Adipose-derived mesenchymal stem cell exosomes ameliorate spinal cord injury in rats by activating the Nrf2/HO-1 pathway and regulating microglial polarization

Yi Luo, You-zhi He, Yong-fu Wang, Yu-xia Xu, Li Yang

Department of Spine Surgery, The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha, Hunan, China

Abstract

As of now, there are no satisfactory treatments for spinal cord injury (SCI), so new therapeutic approaches are necessary to be explored. Adipose-derived mesenchymal stem cell exosomes (ADMSC-Exo), delightfully, show remarkable therapeutic effects. Therefore, we try to investigate the effects and mechanisms of ADMSC-Exo on SCI, as well as to provide novel approaches for the treatment of SCI. Adipose-derived mesenchymal stem cells (ADMSC) were isolated from rats and then exosomes (Exo) were extracted from the cells. The extracted Exo were identified by flow cytometry, transmission electron microscopy and nanoparticle tracking analysis (NTA). Then, the SCI rat model was established by the spinal cord impactor and injected with 200 µl PBS or Exo into their tail veins at 30 min, 24 h, and 48 h after surgery. The rats in the Control group and Exo group only exposed the spine. Motor function recovery was assessed on days 0, 7, 14, 21, and 28; histopathological changes and apoptosis levels in spinal cord tissues were observed by HE staining and TUNEL staining; the levels of inflammatory cytokines TNF-α, IL-6, and MCP-1 in spinal cord tissues were measured by ELISA; the expression levels of iNOS, IL-12, Arg1, and Mrc1 in spinal cord tissues were detected by qRT-PCR; and Nrf2, HO-1, and NQO1 protein expression in spinal cord tissues were detected by Western blot. ADMSC-Exo were successfully isolated and identified. ADMSC-Exo significantly relieved SCI and promoted motor function recovery in SCI rats. Moreover, ADMSC-Exo inhibited the expression of both inflammatory factors in the spinal cord tissues and M1 microglia, promoted the expression of M2 microglia, and activated the Nrf2/HO-1 pathway. Altogether, ADMSC-Exo can not only ameliorate SCI, but also promote the motor function recovery of rats. And the mechanism of ADMSC-Exo improving SCI may be achieved by activating Nrf2/HO-1 pathway and microglial polarization.

Key words: *spinal cord injury, adipose-derived mesenchymal stem cells, exosomes, microglial polarization, Nrf2/HO-1 pathway.*

Introduction

Spinal cord injury (SCI), a traumatic injury to the spine, may result in permanent impairment of sensory and motor function [17]. Surveys have reported that more than 25,000 people worldwide suffer from SCI each year, mainly caused by tumours, traffic accidents, trauma, and other sports injuries. Since 2012, there has been an increase in recorded cases of traumatic SCI caused by falls going from 16% to 30.5% as a result of the aging population [1]. The worldwide mortality rate for acute SCI is estimated to be between 4.4% and 16.7%. Importantly, SCI seriously affects the quality of life of patients. The recovery of physical function after SCI has been a major clinical challenge since the spinal cord cannot regenerate [24].

Communicating author:

Yi Luo, Department of Spine Surgery, The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha, Hunan, 410007, China, e-mail: 2018050708@usc.edu.cn

The pathophysiology of SCI includes both primary injury and secondary injury. The secondary injury, mainly due to neuroinflammation, can lead to oedema, cavitation and reactive gliosis. And the primary focus of SCI treatment is the subsequent injury in which microglia play a crucial role [3]. Microglia, a type of glial cells, shares similarities with macrophages in the brain and spinal cord and serves as the first and most important line of immune defence in the central nervous system (CNS) [13]. The stable and sustained inflammatory response in SCI will limit the repair and regeneration of tissues as well as the recovery of neurological function. However, by effectively inducing microglia to polarize from pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype and releasing anti-inflammatory cytokines to change the inflammatory microenvironment, the inflammatory response is attenuated, the secondary injury relieved, and the motor function recovery promoted [22]. Therefore, regulating the polarization of microglia is effective to treating SCI. Activation of the nuclear factor-erythroid 2 related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) signalling pathway has been found to promote macrophage polarization to M2 phenotype, while SnPP, an HO-1 inhibitor, encourages macrophage polarization to M1 phenotype [19,21]. In a nutshell, the Nrf2/HO-1 signalling pathway has an important regulatory effect on microglial polarization.

With the advancements in stem cell technology, the immunomodulatory function of stem cell transplantation, especially of the transplantation of mesenchymal stem cells (MSCs), which are also known as mesenchymal stromal cells, has become hotly discussed in the field of SCI treatment [14]. Zhou et al. found that transplantation of adipose-derived mesenchymal stem cells (ADMSC) prevented neuroinflammation, supported neural tissue preservation, and ultimately promoted functional recovery in SCI rats [29]. ADMSC exert their effects mainly through exosomes (Exo), the paracrine substances of stem cells. Exo, the vesicles (30-220 nm in diameter) released by organelle fusion, can serve as stable and reliable carriers to deliver specific proteins, lipids, and genetic material (mRNAs, miRNAs, other small non-coding RNAs and DNA) to target tissues or organs [2,10]. Recent years have witnessed a surge in studies on the application of MSCs-derived Exo (MSCs-Exo) in the fields of tissue engineering, inflammatory regulation, and regenerative medicine. MSCs-Exo, of which bone marrow-derived MSCs-Exo have been widely studied in the SCI treatment, can avoid some adverse reactions that occur when stem cells are directly transplanted. The hypoxia-treated ADMSC-derived Exo (ADMSC-Exo) have also been demonstrated to relieve SCI by inhibiting microglial inflammatory responses [23,27]. Therefore, ADMSC-Exo containing various paracrine mediators may become one of the treatment options for SCI, but the exact specific mechanism of action remains unknown. Based on this, we confirmed the therapeutic effect of ADMSC-Exo on SCI by constructing a SCI rat model, and preliminarily explored its mechanism of action. Importantly, this study is expected to provide an exact theoretical basis for the clinical application of ADMSC-Exo.

Material and methods

Isolation and culture of adipose-derived mesenchymal stem cells

ADMSC cells were isolated as previously described [15]. Adipose tissues were extracted from the inguinal region of 3 female Sprague-Dawley (SD) rats with 6-8 weeks of age and 20-25 g of weight. The extracted fat was incubated with 0.2 U/ml collagenase type IV for 3 h at 37°C, followed by neutralization with 10 ml foetal bovine serum. Upon centrifugation, the cell debris was removed. And vascular cells, fibroblasts, and adipocytes obtained by previous centrifugation were resuspended in Hank's Balanced Salt Solution. The cell resuspension was subsequently filtered through a 40-μm membrane. Then, the cells were centrifuged at 274 × g for 10 min, and cell debris was removed again. After this, the precipitate was resuspended in 1 ml of the complete medium, where the cells were developed to sub-confluence. Again, the cells were resuspended in the culture medium, digested with trypsin, and filtered. After the filtrate was collected in a cell culture flask, primary ADMSC culture was obtained and then became visible large flat cells after three passages. ADMSC at the third passage were chosen to continue the subsequent experiments.

Flow cytometry

Before detection, ADMSC were washed three times with phosphate buffered saline (PBS), centrifuged and resuspended. The cells were first incubated with CD90, CD44, and CD45 antibodies in the dark for 30 min and washed with PBS. Next incubation was performed with different fluorescent secondary antibodies for 30 min. After washing with PBS again, 2% paraformaldehyde diluted in PBS was added. Lastly, the expressions of ADMSC surface antigens were detected by flow cytometry (FACSymphony A5).

Extraction of adipose-derived mesenchymal stem cell exosomes

ADMSC were seeded at 5×10^5 cells in a 10 cm dish. Twenty-four hours later, the medium collected from the dish was centrifuged at 3,000 g for 15 min, followed by the removal of the cells and cellular debris. Extraction of

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Exo was performed following the steps described in the ExoQuick kit instructions (System Biosciences, CA, USA). At first, ADMSC-Exo were separated by adding 2 ml of Exosome Concentration Solution (ECS) into the impurity-free centrifuged supernatant. After incubation at 4°C overnight and centrifugation again, the supernatant was discarded, and the Exo precipitate was evenly blown with PBS. Subsequently, the resuspension was centrifuged to take the supernatant which was next transferred into the upper chamber of the Exosome Purification Filter (EPF). Upon centrifugation, the liquid collected from the bottom of the EPF was the purified ADMSC-Exo. Finally, the liquid was completely suspended in 50 µl of PBS and together stored at −80°C for further study.

Transmission electron microscopy

Ten microliters Exo liquid was placed on 400 mesh copper grids coated with formvar and incubated for 5 min before discarding excess liquid. Later, uranyl acetate was supplemented to the grid for negative staining for 1 min and excess liquid was also abandoned. The resulting sample was then observed using a transmission electron microscope at 100 kV.

Nanoparticle tracking analysis

Size determination and concentration measurement of Exo were performed on the NanoSight LM10-HS (NanoSight Ltd, Amesbury, United Kingdom) equipped with a 638 nm laser. The sample was diluted to 1 ml in clean PBS prior to analysis. The diluted sample was drawn into the sample chamber with a sterile syringe until the sample reached the outlet of the chamber. At last, the collected data were analysed using nanoparticle tracking analysis (NTA) software. By the way, NTA software could provide high-resolution particle size distribution profiles and concentration measurement values of outer vesicles.

Animal grouping and processing

The 6-week-old female SD rats were habituated for 2 weeks before carrying out the experiment (8-week-old). The rats were cared for in accordance with the protocols approved by the Institutional Animal Care and Use Committee. All experimental protocols were approved by the Ethics Committee for Animal Use of The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China (2021-S0146).

A total of 20 rats were randomly divided into four groups: Control group, Exo group, SCI group, and SCI + Exo group (*n* = 5 in each group). Noticeably, rats in the Control and Exo groups were only exposed to the spine during modelling. SCI rats were injected with 200 µl PBS (SCI group) or Exo (SCI + Exo group), respectively, *via* the tail veins at 30 min, 24 h, and 48 h after surgery.

As for surgery, rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital at a dose of 50 mg/kg. Then, the rats were placed on an operating table and disinfected. A 2-3 cm incision was made along the dorsal midline centred on the spinous process of T10 vertebra. Following the dissection of the surface muscles, the vertebrae were exposed and the spinous processes and laminae were removed to completely expose the T10 vertebra. In this process, the dura mater was completely preserved. A standard impactor was used to strike the T10 vertebra with a force of 2 N. The wound was subsequently rinsed with penicillin saline and sutured.

The successfully established SCI rat model is characterized by congestive oedema at the level of T10, twitching of the tail and hindlimb, and a Basso Beattie Bresnahan (BBB) score less than 10 points [25]. Twenty-eight days after surgery, the rats were euthanized by intraperitoneal injection of 1% sodium pentobarbital at an excessive dose of 120 mg/kg, and their spinal cord tissues were collected.

Basso Beattie Bresnahan score

As described in the previous study [25], the Basso Beattie Bresnahan (BBB) score was used to detect changes in hindlimb motor function in rats at 0, 7, 14, 21, and 28 days after SCI. The BBB score for SCI rats ranged from 0 to 21 based on the scoring indicators, including hindlimb joint mobility, walking ability, paw and tail posture, limb coordination, and limb stability.

HE staining and TUNEL staining

Twenty-eight days after surgery, the rats were euthanized, and some spinal cord tissues (approx. 0.5 cm around the injury site) were collected from three rats of each group. Next, the tissues were fixed in 4% paraformaldehyde for 24 h, followed by routine dehydration, clearing, waxing, embedding and sectioning with a section thickness of 6 µm. Some sections were stained with HE, dehydrated with alcohol and xylene, and the sections were sealed with neutral resin. Later on, histopathological changes were observed under a light microscope (Olympus, Japan). Some other sections were stained with TUNEL. Upon adding 3% hydrogen peroxide solution for incubation for 5 min, 0.05 mol/l citrate buffer solution was supplied for recovery for 10 min at 100°C. Later, the sections were blocked in 15% non-fat dry milk solution for 1 h. Subsequently, TUNEL detection solution (1 : 500) was supplemented for incubation at ambient temperature for 90 min, fluorescein antibody (1 : 500) added for incubation at 37°C for 60 min, and PI staining solution dropped for reaction for 5 min. Then, the sections were dried

at 60°C, mounted, and finally observed and photographed under a fluorescence microscope. The number of bright green cells under the visual field was counted using ImageJ software. Attentively, the procedure after adding TUNEL detection solution requires protection from light.

ELISA

Some spinal cord tissues were extracted from five rats in each group 28 days after surgery. Subsequently, the tissues were homogenized in pre-chilled PBS, centrifuged at 3,000 revolutions per minute (15 minutes; 4°C), and then the supernatants were collected for testing. The expression of interleukin 6 (IL-6), tumour necrosis factor α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) were finally quantified on the microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) following the instructions of ELISA kits (Thermo Fisher Scientific, USA).

qRT-PCR

Similarly, some spinal cord tissues were collected from five rats in each group, and the total RNA was extracted using the RNeasy Mini Kit. Then, NanoDrop was applied to detect the concentration and purity of RNA, cDNA was prepared according to the random primer reverse transcription kit (Thermo Fisher Scientific, USA), and qPCR was performed using real-time PCR device following the instructions of the SYBR Premix EX Taq kit (TaKaRa, Japan). All the primers were listed in Table I. The relative expression levels of target genes were calculated by $2^{-\Delta\Delta Ct}$ method, in which GAPDH was the internal control gene.

Western blot

Again, a part of spinal cord tissues of three rats of each group were collected to extract total protein using RIPA lysate (Beyotime, Wuhan, China). The concentration of the extracted protein was determined with a BCA kit (Thermo Fisher Scientific, USA). Initially, 20 μg of protein was boiled and denatured in 5 × loading buffer, followed by separation of the protein with SDS-PAGE. The separated protein was then transferred to PVDF membrane, and blocked in 5% skimmed milk powder for 2 h. Next, the membrane was incubated with primary antibody (Abcam) (CD81, ab79559; CD63, ab134045; TSG101, ab125011; Nrf2, ab62352; HO-1, ab305290; NQO1, ab80588) overnight at 4°C. Furthermore, the membrane was incubated with secondary antibody at ambient temperature for 1 h, and washed with TBST three times. ECL chemiluminescence reagent was introduced to the membrane which would later be placed in a gel imaging system (Alpha Innotech, USA)

to develop and collect images. Image J software was used to analyse the grey level of the protein bands, and GAPDH was considered as an internal reference to calculate the relative protein expression.

Data analysis

SPSS 26.0 statistical software was adopted. Measurement data were expressed as mean ± standard deviation (SD). One-way analysis of variance was applied to compare multiple groups, while independent *T* test to compare two groups. *P* < 0.05 was defined as statistically significant.

Results

Identification of adipose-derived mesenchymal stem cells and adipose-derived mesenchymal stem cell exosomes

To better determine the effect of ADMSC-Exo, ADMSC were isolated from adipose tissues collected from female SD rats. First, we could obviously observe that the expressions of ADMSC surface antigen CD90 and CD44 were positive and CD45 expression was negative (Fig. 1A-C). Subsequent to isolation and purification of ADMSC-Exo, ADMSC-Exo were found to present a round or oval shape and have a typical bilayer membrane structure under electron microscope (Fig. 1D). According to the outcomes of NTA, the diameters of ADMSC-Exo were predominantly concentrated at 60-130 nm (Fig. 1E). Moreover, Western blot results indicated expressions of Exo signature proteins CD81, CD63, and TSG101 in ADMSC-Exo (Fig. 1F). In conclusion, ADMSC-Exo were successfully obtained.

Table I. Primer sequences for qPCR

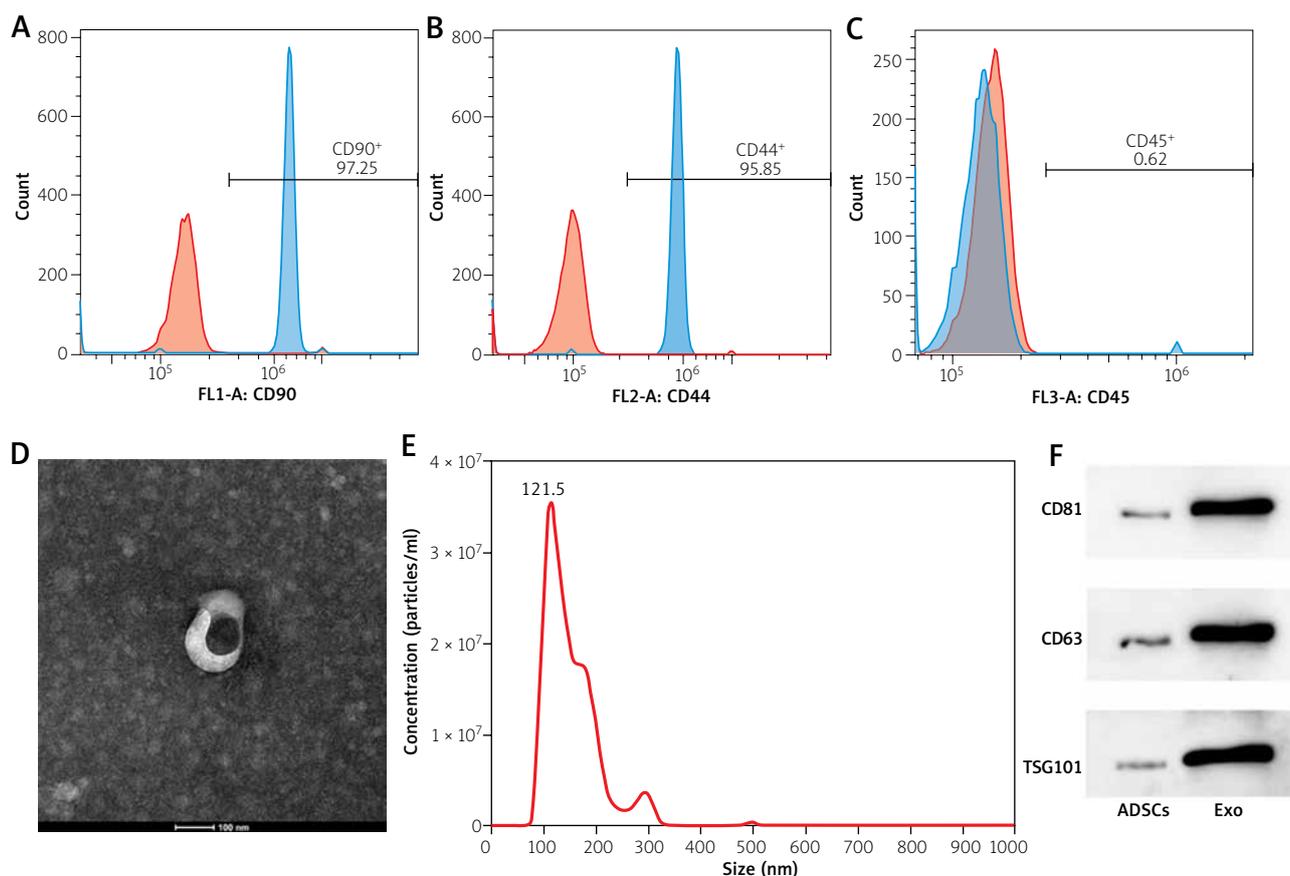| RNA	Sequences (5' to 3')
iNOS	F: 5'-GAGACAGGGAAGTCTGAAGCAC-3'
	R: 5'-CCAGCAGTAGTTGCTCCTCTTC-3'
IL-12	F: 5'-ACGAGAGTTGCCTGGCTACTAG-3'
	R: 5'-CCTCATAGATGCTACCAAGGCAC-3'
Arg1	F: 5'-CATTGGCTTGCGAGACGTAGAC-3'
	R: 5'-GCTGAAGGTCTCTTCCATCACC-3'
Mrc1	F: 5'-GTTCACCTGGAGTGATGGTTCTC-3'
	R: 5'-AGGACATGCCAGGGTCACCTTT-3'
GAPDH	F: 5'-CATCACTGCCACCCAGAAGACTG-3'
	R: 5'-ATGCCAGTGAGCTTCCCGTTCAG-3'

Fig. 1. Identification of adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes (ADMSC-Exo). **A-C)** Flow cytometry results of the expressions of ADMSC surface antigens CD90 (**A**), CD44 (**B**), and CD45 (**C**); **D)** Transmission electron microscopy results of the morphology of Exo after negative staining with uranyl acetate; **E)** NTA results of the diameter of ADMSC-Exo; **F)** Western blot results of the protein expression levels of CD81, CD63, and TSG101 in ADMSC and ADMSC-Exo.

Adipose-derived mesenchymal stem cell exosomes attenuate spinal cord injury and promote motor function recovery in spinal cord injury rats

In order to explore the function of ADMSC-Exo, we constructed models of SCI rats who were injected with 200 μl Exo into their tail veins at different time points (30 min, 24 h, and 48 h) after surgery, followed by assessment of motor recovery in each group of rats on days 0, 7, 14, 21, and 28. The results revealed that the BBS scores of the SCI and SCI + Exo groups had a slowly increasing trend on days 7, 14, 21, and 28, but the BBB scores of the SCI + Exo group were significantly higher than those of the SCI group ($p < 0.01$) (Fig. 2A). After HE staining, the morphology of neuronal cells was found to be essentially normal in the Control and Exo groups. However, extensive neuronal degeneration and necrosis, neuronal vacuolation, and massive inflammatory cell infiltration were observed in the SCI group; compared with the SCI group, the SCI + Exo group exhibited greatly relieved SCI, mild neuronal necrosis and a small amount of infiltrated inflammatory cells (Fig. 2B). Besides, TUNEL staining results indicated that the apoptosis levels of cells in the spinal cord tissues of SCI rats were significantly increased, while Exo significantly reduced the apoptosis levels (Fig. 2C). Collectively, ADMSC-Exo could significantly alleviate SCI and promote motor function recovery in SCI rats.

Adipose-derived mesenchymal stem cell exosomes inhibit spinal cord inflammation in spinal cord injury rats

With the aim of further understanding the effect of ADMSC-Exo on SCI rats, we detected the expression of inflammatory cytokines TNF-α, IL-6 and pro-inflammatory cytokine MCP-1 in the spinal cord tissues of rats in each group by ELISA. The detection results revealed that the
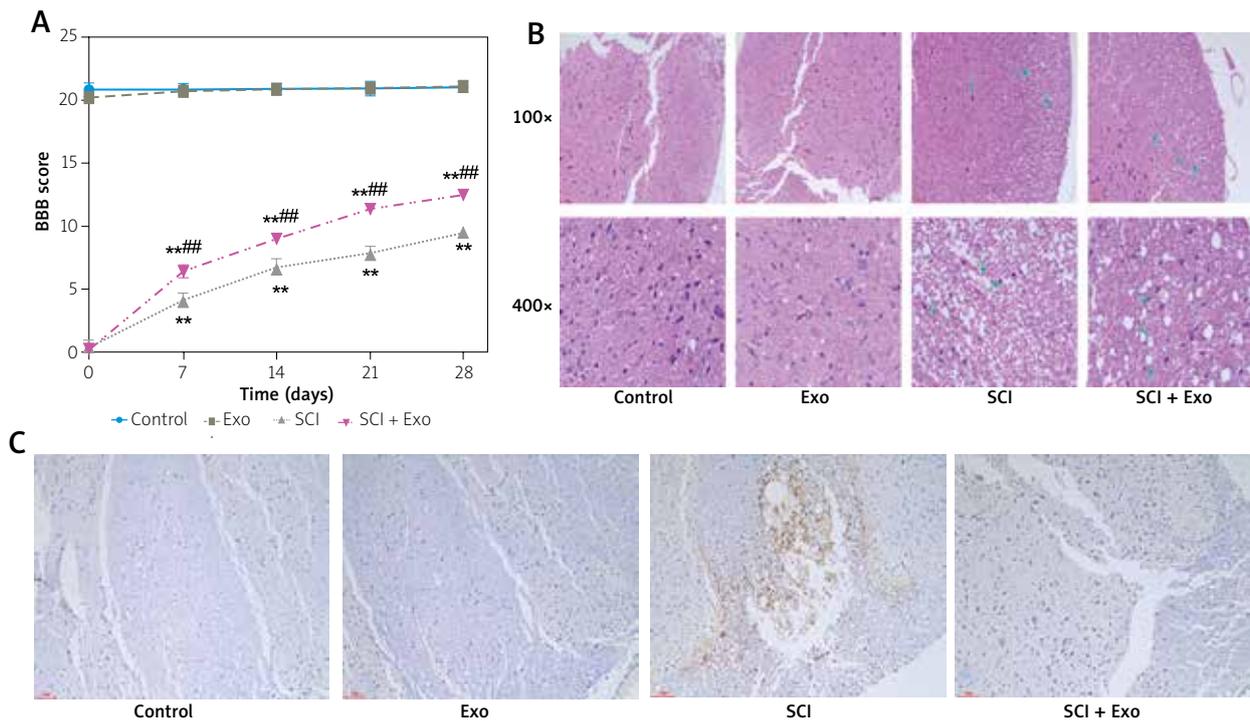
Fig. 2. Adipose-derived mesenchymal stem cell exosomes alleviate spinal cord injury and promote motor function recovery in spinal cord injury rats. **A)** BBB scores of rats in each group on days 0, 7, 14, 21, and 28 to evaluate the motor function recovery; **B)** HE staining results of the histopathological changes of spinal cord tissues in each group (magnification scale: 100× and 400×; bar: 10 µm); **C)** TUNEL staining results of the apoptosis level of cells in spinal cord tissues of rats in each group (magnification scale: 100×; bar: 10 µm). **p < 0.01, vs. control, ##p < 0.01, vs. SCI (n = 5).

levels of TNF-α, IL-6, and MCP-1 in the SCI and SCI + Exo groups were much higher than those in the Control group (p < 0.05); the levels of TNF-α, IL-6, and MCP-1 in the SCI + Exo group were much lower than those in the SCI group (p < 0.05) (Fig. 3). It can be concluded that ADMSC-Exo inhibited spinal cord inflammation in SCI rats.

Adipose-derived mesenchymal stem cell exosomes induce microglial polarization

The M1 microglia markers iNOS, IL-12, and the M2 microglia markers Arg1 and Mrc1 were then examined. According to qRT-PCR results, SCI group displayed a significant increase in the levels of iNOS and IL-12 in the spinal cord tissues of SCI rats compared with the Control group (p < 0.05); while SCI-Exo obviously declined the levels of iNOS and IL-12 and increased the expression levels of Arg1 and Mrc1 in the spinal cord tissues of SCI rats (p < 0.05) (Fig. 4). Briefly speaking, ADMSC-Exo induced microglial polarization, inhibited the expression of M1 pro-inflammatory phenotype, and promoted the expression of M2 anti-inflammatory phenotype.

Adipose-derived mesenchymal stem cell exosomes activate Nrf2/HO-1 pathway in spinal cord tissues of spinal cord injury rats

Ultimately, in an attempt to reveal the mechanism of ADMSC-Exo, we detected the activity of Nrf2/HO-1 pathway in rat spinal cord tissues by Western blot. The results presented that the protein expression levels of Nrf2, HO-1 and NQO1 in spinal cord tissues of the SCI group were apparently down-regulated compared with the Control group (p < 0.01); the protein levels of Nrf2, HO-1, and NQO1 in SCI + Exo group were considerably up-regulated compared with the SCI group (p < 0.01) (Fig. 5A-D). In summary, SCI inhibited Nrf2/HO-1 pathway activity in spinal cord tissues, while ADMSC-Exo activated Nrf2/HO-1 pathway and protected spinal cord tissues.

Discussion

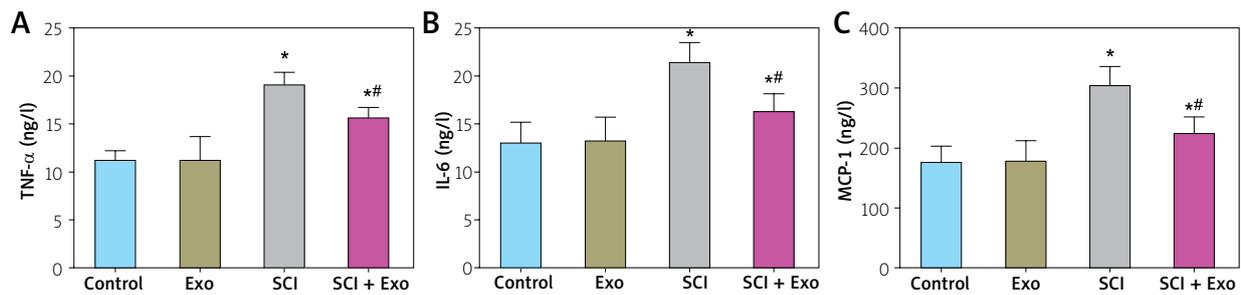Exosomes, containing abundant bioactive substances such as protein, RNA and DNA, play a signifi-

Fig. 3. Adipose-derived mesenchymal stem cell exosomes inhibit spinal cord inflammation in spinal cord injury rats. **A)** ELISA results of the level of TNF-α in the spinal cord tissues of rats in each group; **B)** ELISA results of the level of IL-6 in the spinal cord tissues of rats in each group; **C)** ELISA results of the level of MCP-1 in the spinal cord tissues of rats in each group. *$p < 0.05$, vs. control; #$p < 0.05$, vs. SCI ($n = 5$).
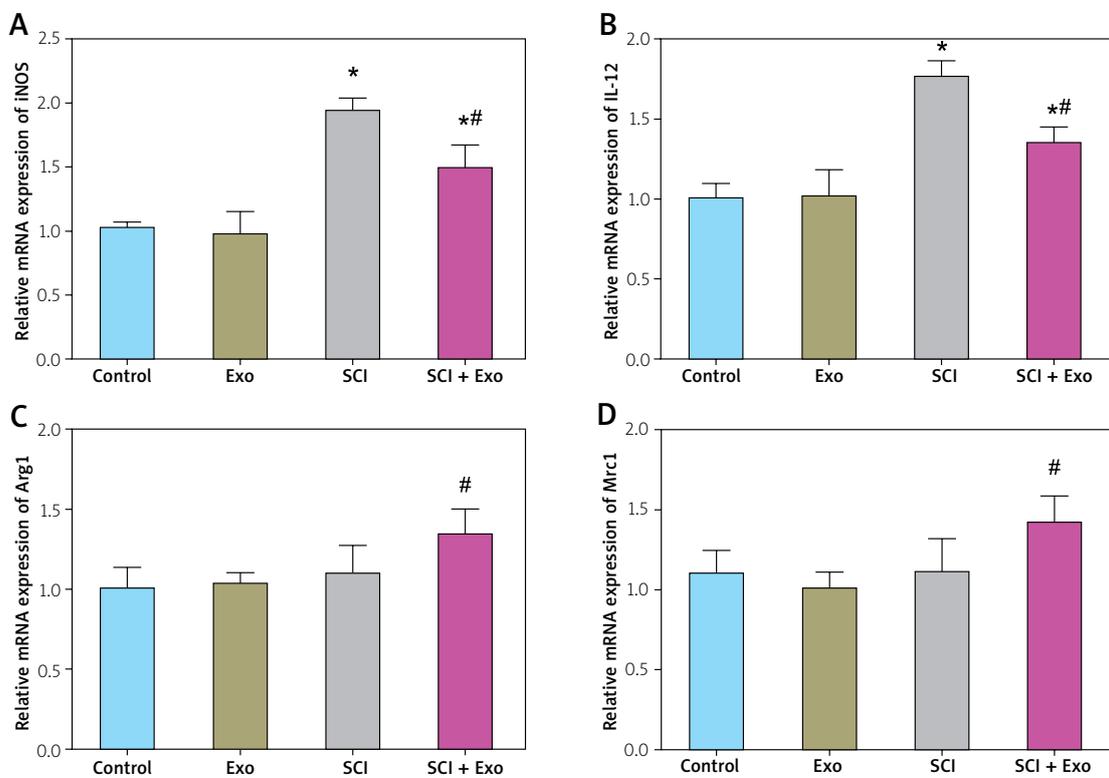

Fig. 4. Adipose-derived mesenchymal stem cell exosomes induce microglial polarization. **A-D)** qRT-PCR results of the expression levels of iNOS (**A**), IL-12 (**B**), Arg1 (**C**) and Mrc1 (**D**) in spinal cord tissues of rats in each group *$p < 0.05$, vs. control; #$p < 0.05$, vs. SCI ($n = 5$).

cant role in transporting and regulating complex signalling molecules. As the main effector of stem cells, Exo have gradually emerged as a new hotspot in the field of stem cell tissue regeneration and repair [12]. In particular, ADMSC-Exo have a positive therapeutic effect on a variety of diseases. He *et al.* claimed that ADMSC-Exo may actively contribute to the healing of skin wounds by targeting miR-124 through activation of the

Wnt/β-catenin pathway [9]. In addition, recent studies have disclosed that ADMSC-Exo can be exploited as a novel anti-inflammatory agent and a current therapeutic target for rheumatoid arthritis because of their multiple immunomodulatory and anti-inflammatory effects [5]. Accordingly, ADMSC-Exo can aid in repairing a variety of physical injuries and may be a promising injury therapeutic agent. The therapeutic effect of
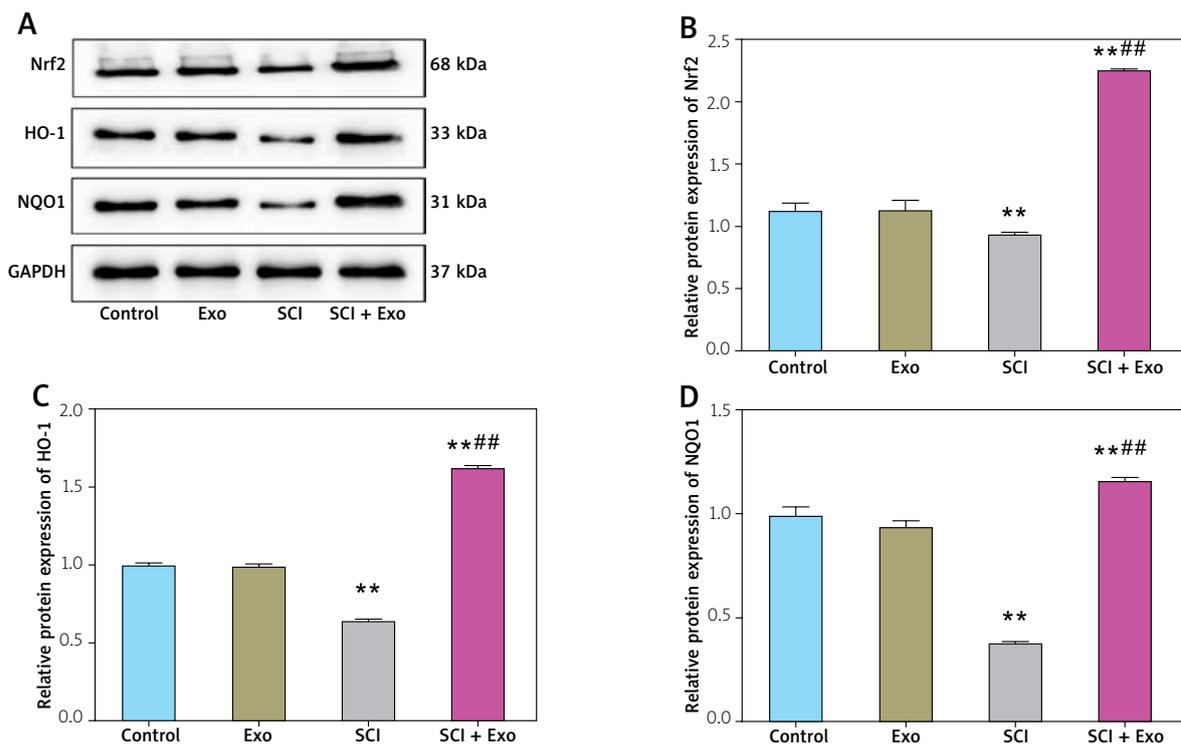
Fig. 5. Adipose-derived mesenchymal stem cell exosomes activated the Nrf2/HO-1 pathway in spinal cord tissues of spinal cord injury rats. **A**) Western blot results of the protein expressions of Nrf2, HO-1, and NQO1 in the spinal cord tissues of rats in each group; **B-D**) ImageJ results of quantitative analysis on the protein expressions of Nrf2 (**B**), HO-1 (**C**), and NQO1 (**D**) in the spinal cord tissues of rats in each group. **p < 0.01, vs. control; ##p < 0.01, vs. SCI (n = 3).

ADMSC-Exo on SCI has previously been documented in investigations [23,27]. To explore the therapeutic effect and mechanism of ADMSC-Exo on SCI, we first isolated ADMSC from rats and then extracted ADMSC-Exo which were subsequently injected into SCI rats. In the end, a conclusion that ADMSC-Exo significantly alleviated SCI and promoted motor function recovery in SCI rats was made in this study where the mechanism of action was further explored too.

The function of ADMSC-Exo in relieving inflammation has already been demonstrated [4,11]. In the study of Cho *et al.*, ADMSC-Exo could ameliorate atopic dermatitis-like symptoms by modulating the inflammation and decreasing the expression of inflammatory cytokines such as IL-4, IL-23, IL-31, and TNF-α [6]. In addition, ADMSC-Exo therapy has also been found to protect organs from ischemia reperfusion (IR) injury, as well as to significantly attenuate inflammation and oxidative stress response in the treatment of IR injury [16]. As for our findings, ADMSC-Exo not only inhibited the expression of inflammatory cytokines TNF-α, IL-6, and MCP-1 in the spinal cord tissues of SCI rats but also attenuated spinal inflammation. Furthermore,

ADMSC-Exo, displayed in previous studies, could translocate into macrophages, increased M2-related arginase-1 and IL-10 expression, and induced macrophage polarization to the anti-inflammatory M2 phenotype [28]. Our study first discovered that ADMSC-Exo were capable of decreasing M1 phenotype expression and promoting M2 phenotype expression in spinal cord tissues, indicating that ADMSC-Exo can induce microglial polarization to inhibit the inflammation in spinal cord tissues.

In an effort to further reveal the mechanism of action of ADMSC-Exo, we investigated signalling pathways in spinal cord tissues. Based on previous research, Nrf2/HO-1 pathway is involved in microglial polarization and inflammation in the nervous system [7]. Nrf2, a cytoprotective factor, regulates the gene expression of encoding antioxidant, anti-inflammatory, and detox proteins [18]. After stimulation by inflammatory or oxidative response, Nrf2 can translocate into the nucleus, activate the expression of HO-1 (a gene downstream of the Nrf2 signalling pathway) and inhibit inflammation via nuclear transcription factor (NF-κB) signalling pathway. HO-1 and its product NQO1 protect cells

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and organs by preventing oxidative damage, regulating apoptosis and inflammation, as well as promoting angiogenesis [8]. It has been discovered that Nrf2 activation in neurons or astrocytes improves spinal cord IR injury by enhancing the antioxidant, anti-apoptotic, and survival capacities of neurons [26]. What is more, knockout of Nrf2 worsens neurological impairments in spinal cord tissues following SCI surgery [20]. Therefore, the Nrf2/HO-1 pathway may be an important target for the treatment of SCI. In our study, ADMSC-Exo significantly increased the protein levels of Nrf2, HO-1, and NQO1 in SCI rats, supporting the idea that ADMSC-Exo may function by stimulating the Nrf2/HO-1 pathway.

Conclusions

Taken together, ADMSC-Exo can activate the Nrf2/HO-1 pathway, regulate microglial polarization, inhibit spinal cord inflammation, thereby improving SCI and promoting motor function recovery in rats. Briefly speaking, ADMSC-Exo can act as a novel target for the treatment of SCI. However, the specific mechanism by which ADMSC-Exo regulate the Nrf2/HO-1 pathway has not been revealed in this study. Besides, the mechanism of action of ADMSC-Exo is very complicated and further exploration is deserved to provide comprehensive data for clinical practice.

Funding

Disclosure

The authors report no conflict of interest.

References

1. Ahuja CS, Wilson JR, Nori S, Kotter MRN, Druschel C, Curt A, Fehlings MG. Traumatic spinal cord injury. Nat Rev Dis Primers 2017; 3: 17018.
2. Breakefield XO, Frederickson RM, Simpson RJ. Gesicles: Microvesicle "cookies" for transient information transfer between cells. Mol Ther 2011; 19: 1574-1576.
3. Brennan FH, Li Y, Wang C, Ma A, Guo Q, Li Y, Pukos N, Campbell WA, Witcher KG, Guan Z, Kigerl KA, Hall JCE, Godbout JP, Fischer AJ, McTigue DM, He Z, Ma Q, Popovich PG. Microglia coordinate cellular interactions during spinal cord repair in mice. Nat Commun 2022; 13: 4096.
4. Chang CL, Sung PH, Chen KH, Shao PL, Yang CC, Cheng BC, Lin KC, Chen CH, Chai HT, Chang HW, Yip HK, Chen HH. Adipose-derived mesenchymal stem cell-derived exosomes alleviate overwhelming systemic inflammatory reaction and organ

damage and improve outcome in rat sepsis syndrome. Am J Transl Res 2018; 10: 1053-1070.
5. Chang TH, Wu CS, Chiou SH, Chang CH, Liao HJ. Adipose-derived stem cell exosomes as a novel anti-inflammatory agent and the current therapeutic targets for rheumatoid arthritis. Biomedicines 2022; 10: 1725.
6. Cho BS, Kim JO, Ha DH, Yi YW. Exosomes derived from human adipose tissue-derived mesenchymal stem cells alleviate atopic dermatitis. Stem Cell Res Ther 2018; 9: 187.
7. Dong L, Dongzhi Z, Jin Y, Kim YC, Lee DS, Huang S, Panichayupakaranant P, Li B. Taraxacum officinale Wigg. Attenuates inflammatory responses in murine microglia through the Nrf2/HO-1 and NF-kappaB signaling pathways. Am J Chin Med 2020; 48: 445-462.
8. Han S, Gao H, Chen S, Wang Q, Li X, Du LJ, Li J, Luo YY, Li JX, Zhao LC, Feng J, Yang S. Procyanidin A1 alleviates inflammatory response induced by LPS through NF-kappaB, MAPK, and Nrf2/HO-1 Pathways in RAW264.7 cells. Sci Rep 2019; 9: 15087.
9. He L, Zhu C, Jia J, Hao XY, Yu XY, Liu XY, Shu MG. ADSC-Exos containing MALAT1 promotes wound healing by targeting miR-124 through activating Wnt/beta-catenin pathway. Biosci Rep 2020; 40: BSR20192549.
10. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 2018; 75: 193-208.
11. Hu J, Jiang Y, Wu X, Wu Z, Qin J, Zhao Z, Li B, Xu Z, Lu X, Wang X, Liu X. Exosomal miR-17-5p from adipose-derived mesenchymal stem cells inhibits abdominal aortic aneurysm by suppressing TXNIP-NLRP3 inflammasome. Stem Cell Res Ther 2022; 13: 349.
12. Joo HS, Suh JH, Lee HJ, Bang ES, Lee JM. Current knowledge and future perspectives on mesenchymal stem cell-derived exosomes as a new therapeutic agent. Int J Mol Sci 2020; 21: 727.
13. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev 2011; 91: 461-553.
14. Kim BG, Hwang DH, Lee SI, Kim EJ, Kim SU. Stem cell-based cell therapy for spinal cord injury. Cell Transplant 2007; 16: 355-364.
15. Li J, Curley JL, Floyd ZE, Wu X, Halvorsen YDC, Gimble JM. Isolation of human adipose-derived stem cells from lipoaspirates. Methods Mol Biol 2018; 1773: 155-165.
16. Lin KC, Yip HK, Shao PL, Wu SC, Chen KH, Chen YT, Yang CC, Sun CK, Kao GS, Chen SY, Chai HT, Chang CL, Chen CH, Lee MS. Combination of adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes for protecting kidney from acute ischemia-reperfusion injury. Int J Cardiol 2016; 216: 173-185.
17. Liu S, Chen Z. Employing endogenous NSCs to promote recovery of spinal cord injury. Stem Cells Int 2019; 2019: 1958631.
18. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol Life Sci 2016; 73: 3221-3247.
19. Luo D, Guo Y, Cheng Y, Zhao J, Wang Y, Rong J. Natural product celastrol suppressed macrophage M1 polarization against inflammation in diet-induced obese mice via regulating Nrf2/HO-1, MAP kinase and NF-kappaB pathways. Aging (Albany NY) 2017; 9: 2069-2082.
20. Mao L, Wang HD, Wang XL, Tian L, Xu JY. Disruption of Nrf2 exacerbated the damage after spinal cord injury in mice. J Trauma Acute Care Surg 2012; 72: 189-198.
21. Naito Y, Takagi T, Higashimura Y. Heme oxygenase-1 and anti-inflammatory M2 macrophages. Arch Biochem Biophys 2014; 564: 83-88.

22. Orihuela R, McPherson CA, Harry GJ. Microglial M1/M2 polarization and metabolic states. Br J Pharmacol 2016; 173: 649-665.

23. Shao M, Jin M, Xu S, Zheng C, Zhu W, Ma X, Lv F. Exosomes from long noncoding RNA-Gm37494-ADSCs repair spinal cord injury via shifting microglial M1/M2 polarization. Inflammation 2020; 43: 1536-1547.

24. Varma AK, Das A, Wallace Gt, Barry J, Vertegel AA, Ray SK, Banik NL. Spinal cord injury: a review of current therapy, future treatments, and basic science frontiers. Neurochem Res 2013; 38: 895-905.

25. Wang C, Liu C, Gao K, Zhao H, Zhou Z, Shen Z, Guo Y, Li Z, Yao T, Mei X. Metformin preconditioning provide neuroprotection through enhancement of autophagy and suppression of inflammation and apoptosis after spinal cord injury. Biochem Biophys Res Commun 2016; 477: 534-540.

26. Xu J, Huang G, Zhang K, Sun J, Xu T, Li R, Tao H, Xu W. Nrf2 activation in astrocytes contributes to spinal cord ischemic tolerance induced by hyperbaric oxygen preconditioning. J Neurotrauma 2014; 31: 1343-1353.

27. Yang ZL, Rao J, Lin FB, Liang ZY, Xu XJ, Lin YK, Chen XY, Wang CH, Chen CM. The role of exosomes and exosomal noncoding RNAs from different cell sources in spinal cord injury. Front Cell Neurosci 2022; 16: 882306.

28. Zhao H, Shang Q, Pan Z, Bai Y, Li Z, Zhang H, Zhang Q, Guo C, Zhang L, Wang Q. Exosomes from adipose-derived stem cells attenuate adipose inflammation and obesity through polarizing M2 macrophages and beiging in white adipose tissue. Diabetes 2018; 67: 235-247.

29. Zhou Z, Tian X, Mo B, Xu H, Zhang L, Huang L, Yao S, Huang Z, Wang Y, Xie H, Xu L, Zhang H. Adipose mesenchymal stem cell transplantation alleviates spinal cord injury-induced neuroinflammation partly by suppressing the Jagged1/Notch pathway. Stem Cell Res Ther 2020; 11: 212.