are not available. Recently, the exogenous MSC transplantation have been used as a suitable approach to promote myelin repair (Tejedor, 2015; Rivera and Aigner, 2012).

There have been lots of attention on oligodendroglial cells during neuroregenerative therapies, because the failure in the remyeliantion process is mostly due to a failing in the proliferation and differentiation of myelinating oligodendrocytes (Tejedor, 2015). In this study, we confirmed that MSC transplantation could increase remyelination in demyelination disorders of the CNS through secretion of trophic factors such as TGF-β and CX3CL1. These results are in line with previous studies. Jaramillo and colleagues (2013) reported that transplantation of MSCs increased the OPC migration to the demyelinated regions and also enhanced remyelination. This effect is due to the secretion of trophic factors (Jaramillo-Merchan et al., 2013). One opposing study reported that MSCs have no major effect on demyelination. However, there were several differences in experimental design, including short duration of investigation after administration of MSCs (Nessler et al., 2013). One possible mechanism that could explain our findings regarding myelination is that MSCs directly impact the viability of oligodendrocytes during apoptosis, OPCs differentiation and they also attract the oligodendrocytes to demyelination regions by secreting CX3CL1 and TGF-β. These results are similar to the study conducted by Cruz-Martinez et al. (2017). They transplanted BMSCs into the lateral ventricles of the cuprizone induced mice model and reported that MSCs can secrete trophic factors into the CSF and stimulate the oligodendrocytes in subventricular zone (SVZ). Additionally, they showed that trophic factors secreted by the MSCs increased proliferation of stem cells in the SVZ. Their results revealed that MSCs injection had a

paracrine effect on the oligodendrocytes of the SVZ which response to the factors released into the CSF and therefore, MSCs induce oligodendrogenesis and remyelination (Cruz-Martinez et al., 2017). Our findings showed that MSCs secreted trophic factors and led to a significant decrease in inflammation via downregulation of pro-inflammatory factors IL-1β and TNF-α.

Isele and colleagues reported that BMSCs protect neurons against amyloidβ induced apoptosis through stimulation of the phosphatidylinositide 3-kinase/protein kinase B signalling in vitro (Isele et al., 2007). Papazian et al. (2018) showed that BMSCs released factors that protected neurons against glutamate excitotoxicity. Glutamate excitotoxicity create neuronal and oligodendroglial injury in neurodegenerative conditions, such as EAE and MS; Thus their findings represent the neuroprotective effects of MSCs (Papazian et al., 2018).

Our results suggest that mesnchymal stem cells treatment could enhance myelination in corpus callosum via glial cells modulation, thus it might be used as a new approach for demyelination and gliosis treatment in MS.

5. Conclusion

Our findings showed that MSCs transplantation could increase CX3CL1 and TGF-β as trophic factors and decrease inflammation, microgliosis and astrocytosis in cuprizone model. MSCs injection could also attract oligodendrocytes in demyelination area and promote remyelination in a chronic cuprizone model. Finally, we could find an approach for improving inflammation and chronic demyelination in neurodegenerative diseases.

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Conflict of interests

The authors declare no conflict of interests regarding the publication of this paper.

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