

# Valproic acid effects on human adipose-derived stem cell differentiation into oligodendrocytes and improved remyelination in a mouse model of Multiple Sclerosis

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**ABSTRACT** Valproic acid (VPA), a neuroprotective agent and inhibitor of GSK3- $\beta$ , along with human Adipose-Derived Stem Cells (hADSCs) have been proposed to be potential therapeutic agents for neurodegenerative disorders. In the present study, we have assessed the effects of VPA alone or in combination with hADSCs on oligodendrocyte differentiation, remyelination, and functional recovery in a mouse model of Multiple Sclerosis (MS). These MS-model mice were randomly divided into cuprizone, sham, VPA, hADSC, and VPA/hADSC groups, with 10 mice considered a control group (healthy mice). The hanging wire test was used to measure motor behavior. To estimate the level of myelination, we performed toluidine blue staining and immunofluorescent staining for OLIG2 and MOG-positive cells. Real-time PCR was used to evaluate the expression of  $\beta$ -catenin, human and mouse Mbp, Mog, and Olig2 genes. Remyelination and motor function improved in mice receiving VPA, hADSCs, and especially VPA/hADSCs compared to the Cup and Sham groups ( $P < 0.01$ ). Additionally, the number of MOG and OLIG2 positive cells significantly increased in the VPA/hADSCs group compared to the Cup and Sham groups ( $P < 0.01$ ). The expression of  $\beta$ -catenin, myelin and the other oligodendrocyte-specific genes was significantly higher in the VPA recipient groups. Valproic acid can enhance the differentiation of stem cells into oligodendrocytes, making it a potential candidate for MS treatment.

**KEYWORDS:** Multiple Sclerosis, valproic acid, human adipose-derived stem cell, neuroprotective agent, remyelination

## Introduction

Stem cells (SCs) are undifferentiated cells with the capacity for self-renewal and the potential to differentiate into other cell types (Zakrzewski *et al.*, 2019). Cell therapy using embryo and adult stem cells has recently emerged as an advanced area of scientific research. In this context, adipose-derived stem cells have garnered particular attention due to their abundance in adipose tissue and ease of isolation. These cells can repair damaged tissue through immunomodulatory and neuroprotective effects, achieved by releasing trophic factors, inhibiting inflammation, and creating a microenvironment conducive to the survival of neurons, axons, and oligodendrocytes (Bateman *et al.*, 2018). Previous studies have also demonstrated the beneficial effects of human adipose-derived stem cell (hADSC) transplantation in structural and functional recovery

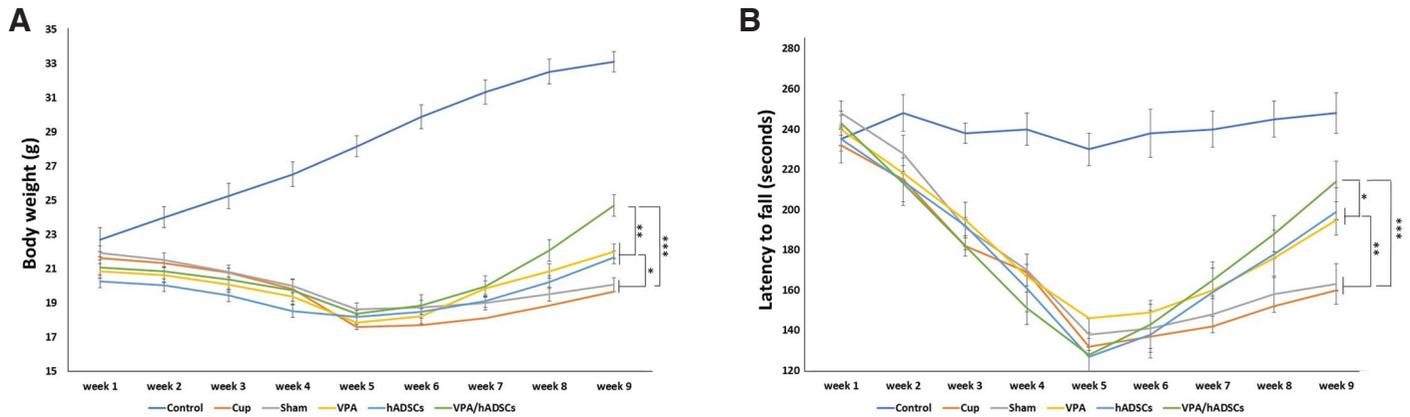
from neurodegenerative disorders such as Multiple Sclerosis (MS) (Ghasemi, 2018; Villoslada, 2016).

MS is a chronic disease characterized by various neurological symptoms resulting from demyelination (Moghadasi, 2020). It is essential to note that, in this abnormal condition, widespread demyelination and the formation of dense glial scars typically occur in various regions of the white matter due to the spread of inflammation. Previously, MS pathology solely focused on the appearance of focal demyelinated plaques in the white matter

**Abbreviations used in this paper:** CC, Corpus callosum; CNS, Central nervous system; CUP, Cuprizone; GSK3- $\beta$ , Glycogen synthase kinase-3 beta; hADSCs, Human Adipose-Derived Stem Cells; iP, Intraperitoneally; MBP, Myelin basic protein; MOG, Myelin oligodendrocyte glycoprotein; MS, Multiple Sclerosis; SCs, Stem cells; VPA, Valproic acid.

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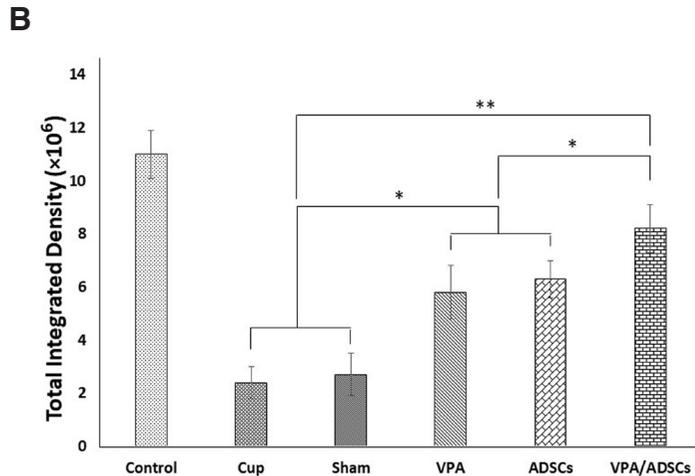
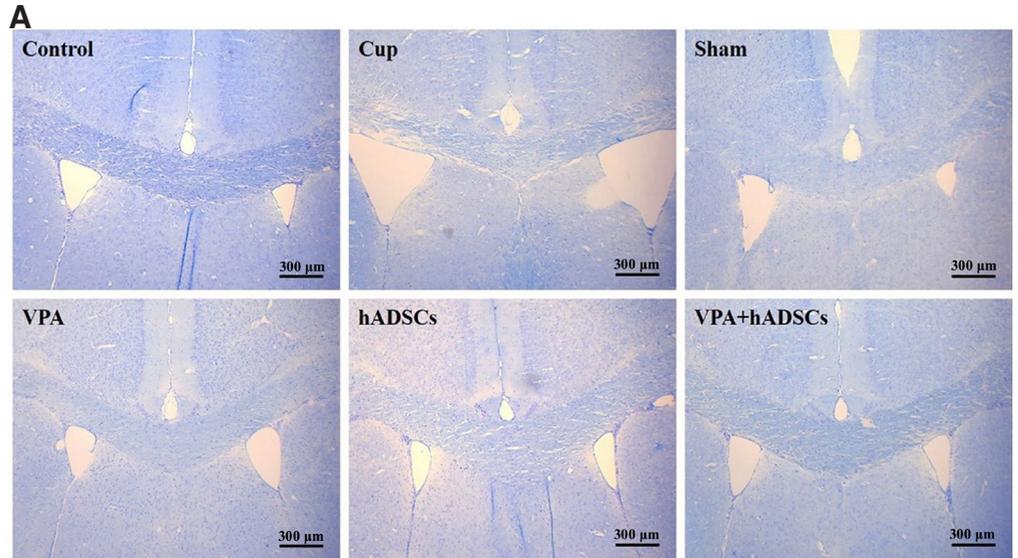


**Fig. 1. Body weight changes of mice during the study. (A)** Comparing the body weight of mice showed that the use of cuprizone led to weight loss, and after received of VPA and hADSCs, body weight increased significantly compared with other groups. **(B)** Hanging wire test also, showed a significant improvement in the motor function of mice after receiving hADSCs and VPA. Groups: Cuprizone (Cup), valproic acid (VPA), human adipose derived stem cells (hADSC), and VPA/hADSC. (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

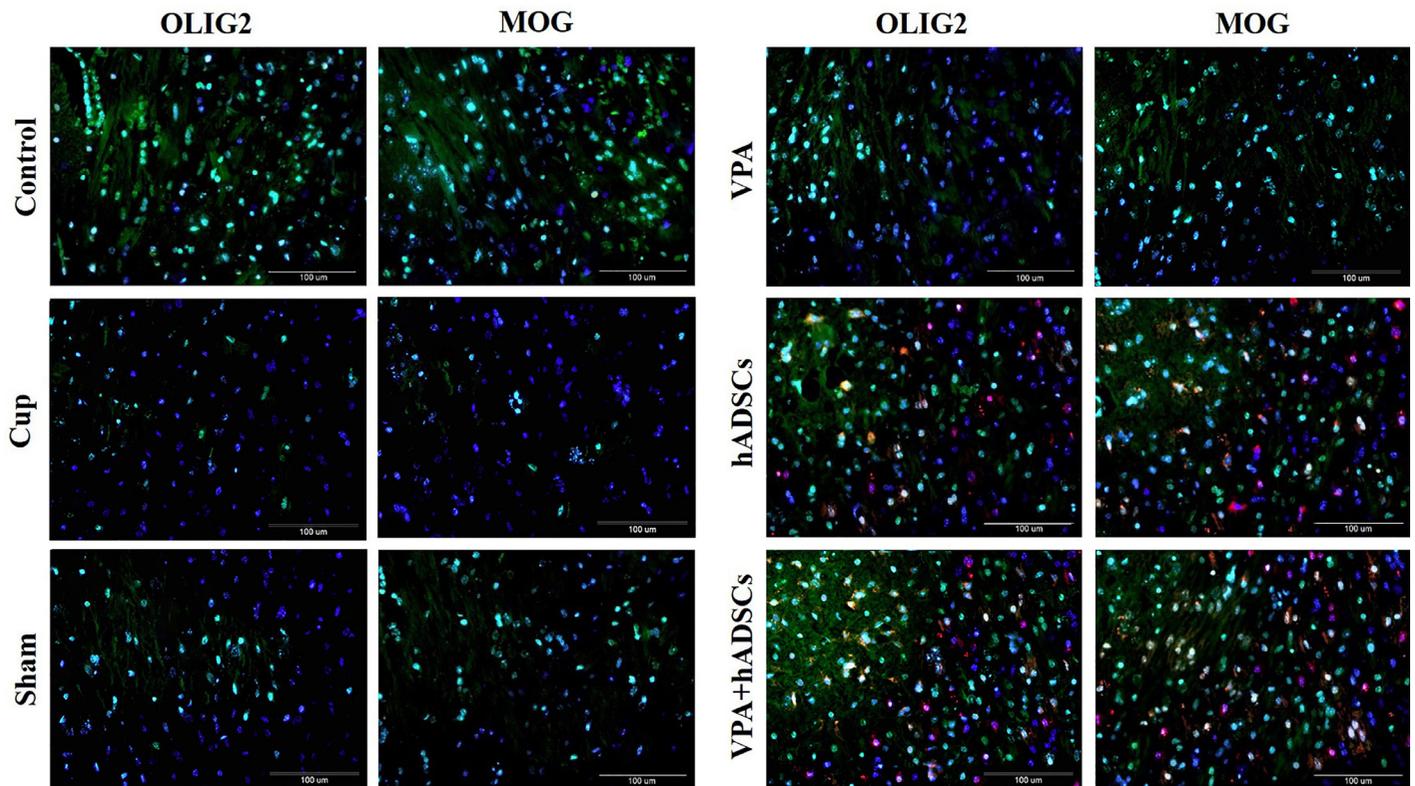
(Lassmann, 2018). However, it is now recognized that demyelination and glial scar formation can also be observed in the gray matter of the brain and spinal cord (Gajofatto and Benedetti, 2015).

Experimental studies' findings in MS treatment revealed that immunomodulatory drugs and neuroprotective agents can protect neurons and enhance remyelination by reducing central nervous system (CNS) inflammation and cell death. Consequently, the formation and regeneration of myelin have been mimicked as a specific biological process in the CNS through experimental therapies (Villoslada, 2016).

Neurotrophic factors, via the activation of signaling pathways such as phosphoinositide 3-kinase, glycogen synthesis kinase-3 $\beta$  (GSK-3 $\beta$ ), mitogen-activated protein kinase, and the nuclear factor- $\kappa$ B pathway, can prevent cell apoptosis, promote cell survival and differentiation, and maintain communication between target cells and neurons. It has been reported that the inactivation of the GSK-3 $\beta$  pathway can lead to brain protection, motor function recovery, neuronal survival, and neurogenesis by stimulating brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), Cyclin D1, and Neuro D1 (Xu et al., 2019; Moon et al., 2018). Valproic acid (VPA), a GSK-3 $\beta$  inhibitor (Zeng et al., 2019), is widely employed clinically as a pharmacological agent in treating neurological diseases.



**Fig. 2. Staining of mouse corpus callosum sections with toluidine blue. (A)** Demyelination was evident in the images of the untreated (Cup and sham) groups. In contrast, myelin density measurement indicated that re-myelination occurred using hADSCs or VPA. **(B)** Myelin density was assessed using Image J software. After receiving hADSCs and VPA, myelin density increased significantly.



**Fig. 3. Immunohistochemical staining of mouse brain sections with oligodendrocyte-specific cell markers.** PKH26- labeled hADSCs (red) were shown around the nucleus, labeled blue with DAPI. The OLIG2 and MOG markers were expressed by PKH26+ cells. Groups: cuprizone (Cup), valproic acid (VPA), human adipose derived stem cells (hADSCs), and VPA/hADSCs. Scale bar, 100  $\mu$ m.

This factor is pivotal in regulating cellular processes, including cell differentiation and proliferation, by influencing intracellular signaling through specific receptors (Moon *et al.*, 2018).

Based on the introduction above, if valproic acid can stimulate the differentiation of stem cells into oligodendrocyte cells, it could be considered a potential candidate for a novel treatment approach for MS. Consequently, this in-vivo study was designed to assess the effects of valproic acid, as a GSK3- $\beta$  inhibitor, on stem cell differentiation into oligodendrocytes and remyelination in a mouse model of multiple sclerosis induced by cuprizone.

Among the various animal models of MS, cuprizone (Cup) has been used recently to induce MS in animals. Cuprizone, as a toxic compound and a copper chelating agent, can cause demyelination in different areas of the brain such as the corpus callosum, by disrupting the metabolism of oligodendrocytes (Zhen *et al.*, 2017; Kopanitsa *et al.*, 2021). Cup can reduce the expression of myelin-related genes and proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein in the corpus callosum (Han *et al.*, 2020). Therefore, in this study, a cuprizone model of MS was used.

## Results

### Characterization of hADSCs

After cell culture, the hADSCs exhibited an elongated morphology similar to fibroblast cells, confirming their mesenchymal nature. Furthermore, upon examination of the cells under a fluorescent microscope, it was confirmed that approximately 80% were labeled with PKH26.

### Effects of hADSCs and VPA on mouse motor function improvement and weight changes

Mouse body weight and motor behavior were assessed and recorded weekly using a hanging wire test. Data evaluation showed that weight loss and a decline in motor function were evident due to the application of cuprizone. However, the use of VPA, hADSCs, and especially VPA/hADSCs ( $P < 0.05$ ,  $P < 0.05$ ,  $P < 0.001$ , respectively) led to a significant increase in body weight compared to the other groups (Fig. 1A). In general, the groups receiving VPA, hADSCs alone, and in combination demonstrated a significant increase in the hanging time of the mice compared to the untreated groups ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.001$ , respectively). Additionally, there was a statistically significant increase observed in the VPA/hADSCs group when compared to the VPA and hADSCs groups ( $P < 0.05$ ) (Fig. 1B).

### Histological analysis

To assess the myelin condition, we stained tissue slides with toluidine blue in the corpus callosum (Fig. 2A). Considerable demyelination was evident in the images of the untreated (Cup and sham) groups. In contrast, myelin density measurement indicated that remyelination occurred using hADSCs or VPA. When comparing the groups, we observed the highest myelin density in the VPA/hADSCs group (\*;  $P < 0.01$ ) (Fig. 2B).

### Immunohistochemistry

Immunohistochemistry using cell type-specific markers (Fig. 3) revealed an increase in MOG and OLIG2 positive cells in the groups treated with VPA, hADSCs, and VPA/hADSCs compared to the untreated groups ( $P < 0.05$ ). However, no significant difference

was observed between the groups receiving VPA and hADSCs compared to the VPA/hADSCs group. The analysis of oligodendrocyte differentiation in the hADSC recipient groups demonstrated that the PKH26/OLIG2+ and PKH26/MOG+ positive cells were significantly fewer in the hADSC group ( $7.2 \pm 0.74\%$  and  $8.6 \pm 0.65\%$ , respectively) than in the VPA/hADSCs group ( $13.42 \pm 0.91\%$  and  $14.8 \pm 1.05\%$ , respectively) ( $P < 0.05$ ).

#### Increase in the expression of $\beta$ -catenin, myelin, and oligodendrocyte-specific genes in response to VPA and hADSC treatments

Five weeks after treatment with VPA and hADSCs, the expression levels of  $\beta$ -Catenin, mouse and human Mbp, Mog, and Olig2 were measured using Real-Time PCR. The results showed that the expression of  $\beta$ -catenin, human and mouse myelin, and oligodendrocyte-specific genes was significantly reduced in the cup and sham groups compared to the treated groups. Meanwhile, a significant increase in gene expression was observed in the VPA and hADSCs treated groups, especially in the group receiving VPA and hADSCs simultaneously (Fig. 4). In the end, no significant difference was observed in the expression of the Mbp gene between the hADSCs group and the VPA/hADSCs group (Fig. 4D). However, significant differences for MOG and OLIG2 were found (Fig. 4E).

#### Discussion

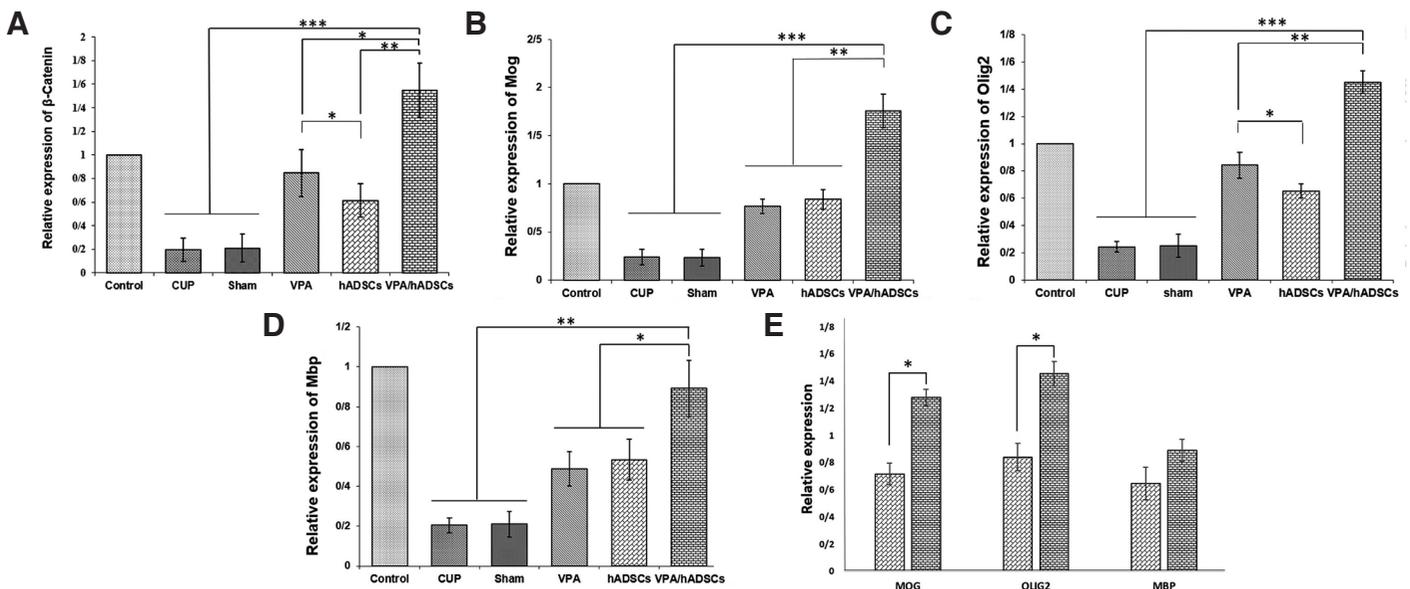
The destruction of the myelin sheath can occur in various CNS disorders, such as multiple sclerosis (MS). In MS disease, the myelin sheath and oligodendrocytes are subject to destruction due to infiltration by immune cells (Dehghan et al., 2016). This abnormal condition can induce severe physical or cognitive disabilities owing to the formation of MS plaques and disturbances in the transmission of nerve impulses. Given that remyelination and recovery in MS are often limited and incomplete, there is a growing concern about the treatment of MS. Current published

data suggests that cell-based therapy employing stem cells and neurotrophic factors has introduced a potential new paradigm for MS treatment. For instance, it has been demonstrated that the intravenous injection of ADSCs can promote remyelination in the corpus callosum (Killer et al., 2017).

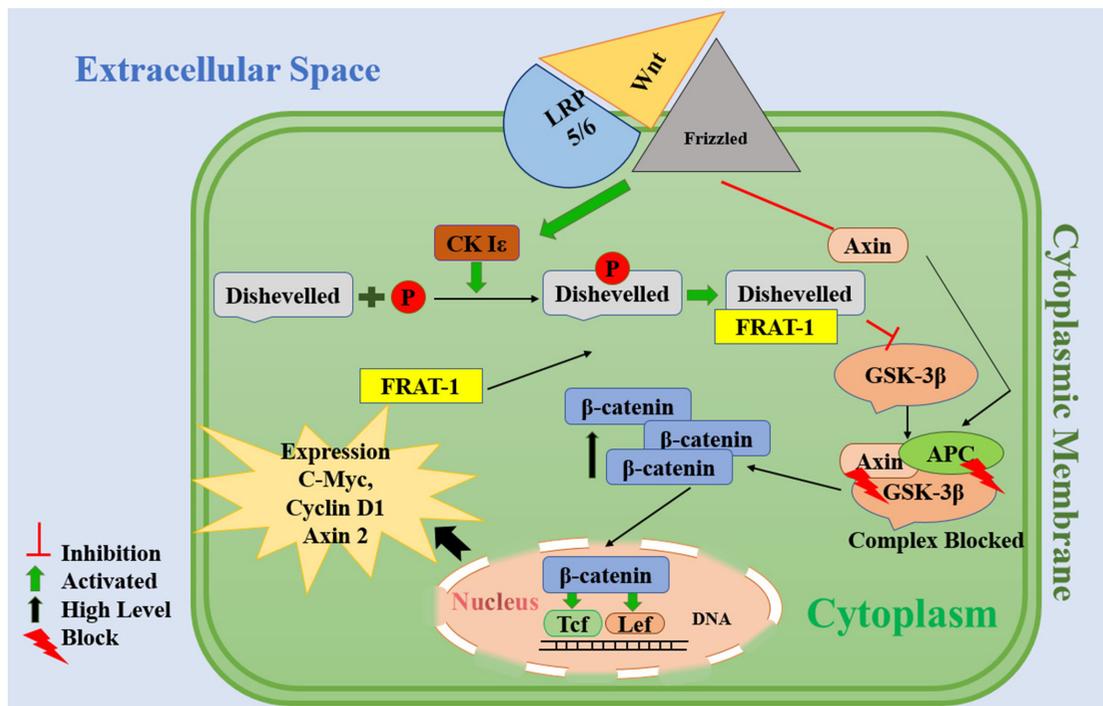
Moreover, the transplantation of hADSCs into a mouse model of MS has led to improved motor coordination, balance, and spinal cord myelination (Talwadekar et al., 2017). Additionally, applying certain drugs or factors can enhance the differentiation of stem cells into target cells. Recent studies have indicated that combining stem cells with neuroprotective agents such as edaravone, pregnenolone, and VPA can increase the differentiation of oligodendrocytes and induce remyelination (Pazhoohan et al., 2014; Bakhtiari et al., 2021; Ganji et al., 2020).

Other studies report that the motor performance of animals improved with the appropriate dose of PLX3397. It is possible that PLX3397 could maintain myelination by regulating the myelin environment in the MS model. Experimental results have shown that the number of microglia significantly decreased following MSC transplantation. Furthermore, the oligodendrocyte population increased, leading to enhanced remyelination in the corpus callosum of the cuprizone model of MS. Consequently, PLX3397 treatment and MSC injection reduced astrogliosis and microgliosis. Another study has confirmed that applying PLX for 21 days increased remyelination by enhancing oligodendrocytes and reducing microglia in the demyelination model (Tahmasebi et al., 2021b; Tahmasebi et al., 2019; Tahmasebi et al., 2021a).

Previous studies have demonstrated that VPA plays a significant role in the regeneration, remyelination, and regulation of the peripheral and central nervous systems through intracellular signaling via specific receptors (Pazhoohan et al., 2014; Jang and Jeong, 2018; Zhu et al., 2021; Azuchi et al., 2017). Since neurotrophic factors are emerging as therapeutic agents for restoring and maintaining the function of oligodendrocytes, our research evaluated the dif-



**Fig. 4. The relative expression of  $\beta$ -catenin, myelin and oligodendrocyte-specific genes in the corpus callosum. (A-D)** Results showed a significant upregulation of  $\beta$ -catenin, Mbp, Olig2 and Mog in VPA and hADSC treated groups, compared with untreated groups. **(E)** Comparison of the expression of human MBP, OLIG2 and MOG found in the hADSC and VPA/hADSC groups. The simultaneous use of VPA/hADSC had a significant increase in the expression of MOG and OLIG2 genes. Groups: Cuprizone (Cup), Valproic Acid (VPA), human Adipose Derived Stem Cells (hADSCs), and VPA/hADSCs. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



**Fig. 5. Proposed mechanism of action of valproic acid.** The Wnt signaling pathway is activated upon wnt ligand binding to the transmembrane receptor Frizzled and co-receptor LRP-5/6. After forming the Wnt-Frizzled-LRP5/6 complex, Dishevelled (DVL) is employed/recruited. Subsequently, DVL is phosphorylated by casein kinase Iε (Ck Iε) and connection of DVL with Frat inhibits GSK-3β activity. Additionally, the LRP5/6 receptor from the Wnt-Frizzled-LRP5/6 complex will degrade Axin. The complex of adenomatous polyposis coli (APC), GSK-3β and Axin is not formed and as a result, GSK-3β is inhibited. Thereafter, the inactivation of this complex causes the blockage of β-catenin protein phosphorylation and promotes the cytoplasmic concentration of β-catenin. When β-catenin passes through the nucleus membrane, it activates the Tcf / Lef transcription factor leading to the transcription and expression of cyclin D1, Axin 2 and c-Myc.

differentiation of stem cells into oligodendrocytes and remyelination of the corpus callosum area in cuprizone-induced mice 5 weeks after treatment with hADSCs and VPA.

We have demonstrated that VPA can mitigate the adverse effects of cuprizone, including demyelination, oligodendrocyte death, muscle weakness, and weight loss in a mouse model of MS. Cuprizone can induce apoptosis of oligodendrocytes by inhibiting cytochrome oxidase and inducing mitochondrial disorder in the energy production cycle (Mohammadi-Rad *et al.*, 2019). However, VPA can mitigate these adverse effects through its neuroprotective properties and by enhancing remyelination via the secretion of neurotrophic factors and neuroprotective functions (Chen *et al.*, 2016; Ghasemi *et al.*, 2014).

Furthermore, injecting hADSCs and applying VPA can increase the mean percentage of Olig2 and MOG-positive cells. According to these findings, VPA may operate within the therapeutic range by either promoting the differentiation of hADSCs into oligodendrocytes or enhancing cell proliferation and protection. Consequently, it increases the population of myelin-generating oligodendrocytes, thereby improving motor behavior and remyelination. Other results have indicated that VPA can play a significant role in nerve regeneration by promoting the differentiation of ADSCs into neurons. Additionally, in the mouse MS model, the expression of Olig2 and MBP genes increased with hADSCs, leading to stimulation of remyelination and improved motor behavior (Bakhtiari *et al.*, 2021; Kim *et al.*, 2015; Santos *et al.*, 2020).

It is worth noting that VPA, as a GSK3-β inhibitor, can regulate the fate of stem cells by altering the expression of specific genes in the Wnt/GSK3-β molecular pathway, such as the transcription of the β-catenin protein (Kim *et al.*, 2015; Soleimani and Ghasemi, 2017). Moreover, increasing the level of β-catenin in the cell appears to be a potential new therapeutic target for treating MS (Chiu and Chuang, 2011). β-catenin is crucial in the Wnt pathway for initiating cellular processes such as proliferation, differentiation, and maturation of stem cells. Additionally, it plays a neuroprotective role (Kühl and Kühl, 2013; Miki *et al.*, 2011). As depicted in Fig. 4E, in VPA-treated groups, the expression of the β-catenin gene and the genes associated with oligodendrocytes (Mog and Olig2) and myelin production (Mbp) increased.

Therefore, VPA may inhibit the phosphorylation of β-catenin through the inactivation of GSK-3β, which in turn could enhance the expression of anti-apoptotic genes and the transcription of growth factors by raising the cytoplasmic level of β-catenin and facilitating its translocation into the nucleus. As a result, VPA improves motor behavior and enhances remyelination by reducing oligodendrocyte death, promoting cell survival and proliferation, and facilitating the differentiation of transplanted cells into myelin-producing cells (Fig. 5).

In summary, our research results demonstrate that valproic acid can effectively promote the differentiation of human adipose-derived stem cells into oligodendrocytes and enhance remyelination in multiple sclerosis (MS) diseases by inactivating GSK-3β func-

tion and phosphorylating  $\beta$ -catenin. However, further studies are required to investigate the detailed mechanism underlying these therapeutic effects.

## Materials and Methods

### Experimental animals

We employed adult female C57BL/6 mice (Laboratory Animal Center, Royan Center, Isfahan, Iran), 8 weeks old and weighing 18 - 22 g, for our study. This research received approval from the IUMS Ethical Committee and was conducted using cuprizone mice models at the Central Laboratory of Isfahan University of Medical Sciences (Liu *et al.*, 2018). The mice were provided free access to food and water and maintained under suitable health conditions and temperature control ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ). They were divided into five groups: cuprizone (Cup,  $n=8$ ), sham ( $n=8$ , received normal saline for 4 weeks intraperitoneally (IP)), human adipose-derived stem cells ( $n=8$ ,  $1 \times 10^6$  labeled hADSCs, administered intravenously (IV)), valproic acid (Azuchi *et al.*, 2017) (VPA,  $n=8$ , 300 mg/kg/day VPA, IP), and VPA/hADSCs ( $n=8$ , 300 mg/kg/day VPA, IP for 4 weeks and  $1 \times 10^6$  labeled hADSCs, IV) groups. A healthy control group ( $n=8$ ) was also included for comparison purposes.

### Induction of demyelination

All mice (except the healthy control group) were administered a dose of cuprizone. They received 0.2% cuprizone (Sigma-Aldrich Inc. C9012) for 5 weeks to induce demyelination (Liu *et al.*, 2018). Throughout the study, we measured the body weight and monitored behavioral changes in all mice to assess the effects of cuprizone.

### Thawing and culture of hADSCs

We retrieved the frozen cells (hADSCs in passages 2: P2) from the nitrogen tank and thawed them following the procedure outlined in our previous study (Bakhtiari *et al.*, 2021). Subsequently, we placed the cell suspension into T75 flasks and cultured the cells in the presence of DMEM/F12 (Bioidia, B11027) containing 10% fetal bovine serum (FBS) (Gibco, 10270106) and a penicillin/streptomycin solution. We used  $1 \times 10^6$  labeled hADSCs from passages 4 (P4) for injection into the tail vein of the mice.

### Cell membrane labeling with PKH26

Seventy-two hours before cell transplantation, human Adipose-Derived Stem Cells (hADSCs) were labeled with the *in vitro* cell membrane labeling agent PKH-26 (Sigma-Aldrich, MINI26). To achieve this, we placed cells in a conical tube and washed them once with a serum-free medium to remove proteins and lipids. Subsequently, we centrifuged the cells at 1500 rpm for 5 minutes. PKH26 was added to the cells washed with a phosphate buffer solution (PBS). After 5 minutes, 1% bovine serum albumin (BSA) (Sigma-Aldrich A2153) was utilized to halt the cell labeling process. The cells were washed three times with DMEM/F12 medium to eliminate any excess dye, and fluorescent microscopy (Olympus BX51, Japan) was employed to assess staining efficiency. (Ganji *et al.*, 2020; Ghasemi *et al.*, 2014; Razavi *et al.*, 2018)

### Behavioral test (Wire-Hanging test)

The wire-hanging test was conducted throughout the study to assess motor behavior and muscular strength. For this purpose, a metal wire with a 2 mm diameter was utilized. Mice were suspended

TABLE 1

### THE LIST OF PRIMERS USED IN THIS STUDY

Gene	Primer sequences
B-actin-m-F	5'-CGGTTCCGATGCCTGAGGCTCTT-3'
B-actin-m-R	5'-CGTCACACTTCATGATGGAATTGA-3'
B-ACTIN-H-F	5'-AGCCTCGCCTTTGCCGATCC-3'
B-ACTIN-H-R	5'-ACATGCCGGAGCCGTTGTGCG-3'
Mbp-m-F	5'-AACATTGTGACACCTCGAACA-3'
Mbp-m-R	5'-TGTCTCTTCC TCCCCAGCT-3'
MBP-H-F	5'-ACTATCTCTTCTCCAGCTTAAAAA-3'
MBP-H-R	5'-TCCGACTATAAATCGGCTCACA-3'
Mog-m-F	5'-TGATTTCCCTCCCTCAACTG-3'
Mog-m-R	5'-CGTATCCTGGTTGGCAGAAAT-3'
MOG-H-F	5'-ACCAGGCACCTGAATATCGG-3'
MOG-H-R	5'-CAGGGCTCACCCAGTAGAAAG-3'
Olig2-m-F	5'-TGGAGAGATGCGTTCGTTCC-3'
Olig2-m-R	5'-GTGCTCTGCTCCTCTCTAA-3'
OLIG2-H-F	5'-AGATCGACGCCGACACCCAGCG-3'
OLIG2-H-R	5'-TCGGACCCGAAAACTGGATGCG-3'
$\beta$ catenin-m-F	5'-GTACGCACCATGCAGAATAC-3'

from the wire for 5 minutes. If a mouse lost its balance and fell off the wire during the experiment, it was placed on the wire twice, and the hanging time was calculated. Mice displaying a decrease in hanging time of more than 60 seconds were categorized as having significant motor dysfunction compared to before the injury, while those with slight motor dysfunction exhibited a decrease in the longest hanging time of 30 to 60 seconds. Mice with a decrease in the longest hanging time of less than 30 seconds were considered to have no motor disability (Yamazaki *et al.*, 2021).

### Toluidine blue staining

Immediately following the study's conclusion, mice were deeply anesthetized using ketamine/xylazine, and cardiac perfusion was performed to fix the brain, as previously described (Yu *et al.*, 2017). The brains of the mice were then immersed in 4% paraformaldehyde for 24 hours and subsequently embedded in paraffin. Sections with a thickness of 5  $\mu\text{m}$  were prepared using a microtome. Finally, the slides were stained with toluidine blue, and an Olympus Provis light microscope was employed to examine the stained samples (Zhang *et al.*, 2020).

### Immunohistochemistry (IHC)

In this study, Olig2 and myelin oligodendrocyte glycoprotein (MOG) were utilized as specific markers for oligodendrocytes. Tissue samples were deparaffinized and dehydrated. In order to evaluate oligodendrocyte and myelin markers, we prepared 5  $\mu\text{m}$  thick sections of the corpus callosum using a conventional microtome. Antigen retrieval was done by adding sodium citrate buffer and 1 mM EDTA buffer for 10 minutes at  $90^{\circ}\text{C}$  (Gibco, USA). Primary antibodies, including anti-Olig2 antibody (1:1000; Abcam, Cambridge, MA, USA) and anti-MOG antibody (1:1000; Abcam, Cambridge, MA, USA), were applied, and the slides were incubated overnight at  $4^{\circ}\text{C}$ . Following a PBS wash, the slides were incubated at room temperature for 1 hour with FITC-conjugated secondary antibodies (1:500; Abcam, Cambridge, MA, USA). Subsequently, sections were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, D9542) for 2 minutes to label cell nuclei. Finally, using a fluorescent microscope and ImageJ software, the aver-

age percentage of cells expressing MOG and Olig2 markers was determined from 200 cells per slide (Bakhtiari *et al.*, 2021).

### RT-PCR analysis

The levels of  $\beta$ -catenin, human and mouse Mbp, Mog, and Olig2 genes in CC were assessed using real-time RT-PCR. After RNA extraction with the Total RNA Prep Kit (BIOFACT, RP101–050, Korea), 2  $\mu$ g of RNA were utilized for cDNA synthesis using the BioFact™ 5X RT Pre-Mix cDNA Synthesis Kit (BIOFACT, 25081), following the manufacturer's protocol. The expression of the target genes was measured using the BioFact™ 2X Real-Time PCR Master Mix Kit (BIOFACT, DQ385–40) in a total volume of 20  $\mu$ L, containing Power SYBR Green master mix (2x), forward and reverse primers (0.5  $\mu$ M), cDNA (30 ng/ $\mu$ L), and H<sub>2</sub>O, with the following cycling conditions on the Step One Plus™ quantitative real-time PCR detection system (Applied Biosystems): 1 cycle of denaturation (95°C for 5 minutes), followed by a 40-cycle amplification (at 95°C for 15 seconds) and the extension cycle (at 60°C for 40 seconds).  $\beta$ -actin was used as the housekeeping gene. The sequences of the primers used are listed in Table 1. (Bakhtiari *et al.*, 2021; Razavi *et al.*, 2021)

### Statistical analysis

Statistical differences between the groups were determined using a one-way ANOVA followed by the LSD test through SPSS software version 25.0. Results were presented as mean  $\pm$  standard error, and p-values less than 0.05 were considered significant.

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### Author contributions

The conception, design, statistical analysis, and drafting of the manuscript were participated by Sahar Ghosouri and Nazem Ghasemi. Sahar Ghosouri and Mohammad Bakhtiari collected data. Mitra Soleimani participated in revision of the manuscript.

### Ethics approval

Female C57BL/6 mice model were used in this experimental of study on of MS at the Central Laboratory of Isfahan University of Medical Sciences. however, the Ethics Committee approved all process of research were carried out according to the guidelines of the Iranian Committee of Animal care (Ethics#IR.MUI.MED.REC.1399.925).

### Availability of Data and Materials

Data are available from the corresponding authors upon reasonable request.

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