Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(2): 40
https://doi.org/10.31083/j.jin2202040

Original Research

Effects of Bone Marrow Mesenchymal Stem Cells on Myelin Repair and Emotional Changes of a Cuprizone-Induced Demyelination Model

Chu Lei[1], Haowei Chen[2], Kangning Chen[3,*]

[1]Department of Prevention & Healthcare, Southwest Hospital, Third Military Medical University, 400038 Chongqing, China
[2]Department of Hepatobiliary & Pancreatic Surgery, The Fifth People's Hospital of Chongqing, 400062 Chongqing, China
[3]Department of Neurology, Southwest Hospital, Third Military Medical University, 400038 Chongqing, China
*Correspondence: ckn_640827@126.com (Kangning Chen)
Academic Editor: Marcello Ciaccio
Submitted: 24 August 2022 Revised: 27 September 2022 Accepted: 8 October 2022 Published: 16 February 2023

Abstract

Background: Multiple sclerosis (MS) is an autoimmune disease for which bone marrow mesenchymal stem cells (BM-MSCs) have become one of the most promising tools for treatment. Cuprizone(CPZ) induces demyelination in the central nervous system and its use has established a demyelination sheath animal modelwhich is particularly suitable for studying the effects of BM-MSCs on the remyelination and mood improvement of a demyelinating model mice. **Methods**: 70 C57BL/6 male mice were selected and divided into 4 groups: the normal control ($n = 20$), chronic demyelination ($n = 20$), myelin repair ($n = 15$) and cell-treated groups ($n = 15$). Mice in the normal control group were given a normal diet; the chronic demyelination group mice were given a 0.2% CPZ mixed diet for 14 weeks, mice in the myelin repair and cell-treated groups mice were given a 0.2% CPZ diet for 12 weeks and normal diet for 2 weeks, while the cell-treated group mice were injected with BM-MSCs from the 13th week. The cuprizone-induced demyelination model was successfully established and BM-MSCs extracted, behavioural changes of the mice were detected by open field test, elevated plus maze test and tail suspension test, demyelination and repair of the corpus callosum and astrocyte changes were observed by immunofluorescence and electron microscopy and the concentrations of monoamine neurotransmitters and their metabolites detected by enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography-electrochemistry (HPLC-ECD). **Results**: Results suggest BM-MSCs were successfully extracted and cultured, and migrated to the demyelinating area of brain tissue after cell transplantation. Compared with the normal control group, the mice in the chronic demyelination group showed obvious anxiety and depression behaviours ($p < 0.05$); compared with the chronic demyelination group, the anxiety and depression behaviours of the cell-treated group mice were improved ($p < 0.05$); compared with the normal control group, the demyelination of the corpus callosum region of the chronic demyelination group mice was significant ($p < 0.01$), while the myelin sheath of the cell-treated and myelin repair groups was repaired when compared with the chronic demyelination group ($p < 0.05$), and the cell-treated group had a more significant effect than the myelin repair group ($p < 0.05$). Compared with the normal control group, the number of astrocytes in the corpus callosum of the chronic demyelination group mice was significantly increased ($p < 0.01$), and the expression of glial fibrillary acidic protein (GFAP) in the cell-treated group was lower than that in the chronic demyelination and myelin repair groups ($p < 0.05$); the serum concentrations of norepinephrine (NE), 5-hydroxytryptamine (5-HT) and 5-Hydroxyindole-3-acetic acid (5-HIAA) between the normal control and the chronic demyelination groups were significantly different ($p < 0.05$). **Conclusions**: The CPZ-induced model can be used as an experimental carrier for MS combined with anxiety and depression, and BM-MSC transplantation promotes the repair of myelin sheath and the recovery of emotional disorders in the model.

Keywords: Cuprizone; BM-MSCs; multiple sclerosis; demyelination; remyelination; monoamine neurotransmitters

1. Introduction

In the central nervous system, the myelin sheath of neurons is a membrane structure formed by oligodendrocytes which wraps around and protects axons, conducts nerve impulses, and insulates. Under the action of various factors such as immunity, poisoning, infection, metabolism, inflammation, and genetics, demyelination caused by the disintegration and death of oligodendrocytes is closely related to many neurological and mental diseases [1].

Multiple sclerosis (MS) is a type of autoimmune disease, the main pathological feature of which is the demyelination of white matter [2]. The repeated course and multiple lesions can easily lead to severe neurological defects and psychiatric symptoms such as depression and anxiety [3,4], thus affecting a patient's compliance with treatment. Therefore, MS patients with depression and anxiety have been brought increasingly to the attention of researchers and clinicians. The promotion of remyelination is the key treatment for MS patients with anxiety and depression and other affective disorders.

Bone marrow mesenchymal stem cells (BM-MSCs) are pluripotent stem cells with strong proliferation, multidirectional differentiation potential, and non-obvious surface antigens [5–7]. Compared with other stem cells, BM-

MSCs are easier to obtain and subculture. Therefore, they have become one of the most promising methods for the treatment of MS with affective disorders due to their unique biological characteristics and clinical research background. CPZ is a selective copper ion chelating agent [8], it targets and damages oligodendrocytes, inducing demyelination in the central nervous system and establishes the demyelination sheath animal model [9,10]. Some regard this model suited to modelling mental disorders [11]. Therefore, it is particularly suitable for studying the effects of BM-MSCs on the remyelination and mood improvement of the demyelinating model mice without immunoregulation and for comparison of the biological characteristics and myelin repair abilities of BM-MSCs from different sources. It thus provides a new method for the clinical treatment of demyelinating diseases with emotional disorders and may be suited to more generally become a model in the treatment of mental illness [12].

2. Materials and Methods

2.1 Animal Model Establishment

All animal handling and procedures were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (AMUWEC202112128) and performed under protocols approved by the Animal Care and Use Committee that are in accordance with the Chinese Council on Animal Care. All efforts were made to reduce the numbers of animals used and minimize their suffering. Healthy adult C57BL/6 male mice were used for all experiments. They were obtained from the Third Military Medical University Experimental Animal Centre (Chongqing, China).

Seventy eight-week-old C57BL/6 male mice were divided into four groups: the normal control group (n = 20), the chronic demyelination (n = 20) group, the myelin repair group (n = 15) and the cell-treated group (n = 15). The normal control group mice were given normal diet; the chronic demyelination groups of mice were given a 0.2% CPZ mixed diet for 14 weeks, the myelin repair and the cell-treated group mice were both given a 0.2% CPZ diet for 12 weeks and normal diet for two weeks, while the cell-treated group mice were injected with BM-MSCs from the 13th week.

2.2 Isolation, Culture, Identification, and Transplantation of BM-MSCs

2.2.1 Isolation and Culture of BM-MSCs

Five C57BL/6 male mice from the normal group were anaesthetized, the femur and tibia were separated, bone marrow tissue in the bone marrow cavity was flushed out, centrifuged by low temperature centrifuge (3000 rpm/min, 10 min). Cells were collected and resuspended in Dulbecco's modified Eagle's medium (C11885500BT, GIBCO BRL life Technologies, Grand Island, NY, USA) containing 10% foetal bovine serum (FBS), (10099-141, GIBCO BRL life Technologies, Grand Island, NY, USA) supplemented with 100 U/mL penicillin and streptomycin (15140-22,GIBCO BRL life Technologies, Grand Island, NY, USA). Cells were plated and incubated at 37 °C, 5% CO_2 constant temperature and humidity. The culture medium was changed every three days. Cells were subcultured when they covered about 90% of the base of the culture flask. Adherent cells were digested and centrifuged with 0.25% trypsin digestion solution, passaged at a ratio of 1:2, and inoculated into a new cell culture flask. The culture was continued to further purify BM-MSCs.

2.2.2 Identification of BM-MSCs

Second-generation BM-MSCs were taken for cell climbing. When the cells fused to about 90% of the slide, they were performed immunofluorescent staining for CD29 and CD90. The glass slides loaded with cells were removed, rinsed, placed on a coverslip rack, and fixed with 4% paraformaldehyde fixative for 15 minutes; after rinsing, blocked with 1% BSA/0.4% Triton-X100 (9002-93-1 Sigma-Aldrich, St. Louis, MO, USA) solution for 30 minutes; CD29 rabbit anti-mouse monoclonal antibody (ab273724, Abcam, Cambridge, UK) was then added. Simultaneously, a control group was set up and incubated at 37 °C for one hour; after rinsing, FITC-labelled goat anti-rabbit green fluorescent secondary antibody was added drop-wise and slides were incubated at room temperature for one hour; after rinsing with buffer, DAPI staining solution was added drop-wise to the cell slide, the staining solution was washed off for one minute and mounted with fluorescent anti-quencher; CD90 cell immunofluorescence staining method was identical (primary anti-CD90 rabbit anti-mouse monoclonal antibody) (ab133350, Abcam, Cambridgem, UK) Cell staining was then observed by fluorescence microscope (Nikon Eclipse90i) and photographs collected.

2.2.3 Transplantation of BM-MSCs

Second-generation BM-MSCs were digested in a 0.25% trypsin cell digestion solution (C0201, Beyotime, Biotechnology, Shanghai, China), resuspended in fresh medium after centrifugation, an appropriate amount of prepared CSFE fluorescent dye was added, and the solution was incubated at 37 °C for 30 minutes, the cell suspension was then centrifuged for five minutes, washed with resuspended cells in 0.01 mol PBS solution to a final concentration of 5×10^6 cells/mL. Five mice in the chronic demyelination group were randomly selected and each mouse was injected with 200 μL of cell suspension through the tail vein; the mice were anesthetized 48 hours after cell transplantation and fixed with 4% paraformaldehyde fixative by perfusion in the heart. Brain tissue was post-fixed in 4% paraformaldehyde fixative for 48 hours, dehydrated with 30% sucrose solution, sliced with a cryostat (20 μm thick), stained with DAPI for one minute after patching, and then mounted, observed and photographed under a fluorescence microscope.

2.3 Bodyweight Recording and Behavioural Experimental Methods

From the establishment of the demyelination model, the body weights of each group of mice were tested and recorded (twice/week) and the body-weight curve was recorded following establishment of the demyelination model.

Mice were tested with behavioural experiments by the 14th week. *Open field experiment:* Spontaneous activities and exploration by the mice in each group of mice were recorded (XR-XZ301, Shanghai Xinruan Information Technology Co., Ltd.) in the open field environment. The central time, central distance, total movement time and total movement distance of each mouse were analysed by real-time analysis software and the corresponding ratio calculated. *Elevated plus maze experiment*: The activity of each mouse (placed in the central area facing the open arm) in every arm was recorded for five minutes (XR-XG201, Shanghai Xinruan Information Technology Co., Ltd.). The percentage of the open-arm retention time (OT%) and the percentage of open-arm entry time (OE%) of mice was calculated by the monitoring system. *Tail suspension experiment:* The tail of each mouse in each group was hung on the tail suspension instrument for six minutes and the mouse immobility time was recorded as the evaluation index from the second minute of the tail suspension.

2.4 Sample Collection and Testing

Brain tissue was taken from five mice of each group. After 48 hours, brain tissues were fixed by paraformaldehyde, dehydrated with 30% sucrose solution and sectioned (thickness 20 μm). These tissue slices were blocked with 1% BSA/0.3% TritonX-100, rat anti-mouse MBP (1:200) primary antibody (ab7349, Abcam, UK) and rabbit anti-mouse GFAP (1:200) primary antibody (ab7260, Abcam, UK) were added and incubated at 4 °C overnight, slices were then put into a fluorescent secondary antibody, incubated at room temperature for two hours, nuclei stained by DAPI and slides then mounted after rinsing. Slides were then observed and photographed under a fluorescence microscope (Nikon Eclipse90i). The number of cells were counted and the OD value obtained with Image-Pro Plus 6.0(Media Cybernetics, Inc., Rockville, MD, USA).

The brain tissue of four mice from each group was taken. After fixing by 2.5% glutaraldehyde solution for 24 hours, the corpus callosum was separated and cut into small pieces (1 mm^3); then fixed in 1% osmium acid solution; dehydrated by a pyruvic acid gradient at room temperature; embedded in an epoxy resin plate and ultra-thin sectioned; these slices were then double stained with a lemon lead and uranyl acetate staining solution. The slices were observed and photographed by transmission electron microscope (TEM, TECNAI-10, 80 kV, Philips, Holland).

Blood of six mice from each group was taken into a coagulation tube and centrifuged (3000 rpm, 10 min). The upper serum sample was obtained. Standard stock solutions of NE, E, DA, 5-HT, 5-HIAA and DOPAC with a concentration of 0.10 g/L were prepared. Ultrapure water was then used to prepare a mixed standard stock solution with concentration gradient of 0.5, 1.0, 2.0, 5.0, 10, 40, 80, 200 ng/mL, two sub-wells were set for each concentration and 20 μL of sample added. The average of each pair of wells was used to make a standard curve; mouse serum was taken from each group and 0.5 mol/L perchloric acid was then added to a centrifuge tube in proportion to vortex and mix (10:1). After 20 minutes of protein precipitation, the supernatant was decanted after low temperature centrifugation and filtration, a high-performance liquid-electrochemical detector (model 5600A, ESA company) was used to detect the concentrations of serum monoamine neurotransmitters and metabolites (ng/L). The concentration gradient of the diluted standard substance as 90 ng/L, 60 ng/L, 30 ng/L, 15 ng/L, 7.5 ng/L, and a standard curve was made; blank wells and sample wells were set and serum samples taken from the mice of each group as required and placed in the sample wells of the enzyme-coated plate, sealed, and incubated at 37 °C for 30 minutes. They were then washed and enzyme labelling reagents were added, solutions were incubated at 37 °C with shaking, washed again, and solution A and solution B were added to each well then incubated at 37 °C for 15 minutes, a stop solution was then added to stop colour development, the absorbance was measured at 450 nm with microplate reader (Model 680, Hercules, Bio-Rad, USA) and the concentration (ng/L) of 5-HT and NE in each serum sample was calculated for the mice in each group.

2.5 Statistical Analysis

Data were statistically analysed by SPSS18.0 (Version 18.0, SPSS Inc., Chicago, IL, USA). Data after a normality test were expressed in the form of $x \pm s$. After a multi-sample homogeneity of variance test, the comparison between groups was performed by analysis of variance. If $p < 0.05$, the difference was considered to be statistically significant.

3. Results

3.1 Culture, Identification, Transplantation of Mice BM-MSCs

3.1.1 Identification of BM-MSCs

BM-MSCs extracted from mice bone marrow have irregular shapes such as fusiform and polygonal shapes. Their expression of surface markers CD29 and CD90 has a high positive rate (about 90%) (Fig. 1).

3.1.2 Transplantation of BM-MSCs

It can be seen that the labelled green fluorescent BM-MSCs can migrate to the corpus callosum of the brain tissue (Fig. 2). This indicates that BM-MSCs can be transplanted to a demyelinated area.

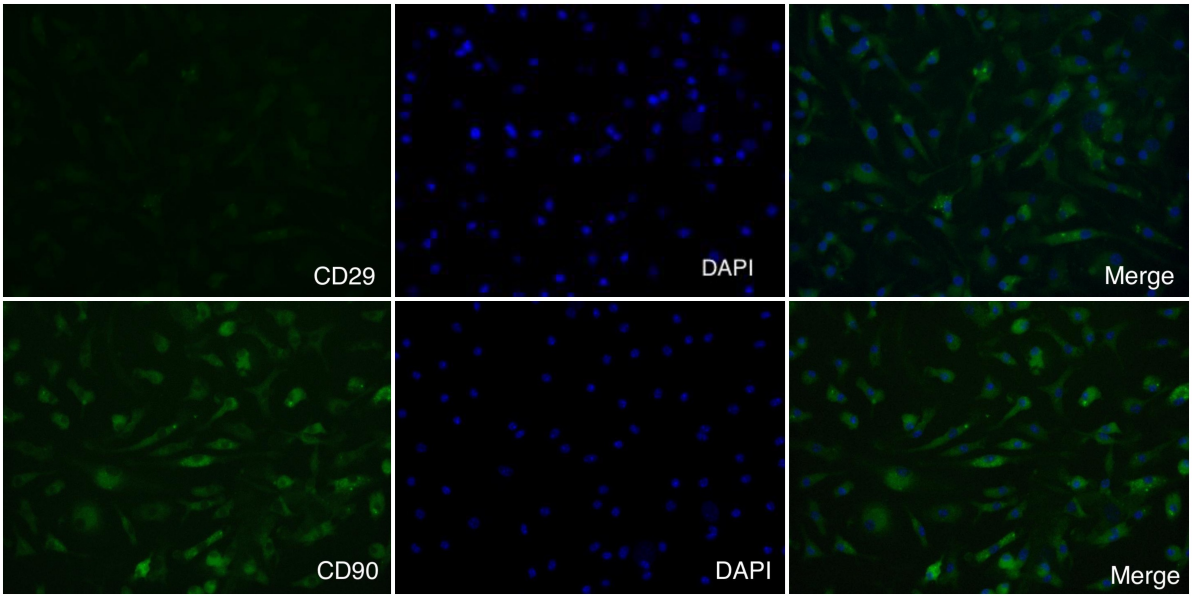

Fig. 1. Immunofluorescence staining of CD29 and CD90 on the surface of BM-MSCs (×200). Immunofluorescence staining of the surface markers CD29 and CD90 on the extracted cells showed the positive rate of these markers were high (about 90%), indicating that the extracted and cultured cells were bone marrow mesenchymal stem cells.

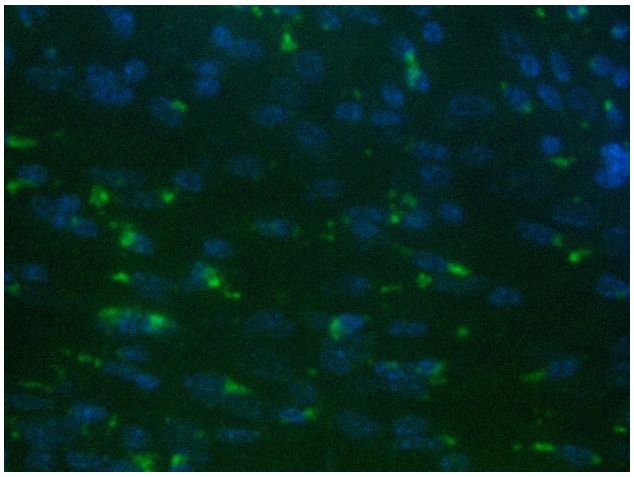

Fig. 2. BM-MSCs labelled with green fluorescence in the corpus callosum region of mice brain tissue (×200). BM-MSCs were labelled with CFSE then transplanted into the model mice. It can be seen that the BM-MSCs labelled with green fluorescence migrate to the corpus callosum area of the mouse brain tissue, indicating that BM-MSCs can be transplanted into demyelinated areas.

3.2 Bodyweight Changes

At the beginning of the model induction, there was no significant difference in the body weight of the mice in each group. After induction, the body weight of mice in the model group increased more slowly than the normal control group, with a significant difference apparent at the 12th week ($p < 0.05$). After BM-MSC transplantation, the bodyweight of the cell-treated group mice was significantly higher than the chronic demyelination group ($p < 0.05$), but there was no significant difference from the normal control group ($p > 0.05$) (Table 1). These results suggest that BM-MSCs improve the bodyweight loss of demyelinating model mice.

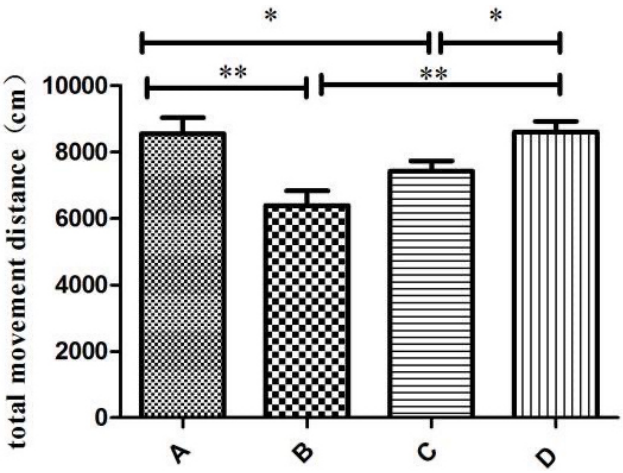

Fig. 3. Total movement distance of mice in each group (*$p <$ 0.05, **$p < 0.01$). The open field experiment showed that compared with the normal control group, the total movement distance of the mice in the chronic demyelination group was significantly reduced ($p < 0.01$), while the total movement distance of mice in the cell-treated group was significantly higher than that of the chronic demyelination ($p < 0.01$) and the myelin repair groups ($p < 0.05$). (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

Table 1. Bodyweight changes of the mice in each group (g, x ± s).

Group	3rd week	6th week	9th week	12th week	15th week
The normal control group	20.90 ± 2.54	24.33 ± 1.92	27.29 ± 2.08	29.75 ± 1.84	33.85 ± 1.70
The chronic demyelination group	19.5 ± 1.28	21.9 ± 1.76^b	23.78 ± 1.86^b	26.01 ± 2.52^b	27.98 ± 3.31^b
The myelin repair group	18.88 ± 2.55	20.82 ± 2.81^b	24.26 ± 2.12^b	25.68 ± 2.85^b	29.93 ± 2.74^a
The cell-treated group	19.53 ± 0.45	21.47 ± 0.76^b	24.77 ± 1.55^b	26.91 ± 2.00^b	32.33 ± 2.96^c

a: $p < 0.05$, compared with the normal control group; b: $p < 0.01$, compared with the normal control group; c: $p < 0.01$, compared with the chronic demyelination group.

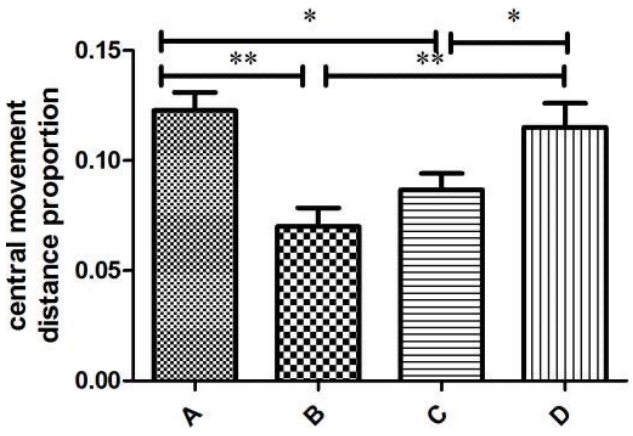

Fig. 4. Central movement distance proportion of mice in each group (*$p < 0.05$, **$p < 0.01$). The open field experiment showed that compared with the normal control group, the central movement distance proportion of the mice in the chronic demyelination group was significantly reduced ($p < 0.01$), while the central movement distance proportion of the mice in the cell-treated group was significantly higher than that of the chronic demyelination ($p < 0.01$) and myelin repair groups ($p < 0.05$). (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

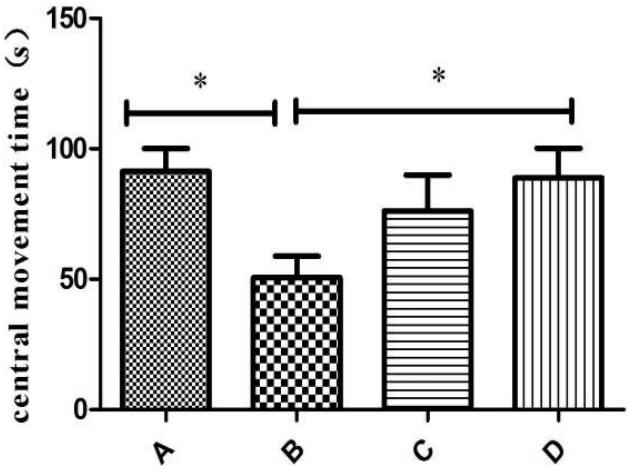

Fig. 5. Central movement time of mice in each group c (*$p < 0.05$, **$p < 0.01$). The open field experiment showed that compared with the chronic demyelination group, the central movement time of mice in the normal control group and the cell-treated group significantly increased ($p < 0.05$). (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

3.3 Behavioural Changes

3.3.1 Open Field Experiment

Compared with the normal control group, the central movement distance ratio and total movement distance of the mice in the chronic demyelination group was significantly reduced ($p < 0.01$); the central movement distance proportion and total movement distance of the mice in the cell-treated group were increased more than both the demyelination ($p < 0.01$) and myelin repair groups ($p < 0.05$) (Figs. 3,4); compared with the chronic demyelination group, the central movement time of the mice in the normal control and the cell-treated groups significantly increased ($p < 0.05$) (Fig. 5). These results suggest that the demyelination model mice show decreased movement ability and obvious anxiety-like behaviours, and that such behaviours can be improved by BM-MSCs.

3.3.2 Elevated Plus Maze Experiment

Compared with the normal control group, the OT% of mice in the chronic demyelination and myelin repair groups were significantly reduced ($p < 0.01$), and the OT% of the mice in the cell-treated group was significantly increased compared with the chronic demyelination group ($p < 0.01$) (Fig. 6). Compared with the chronic demyelination group, the OE% of the mice in the normal control group ($p < 0.01$), the cell-treated group ($p < 0.01$) and the myelin repair group ($p < 0.05$) increased significantly (Fig. 7). These results further indicate that the demyelination model mice have anxious behaviour and BM-MSCs can relieve their anxiety.

3.3.3 Tail Suspension Experiment

Compared with the normal control group, the immobility time of mice in the chronic demyelination and myelin repair groups was significantly increased ($p < 0.01$). The immobility time of the mice in the cell-treated group was lower than that of the mice in the chronic demyelination and the myelin repair groups ($p < 0.05$) (Fig. 8). These re-

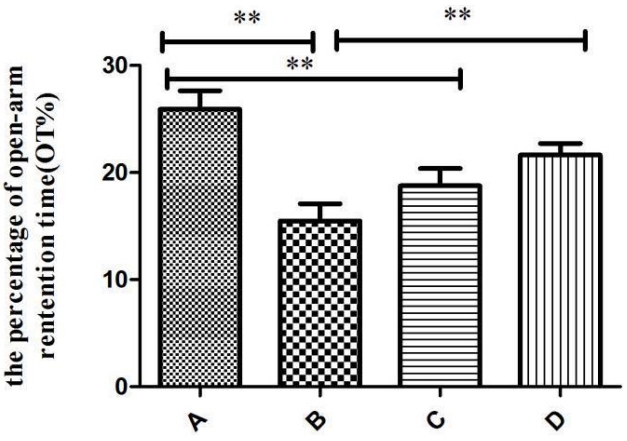

Fig. 6. The percentage of open-arm retention time (OT%) of mice in each group (*$p < 0.05$, **$p < 0.01$). The elevated plus maze experiment showed that compared with the normal control group, the OT% of mice in the chronic demyelination and myelin repair groups were significantly reduced ($p < 0.01$), and the OT% of the mice in the cell-treated group was significantly increased compared with the chronic demyelination group ($p < 0.01$). (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

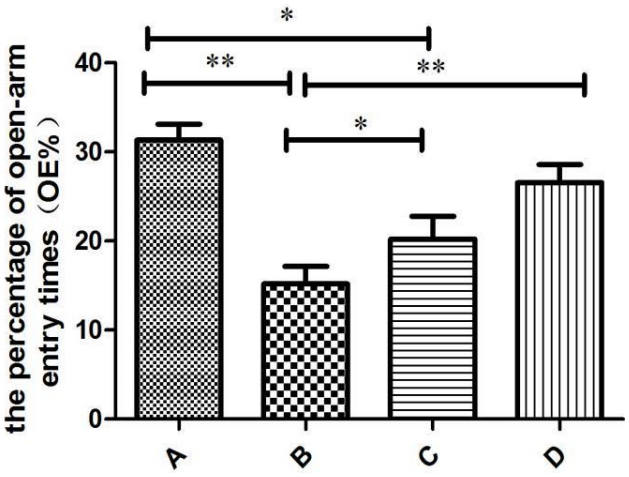

Fig. 7. The percentage of open-arm entry times (OE%) of mice in each group (*$p < 0.05$, **$p < 0.01$). The elevated plus maze experiment showed that compared with the chronic demyelination group, the OE% of mice in the normal control ($p < 0.01$), cell-treated ($p < 0.01$), and myelin repair groups ($p < 0.05$) increased significantly. (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

sults suggest that demyelination model mice show obvious depressive behaviour, and that BM-MSCs improve such behaviour.

3.4 Morphological Changes of Brain Tissue

MBP immunofluorescence staining of brain tissue sections of the mice in each group showed that the corpus

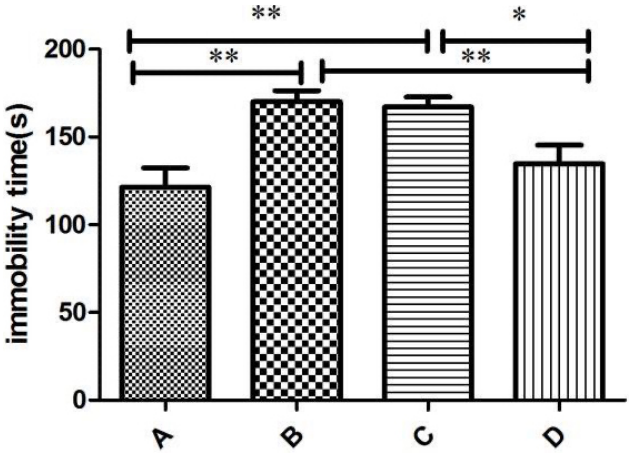

Fig. 8. The immobility time of mice in each group (*$p < 0.05$, **$p < 0.01$). The tail suspension experiment showed that compared with the normal control group, the immobility time of the mice in the chronic demyelination and the myelin repair groups was significantly increased ($p < 0.01$). The immobility time of the mice in the cell-treated group was lower than the mice in the chronic demyelination and myelin repair groups ($p < 0.05$). (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

callosum of the normal control group was thick and dense, while the area of corpus callosum of the chronic demyelination group decreased by 78% ($p < 0.01$) compared with the normal group and that the area of corpus callosum of the myelin repair group decreased by 65% ($p < 0.01$) compared with the normal group. The area of corpus callosum of the cell-treated group increased 73% ($p < 0.01$) compared with the chronic demyelination group and increased 28% ($p < 0.05$) compared with the myelin repair group (Fig. 9).

The ultrastructure of the myelin sheath of the corpus callosum of each group of mice observed by transmission electron microscopy showed that the proportion of myelinated nerve fibres in the corpus callosum of the normal control group was larger ($80.28 \pm 8.75\%$), the structure was more compact and that of the demyelination group was reduced ($19.68 \pm 13.16\%$), the difference was significant ($p < 0.01$), while that of the myelin repair group was increased ($32.44 \pm 16.28\%$, $p < 0.05$) (most of the axons were still not surrounded by myelin sheath). Compared with the myelin repair group, there were relatively more myelinated nerve fibres ($54.67 \pm 17.89\%$, $p < 0.01$) in the corpus callosum of mice in the cell-treated group, however, the proportion of myelinated nerve fibres and the density of myelin in the cell-treated group were significantly lower than those in the normal control group ($p < 0.01$) (Fig. 10).

By immunofluorescence staining of GFAP, a marker of astrocytes, it was found that the amount of activated astrocytes in the corpus callosum of mice in the normal control group (21.56 ± 10.63) was significantly reduced when compared to that of the chronic demyelination group

Table 2. Concentrations of monoamine neurotransmitters and their metabolites in the mice of each group (ng/L, x ± s).

Group	NE	DA	5-HT	E	5-HIAA	DOPAC
the normal control group	1.32 ± 0.92	2.65 ± 1.30	126.04 ± 22.43	2.37 ± 2.01	27.26 ± 3.17	0.94 ± 0.33
the chronic demyelination group	2.82 ± 1.01^{a}	3.67 ± 2.43	159.68 ± 23.1^{ab}	2.69 ± 0.63	43.89 ± 6.59^{ab}	1.59 ± 0.93
the myelin repair group	1.90 ± 0.91	1.72 ± 0.91	146.37 ± 12.19	1.99 ± 1.33	38.22 ± 10.42	0.97 ± 0.14
the cell-treated group	1.72 ± 1.30	1.87 ± 1.01	132.66 ± 20.91	2.68 ± 1.17	27.77 ± 5.81	1.10 ± 0.76

a: $p < 0.05$, compared with the normal group; b: $p < 0.05$, compared with the cell-treated group.

NE, norepinephrine; DA, dopamine; 5-HT, 5-hydroxytryptamine; E, epinephrine; 5-HIAA, 5-Hydroxyindole-3-acetic acid; DOPAC, dihydroxyphenylacetic acid.

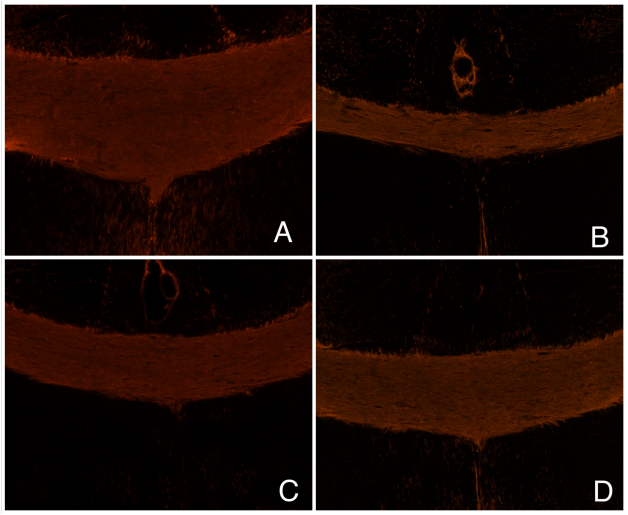

Fig. 9. Changes of MBP in the corpus callosum of the brain tissue of mice in each group (×100). The immunofluorescence staining of MBP on the brain tissue sections of mice in each group showed that the corpus callosum of mice in the normal control group was thick and dense in structure, while the corpus callosum of the chronic demyelination and the myelin repair groups was thinner and looser, the corpus callosum area of the mice in the cell-treated group was enlarged compared with the myelin repair and the chronic demyelination groups. (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

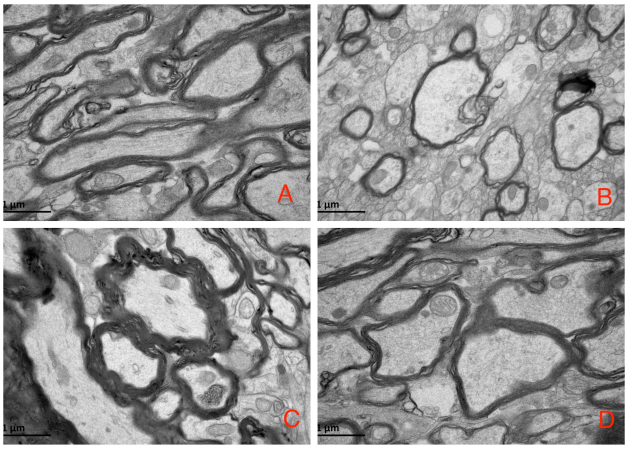

Fig. 10. The ultrastructure of the myelin sheath of the corpus callosum in the brain tissue of each group of mice observed by transmission electron microscope (×30,000). Transmission electron microscopy was used to observe the ultrastructure of the myelin sheath in the corpus callosum of the brain tissue of the mice in each group. Compared with the chronic demyelination group, the proportion of myelinated nerve fibres in the corpus callosum region of the mice in the sheath repair group increased; Compared with the normal control group, the proportion of myelinated nerve fibres and the density of myelin sheath in the mice were significantly different. (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

(78.12 ± 15.47) ($p < 0.01$), while the amount of activated astrocytes in the corpus callosum of mice in the cell-treated group (41.11 ± 17.42) was significantly reduced when compared to that of the chronic demyelination and the myelin repair groups ($p < 0.01$) (Figs. 11,12).

3.5 The Concentration of Monoamine Neurotransmitters and Their Metabolites

The concentrations of NE (1.32 ± 0.92 ng/L), 5-HT (126.04 ± 22.43 ng/L) and 5-HIAA (27.36 ± 3.17 ng/L) in the normal control group were significantly different from their respective concentrations of 2.82 ± 1.01 ng/L, 159.68 ± 23.1 ng/L and 43.89 ± 6.59 ng/L in the chronic demyelination group ($p < 0.05$); the concentrations of 5-HT (132.66 ± 20.91 ng/L) and 5-HIAA (27.77 ± 5.81 ng/L) in the cell-treated group were higher than those in the chronic demyelination group ($p < 0.05$) (Table 2).

The concentrations of NE and 5-HT in mice of each group were measured by ELISA. The concentrations of NE (50.83 ± 17.64 ng/L) and 5-HT (99.30 ± 29.11 ng/L) in the chronic demyelination group were significantly higher than their respective concentrations 23.82 ± 19.65 ng/L and 39.72 ± 19.16 in the normal control group ($p < 0.05$); the concentrations of NE (25.58 ± 19.33 ng/L) and 5-HT (68.60 ± 33.99 ng/L) in the cell-treated group were significantly lower than those of the chronic demyelination group ($p < 0.05$) (Table 3).

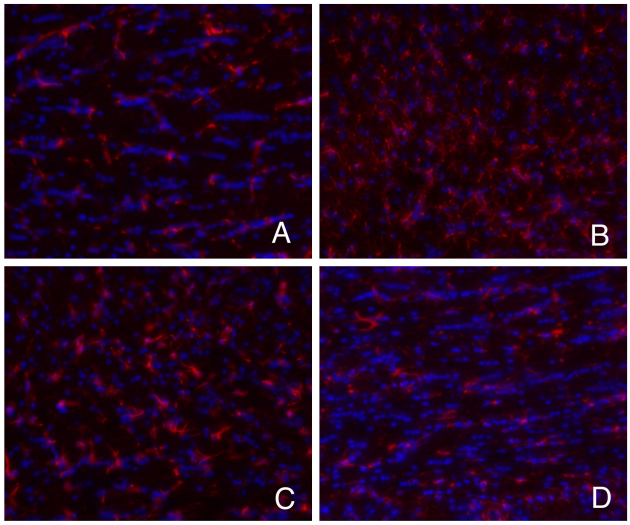

Fig. 11. Activation of GFAP in the corpus callosum of mice in each group (×200). By immunofluorescence staining of astrocyte marker glial fibrillary acidic protein (GFAP), it was found that the number of activated astrocytes in the corpus callosum region of the normal control group was less and the number of activated astrocytes in the corpus callosum region of the chronic demyelination group was significantly increased. Compared with the chronic demyelination and myelin repair groups, the activated astrocytes in the corpus callosum region of the mouse brain tissue in the cell-treated group were significantly decreased. (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

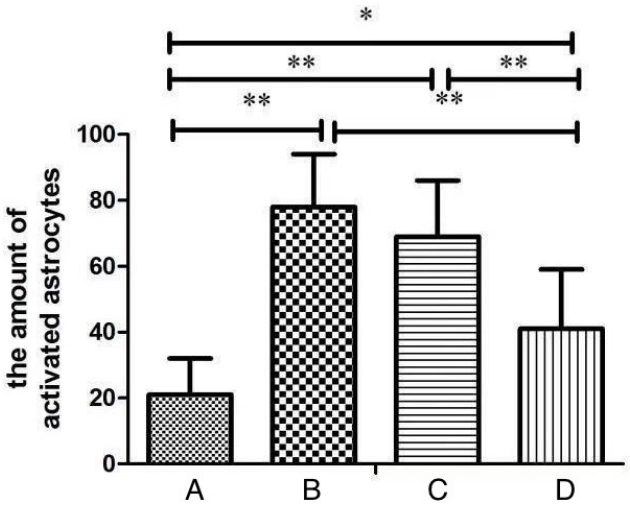

Fig. 12. The amount of activated astrocytes in the corpus callosum. (A) Normal control group; (B) Chronic demyelination group; (C) Myelin repair group; (D) Cell-treated group.

4. Discussion

MS is an inflammatory demyelinating disease of the central nervous system. It has recurring courses, multiple lesions, high disability rate and is often accompanied by mental diseases such as anxiety and depression [13]. Therefore, research into the diagnosis and treatment of affective disorders such as anxiety and depression in MS patients is of top priority. Cuprizone (CPZ) is a copper ion chelating agent which damages mitochondria of oligodendrocytes, induces demyelination of the central nervous system, and provides established animal models of demyelination in the central nervous system. Recently, some investigators have used this model to research MS, schizophrenia and other neuropsychiatric diseases [14], so this model is especially suited for research into central nervous system demyelination and affective disorders.

Table 3. Concentrations of NE and 5-HT of mice in each group (ng/L, x ± s).

Group	NE	5-HT
the normal control group	23.82 ± 19.65	39.72 ± 19.16
the chronic demyelination group	50.83 ± 17.64^{ab}	99.30 ± 29.11^{ab}
the myelin repair group	28.39 ± 18.22	68.60 ± 33.99
the cell-treated group	25.58 ± 19.33	54.83 ± 18.41

a: $p < 0.05$, compared with the normal group; b: $p < 0.05$, compared with the cell-treated group.

BM-MSCs, are a type of pluripotent stem cell population different from bone marrow hematopoietic stem cells. They are easy to access *in vitro*, have strong proliferation, multi-directional differentiation potential, and insignificant surface antigens. They maintain stem cell characteristics after multiple passages, have weak immune rejection when transplanted into the body, all biological properties that make them suitable for the treatment of autoimmune diseases, neurological diseases, blood diseases, bone and cartilage diseases, and liver failure amongst other diseases. They differentiate into a variety of adult cells under specific induction conditions. Currently, the treatment of MS with affective disorders is limited. So as to explore the repair effect of BM-MSCs on myelin and the effect of demyelination and regeneration on emotional changes, BM-MSCs were chosen to be transplanted to CPZ-induced demyelination mice.

Recently, some investigators have found that the white matter myelinated nerve fibres of patients with anxiety and depression have varying degrees of integrity abnormalities when viewed by magnetic resonance diffusion tensor imaging [15,16]. In this study, MBP immunofluorescence staining in a chronic demyelination group showed that the expression of MBP was significantly reduced and transmission electron microscopy showed loose myelin structure and axon atrophy in corpus callosum, indicating serious demyelination. After model mice were injected with BM-MSCs labelled with live tracers, cells could be seen to migrate to the corpus callosum, indicating that BM-MSCs could migrate to damaged parts of the myelin sheath, which has been shown by similar research in EAE model mice [17,18]. The expression of MBP in the corpus callosum

of the mice in a BM-MSC-treated group was significantly higher than that of a chronic demyelination group. Transmission electron microscopy has shown that the myelin density and the proportion of myelinated nerve fibres in the corpus callosum of the mice in a cell-treated group increased. A relatively large number of thick myelinated nerve fibres and some thin myelinated nerve fibres were seen, indicating that BM-MSCs can directly promote the endogenous repair of brain tissue by reducing myelin damage and enhancing myelin regeneration, consistent with previous research [19]. The myelin repair effect mediated by BM-MSCs may be related to soluble factors secreted by brain tissue [20], but further research on the protein factors involved is needed to maximize the potential of BM-MSCs treatment. Additionally, GFAP immunofluorescence staining of the brain tissue of demyelination model mice showed that astrocytes were activated in large numbers and that activated astrocytes may further promote the loss of myelin sheath and inhibit its regeneration, which is one of the important factors for functional reconstruction. The expression of GFAP in the cell-treated group was significantly reduced, indicating that BM-MSCs inhibit astrocyte activation during demyelination. Previous studies have shown that BM-MSCs protects against persistent axon damage, The mechanism may be related to the state of microglia and astrocytes [21], which is consistent with results reported here.

Through monitoring the body weight of mice, it was found that compared with the normal control group, the bodyweight of CPZ-induced demyelination model mice increased slowly, but significantly after injection of BM-MSCs, indicating that BM-MSCs can promote the growth of mice bodyweight and that the mechanism may be related to changes in myelin and mood. In behavioural experiments, demyelination model mice lacked and exploration activities in the open field and elevated plus maze and showed obviously anxious behaviour. In open field and tail suspension experiments, the total distance moved decreased and immobility time increased, which may be related to the demyelination of multiple brain tissues leading to reduced movement activity and depressive behaviours in mice. Therefore, behavioural experiments indicate that chronic demyelination mice have obvious anxiety and depressive behaviours. The central movement distance proportion, total movement distance, open arm retention time, open arm entry times, immobility time and other indicators of the mice in the cell-treated group were significantly improved compared with the chronic demyelination group, indicating that BM-MSCs do repair damaged myelin sheath and improve movement ability and emotional disorders such as anxiety and depression. This suggests that the loss of myelin sheath and the repair process may be related to anxiety or depressive behaviour.

Current research [22] suggests that monoamine neurotransmitters may be the key to pathogenesis and treatment of anxiety and depression. Studies have found that NE and disorders of the central nervous system can lead to depression and anxiety [23,24]. Some investigators have noted that anxiety disorders are related to the activation of 5-HT1A and 5-HT2 receptors in the postsynaptic membrane and that the blocking of 5-HT1A receptors and the activation of 5-HT2 receptors can lead to depression [25,26]. Anxiety and depression often accompany each other, which may be due to the abnormal function of 5-HT and its receptors. In this study, the detection of monoamine neurotransmitters in experimental mice by ELISA and high performance liquid chromatography-electrochemistry found that the serum 5-HT, 5-HIAA, and NE concentrations of the demyelination model mice were significantly higher than those found in the normal control group, indicating that the anxiety and depression in model mice are related to the hyperfunction of 5-HT and NE nerve fibres. The NE, 5-HT, and 5-HIAA concentrations of cell treated mice were significantly reduced, suggesting that BM-MSCs improve the disorder of monoaminergic neurotransmitters in demyelination model mice, thus relieve anxiety and depression disorders. Therefore, it is speculated that BM-MSCs may improve the affective disorders of demyelination model mice by repair of the myelin sheath and regulation of monoaminergic neurotransmitters.

Results reported here show that the anxiety and depression behaviours of the demyelination model mice are significantly related to the damage of myelin and the abnormalities of NE, 5-HT, and metabolites. That means that the anxiety and depression behaviours of mice may be caused by the dual effects of central nervous system myelin damage and monoaminergic neurotransmitter disorders. This supports the view that anxiety and depression disorders are based on structural abnormalities and chemical disorders of the central nervous system [27]. At present, the main treatment for anxiety and depression is still a variety of anti-anxiety and depression drugs and these drugs achieve their therapeutic effect by regulating the abnormalities of neurotransmitters, thus cannot fundamentally repair the damaged nerve myelin sheath. This study therefore provides new ideas for the treatment of MS combined with anxiety and depression and also provides a reference for the use of BM-MSCs for the treatment of mental illness. However, the underlying mechanisms are still unclear and need study.

5. Conclusions

The CPZ-induced model is feasible and can be used as an experimental carrier for MS combined with anxiety and depression, while BM-MSCs transplantation promotes the repair of myelin sheath and the recovery of emotional disorders in this model.

Abbreviations

5-HT, 5-hydroxytryptamine; 5-HIAA, 5-Hydroxyindole-3-acetic acid; BM-MSCs, bone marrow

mesenchymal stem cells; CFSE, carboxyfluorescein succinimidyl amino ester; CPZ, cuprizone; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; DOPAC, dihydroxyphenylacetic acid; E, epinephrine; ELISA, enzyme linked immunosorbent assay; FBS, foetal bovine serum; GFAP, glial fibrillary acidic protein; HPLC-ECD, high-performance liquid chromatography -electrochemical detection; MBP, myelin basic protein; MS, multiple sclerosis; NE, norepinephrine; OD, optical density; OE%, the percentage of open-arm entry time; OT%, the percentage of open-arm retention time.

Author Contributions

CL and HC contributed equally to this work. CL completed the design of work, data analysis, and interpretation of data. HC participated in data analysis, interpretation, and drafting of manuscript. KC participated in design of the work and overall supervision of the work. All authors reviewed and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal handling and procedures were approved by Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (AMUWEC202112128) and performed under protocols approved by the Animal Care and Use Committee that are in accordance with the Chinese Council on Animal Care.

Acknowledgment

We are grateful to the Third Military Medical University animal care facility for providing the animal care and Department of histogenetics (the Third Military Medical University, Chongqing, China).

Funding

This research was funded by Southwest Hospital (grant number: SWH2012LC02).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Takahashi N, Sakurai T, Davis KL, Buxbaum JD. Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. Progress in Neurobiology. 2011; 93: 13–24.

[2] Lemus HN, Warrington AE, Rodriguez M. Multiple Sclerosis. Neurologic Clinics. 2018; 36: 1–11.

[3] Jones KH, Jones PA, Middleton RM. Ford DV, Dalton KT, Jones HL, *et al*. Physical disability, anxiety and depression in people with MS: an internet-based survey via the UK MS Register. PLoS ONE. 2014; 9: e104604.

[4] Marrie RA, Patel R, Bernstein CN, Bolton JM, Graff LA, Marriott JJ, *et al*. Anxiety and depres-sion affect performance on the symbol digit modalities test over time in MS and other immune disorders. Multi-ple Sclerosis Journal. 2021; 27: 1284–1292.

[5] Gao F, Wu Y, Wen H, Zhu W, Ren H, Guan W, *et al*. Multilineage potential research on pancreat-ic mesenchymal stem cells of bovine. Tissue and Cell. 2019; 56: 60–70.

[6] Jiang W, Xu J. Immune modulation by mesenchymal stem cells. Cell Proliferation. 2020; 53: e12712.

[7] GoGel S, Gubernator M, Minger SL. Progress and prospects: stem cells and neurological diseases. Gene Therapy. 2011; 18: 1–6.

[8] Bénardais K, Kotsiari A, Škuljec J, Koutsoudaki PN, Gudi V, Singh V, *et al*. Cuprizone [Bis(Cyclohexylidenehydrazide)] is Selectively Toxic for Mature Oligodendrocytes. Neurotoxicity Research. 2013; 24: 244–250.

[9] Kopanitsa MV, Lehtimäki KK, Forsman M, Suhonen A, Koponen J, Piiponniemi TO, *et al*. Cog-nitive disturbances in the cuprizone model of multiple sclerosis. Genes, Brain and Behavior. 2021; 20: e12663.

[10] Abakumova TO, Kuz'kina AA, Zharova ME, Pozdeeva DA, Gubskii IL, Shepeleva II, *et al*. Erratum to: Cuprizone Model as a Tool for Preclinical Studies of the Effi cacy of Multiple Sclerosis Diagnosis and Therapy. Bulletin of Experimental Biology and Medicine. 2015; 159: 411.

[11] Kondo MA, Fukudome D, Smith DR, Gallagher M, Kamiya A, Sawa A. Dimensional assessment of behavioral changes in the cuprizone short-term exposure model for psychosis. Neuroscience Research. 2016; 107: 70–74.

[12] Zheng K, Wang H, Li J, Yan B, Liu J, Xi Y, *et al*. Structural networks analysis for depression combined with graph theory and the properties of fiber tracts via diffusion tensor imaging. Neuroscience Letters. 2019; 694: 34–40.

[13] Murphy R, O'Donoghue S, Counihan T, McDonald C, Calabresi PA, Ahmed MA, *et al*. Neuro-psychiatric syndromes of multiple sclerosis. Journal of Neurology, Neurosurgery and Psychiatry. 2017; 88: 697–708.

[14] Sun Z, Gu L, Ma D, Wang M, Yang C, Zhang L, *et al*. Behavioral and neurobiological changes in a novel mouse model of schizophrenia induced by the combination of cuprizone and MK-801. Brain Re-search Bulletin. 2021; 174: 141–152.

[15] Paulus MP. The role of neuroimaging for the diagnosis and treatment of anxiety disorders. Depression and Anxiety. 2008; 25: 348–356.

[16] Dalby RB, Frandsen J, Chakravarty MM, Ahdidan J, Sørensen L, Rosenberg R, *et al*. Depres-sion severity is correlated to the integrity of white matter fiber tracts in late-onset major depression. Psychiatry Research: Neuroimaging. 2010; 184: 38–48.

[17] Uccelli A, Benvenuto F, Laroni A, Giunti D. Neuroprotective features of mesenchymal stem cells. Best Practice and Research Clinical Haematology. 2011; 24: 59–64.

[18] Zhang J, Wang H, Fan Y, Yang F. Effect of mesenchymal stem cells transplantation on the changes of oligodendrocyte lineage in rat brain with experimental autoimmune encephalomyelitis. Brain and Behavior. 2021; 11: e01999

[19] S Jyothi P, Divya G, Shilpa Rani S, Anoop Babu V. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PlOS One. 2010; 5: e9016.

[20] Meisel R, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D. Human bone marrow stromal cells inhib-it allogeneic T-cell responses by indoleamine 2,3-dioxygenase–mediated tryptophan degradation. Blood. 2004; 103: 4619–4621.

[21] Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, *et al*. Treatment of Autoimmune Neuroinflammation with a Synthetic Tryptophan Metabolite. Science. 2005; 310: 850–855.

[22] Goddard AW, Ball SG, Martinez J, Robinson MJ, Yang CR, Russell JM, *et al*. Current perspec-tives of the roles of the central norepinephrine system in anxiety and depression. Depression and Anxiety. 2010; 27: 339–350.

[23] Bhatt S, Devadoss T. 5-HT3 Receptor Antagonism: a Potential Therapeutic Approach for the Treat-ment of Depression and other Disorders. Current Neuropharmacology. 2021; 19: 1545–1559.
[24] Li H, Liu Y, Gao X, Liu L, Amuti S, Wu D, *et al*. Neuroplastin 65 modulates anxiety- and de-pression-like behavior likely through adult hippocampal neurogenesis and central 5-HT activity. FEBS Journal. 2019; 286: 3401–3415.
[25] Paul ED, Lowry CA. Functional topography of serotonergic systems supports the Deakin/Graeff hy-pothesis of anxiety and affective disorders. Journal of Psychopharmacology. 2013; 27: 1090–1106.
[26] Stein MB. Neurobiology of Generalized Anxiety Disorder. The Journal of Clinical Psychiatry. 2009; 70: 15–19.
[27] Blier P. Neurotransmitter targeting in the treatment of depression. The Journal of Clinical Psychiatry. 2013; 74 suppl 2: 19–24.