

## Original Article

# Mesenchymal stem cell-derived extracellular vesicles promote nerve regeneration after sciatic nerve crush injury in rats

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**Abstract:** Several studies have demonstrated extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) could promote neural regeneration following central nervous system injury. However, the therapeutic effects of MSC-EVs in peripheral nerve regeneration remain poorly understood. This study was aimed to investigate the therapeutic effects of local application of rat bone marrow mesenchymal stem cell (BMSCs) derived EVs in rat sciatic nerve crush injury model. EVs were isolated from the culture supernatants of BMSCs under serum-free conditioned medium, and identified by transmission electron microscopy (TEM) and scanning electron microscope (SEM). Then, the crush-injured segment of rat was treated by BMSC-EVs or PBS, and assessed the nerve regeneration. Results showed that BMSC-EVs size ranged from 40 to 300 nm and had a typical membrane structure under TEM and SEM. BMSC-EVs treatment promote the function recovery by sciatic function Index, improve the histomorphometric repair in nerve regeneration, and increase the expression of GAP-43, a marker for axon regeneration. The present study demonstrated that BMSC-EVs could promote the functional recovery and nerve regeneration of crush-injured sciatic nerves in rat. BMSC-EVs provide a novel cell-free therapeutic approach to peripheral nerve therapy.

**Keywords:** Bone marrow mesenchymal stem cell, extracellular vesicles, sciatic nerve crush injury

## Introduction

Peripheral nerve injury is a serious complication that can result in complete functional loss or permanent impairment. Despite the continuous improvement of techniques in the repair of peripheral nerves, the outcomes after peripheral nerve injury have been disappointing. In order to recover the injured peripheral nerve to the pre-injury level, investigators have done a lot of research such as allografts [1, 2]. While functional outcomes from reconstructive transplantation are limited by the immunological consequences of allotransplantation such as rejection [3], which is a major challenge in the process of nerve regeneration. Recently, cell-based therapy, particularly mesenchymal stem cells (MSCs), has emerged as a promising approach for enhancing nerve regeneration [4]. Intravenous injection of MSCs has been shown to modulate the local environment with the down-regulation of inflammation and the promotion of axonal regeneration in the mouse

model of sciatic crush [5]. In addition, local injection of MSCs has been shown to increase the number of newborn nerve fibers [6], neurovascular [7] and restore the peripheral nerve function of the injury, with detectable of expression of nerve growth related factors in the supernatant of MSCs [8]. Furthermore, after the transplantation of MSCs, the expression level of nerve growth related factors was significantly increased at the point of sciatic nerve injury [7]. The above study provides good evidence supporting the paracrine effect of stem cell-based therapy. In these contexts, MSCs may deliver critical signals to nerve damage point that contribute to functional recovery and then change the cell behavior or transfer certain cytokines to relieve injury [9-11].

Extracellular vesicles (EVs), as a new mechanism of cell-to-cell communication [12], are nano-sized circular membrane fragments that released by various cell types, including stem cells [13] and progenitors [14] and serve as

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shuttles for the selective pattern of ligands, receptors, enzymes, cytokines, transcription factors, mRNA and microRNA into target cells [15]. The therapeutic effects of EVs have been actively investigated in animal models of various diseases, such as kidney damage, myocardial injury and liver damage [16]. Recently, Intravenous administration of MSC-EVs has been shown to improve functional recovery and enhance neurite remodeling, neurogenesis, and angiogenesis in an ischemic stroke model [17]. However, the role of MSC-EVs on sciatic nerve crush injury has yet to be examined.

In this study, we constructed the sciatic nerve crush injury model in rat, and assessed the therapy effect of EVs from rat bone marrow MSCs (BMSC). Data show that rat BMSC-EVs can protect rat from severe sciatic nerve injury. MSC-derived EVs has great value in implications for a potentially new therapy for the treatment of peripheral nerve injury.

## Materials and methods

### *Cell cultures*

BMSCs were isolated and cultured according to previously described protocol [18]. The cells from 80-100 g Sprague Dawley (SD) rats were collected by flushing the femurs and tibiae with phosphate-buffered saline (PBS), and then the cells were cultured in 25 cm<sup>2</sup> flasks (EuroClone) in low glucose Dulbecco's modified Eagle's medium (L-DMEM; GIBCO-BRL, Carlsbad, CA, USA), with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C in 5% CO<sub>2</sub> atmosphere. The experimental procedure was approved by the Institutional Animal Care Committee of Jiangsu University. After 4 days, nonadherent cells were removed, and the medium was changed every three days. When 70%-80% adherent cells were confluent, they were trypsinized with 0.25% trypsin-EDTA (Invitrogen), harvested, and expanded in larger flasks. A homogenous cell population was usually obtained at passage 3-5 for the experiments.

BMSCs were characterized by the expression of CD105 (endoglin), CD73, and lack of hematopoietic (CD34) and B lymphocyte (CD19) markers was assessed as previously described [19]. Surface markers expressed by BMSCs were detected by FCM using PE anti-CD19, PE anti-

CD34, PE anti-CD73, FITC anti-CD105 mAb (Becton-Dickinson, San Jose, CA, USA). Staining was performed according to the manufacturer's protocol, and at least 10,000 events were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA) using Cell Quest software.

### *Isolation of EVs*

EVs were obtained from the conditioned culture medium of BMSCs, according to a previously described protocol with slight modifications [20]. Briefly, BMSCs were cultured in without serum medium for 48 h, collected the culture supernatant, then centrifuged at 2,000 g for 20 minutes to remove debris. The cell-free supernatants were centrifuged at 100,000 g for 1 h at 4°C (Beckman Coulter Optima L-90K ultracentrifuge). The resultant pellet was washed in PBS, and then was centrifuged again at 100,000 g for 1 hour to pellet the EVs. The pellets were resuspended in 100 µL of PBS and aliquots were stored at -80°C until further use. The protein concentration of the EVs preparations was quantified by the BCA method (Beyotime Biotechnology, China).

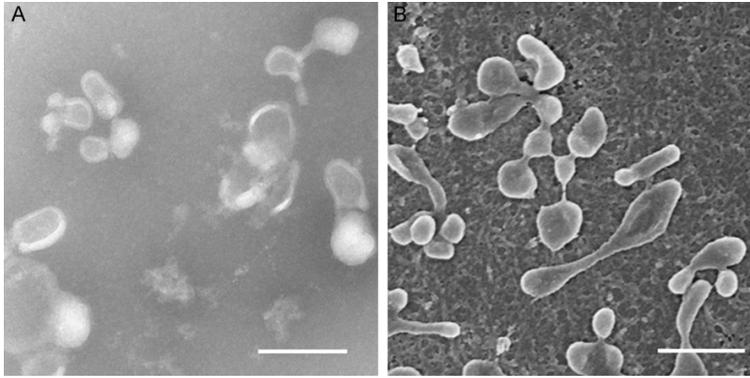
### *Electron microscopy*

For TEM analysis, purified EVs were resuspended in PBS and loaded on a formvar-coated copper grid. Excess fluid was removed with filter paper, and the samples were stained with 1% uranyl acetate for 30 s and then washed, dried and stained with 2% lead citrate for another 30 s. The grids were examined under a transmission electron microscope (Philips, Tecnai12, Netherland) at 80 kV, and electromicrographs were taken and subjected to analyses. For SEM analysis, EVs were fixed in Karnovsky fixative, dehydrated in alcohol, dried on glass surface and then coated with gold by sputter coating. The samples were observed on a scanning electron microscope (Hitachi, S4800-II, Japan). Images were allowed via secondary electron at a working distance of 15 to 25 mm and an accelerating voltage of 20 to 25 kV.

### *Experimental animals and surgical procedures*

Adult male SD rats (200-230 g, n=12) were purchased and maintained in the animal experimental center of Jiangsu University in China. They were kept in individual cages and allowed

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**Figure 1.** Rat BMSC-EVs characterized by transmission electron microscopy and Scanning electron microscope. A. EVs viewed under transmission electron microscope. Scale bar: 0.2  $\mu\text{m}$ . B. EVs viewed under Scanning electron microscope. Scale bar: 0.2  $\mu\text{m}$ .

free access to tap water and standard rat chow in a temperature-controlled room (22-24°C) and relative humidity of 55-70% with 12 h light/dark cycle. This work was carried out in accordance with the ethics Committee of Jiangsu University for the use of laboratory animals (Permit Number: JSU 14-212).

For sciatic nerve crush, the rats were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (10 mg/kg) during all surgical procedures. The right hindquarter was shaved, and then a 10 mm incision was made on the posterior and parallel to the femur. Between biceps femoris and vastus lateralis were bluntly dissected to expose the sciatic nerve. The crush injury was performed 10 mm proximal to its trifurcation using a 3 mm-wide hemostat for 30 s [21]. After creation of the 3 mm-wide crush injury, EVs group rats were immediately injected at a concentration of 45  $\mu\text{g}/30 \mu\text{L}$  of PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  into the lesion using a microinjector (Shanghai Medical Laser Instrument, China), while the control rats received 30  $\mu\text{L}$  of PBS. At the end of the experiment, the animals (6 animals per group per time point) were sacrificed by anesthesia as mentioned before at the fourth week after surgery.

### Functional assessment

Functional evaluation of sciatic nerve was expressed by the sciatic function index (SFI) at 1, 2, 3 and 4 weeks after surgery as previously described [22]. Briefly, the rats hind paws were dipped with ink, and then the rats were allowed to walk through a tunnel so that the footprints could be displayed on a strip of white paper

loaded onto the bottom of the tunnel. The footprints of normal (N) and experimental (E) sides were collected and measured. At least four footprints per animal were taken from each side. Footprints were measured for the following parameters: between the third toe ant heels (PL), first and fifth toe (TS), and second and fourth toe (ITS), and the contra lateral normal side (NPL, NTS, and NITS, respectively). The SFI was calculated according to the following mathematical formula [22]:

$$\text{SFI} = -38.3 \times (\text{EPL-NPL})/\text{NPL} + 109.5 \times (\text{ETS-NTS})/\text{NTS} + 13.3 \times (\text{EITS-NITS})/\text{NITS} - 8.8.$$

Calculated indices from this formula ranged between a score from 0 to 100. Around 0 corresponds to normal function, and -100 corresponds to a total dysfunction.

### Morphological assessment

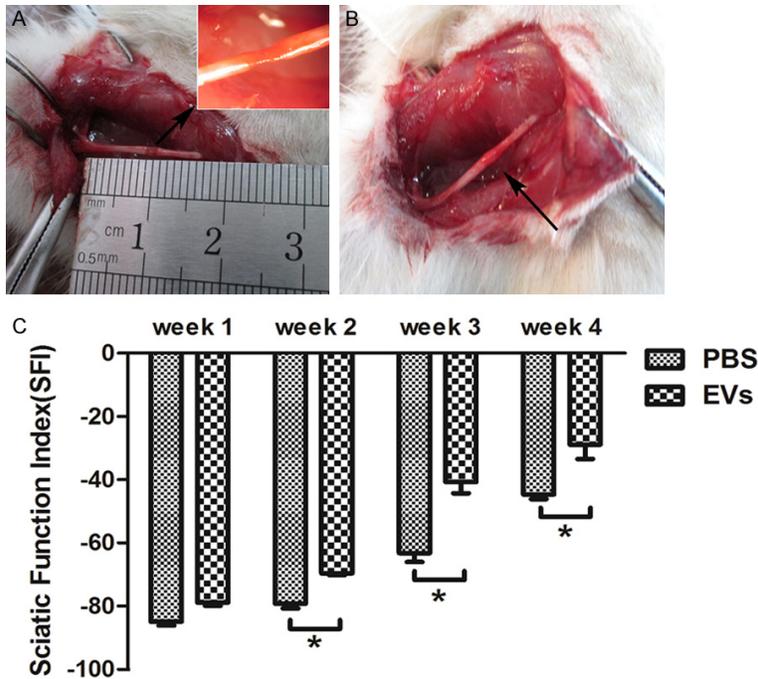
Nerve tissue were harvested and immersed in 4% neutral-buffered formaldehyde at 4°C for 48 hours. The tissue were embedded in paraplast paraffin under light microscopy, and then divided into sections (3 mm thick) and stained with H&E for general histological analysis. Pictures were capture by Nikon Ti-S microscope (Ti-S, Nikon, Japan).

### Immunohistochemical analysis

GAP-43 was used as marker for axon regeneration. Nerve tissue (3 mm thick) were deparaffinized and rehydrated. Briefly, after non-specific immunoreactions were blocked with 1% BSA for 30 min, sections were incubated primary antibody solution (1:200, immunoWay, USA) for 1 h at room temperature, followed by incubation with HRP-conjugated secondary antibodies. Pictures were capture by Nikon Ti-S microscope (Ti-S, Nikon, Japan).

### Statistical analysis

Statistical analysis was performed using the Student's *t*-test with the GraphPad Prism V 5.0 software program (GraphPad, San Diego, CA, USA). *P* value <0.05 was considered statistically significant.



**Figure 2.** Rat BMSC-EVs treatment of sciatic nerve lesion by crush induction. A. Sciatic nerve lesion induction (blank arrow: injure point). B. Rat BMSC-EVs injection followed by the damage point (blank arrow: injection point). C. Rat BMSC-EVs improved the sciatic nerve function index (SFI). SFI for each group at each time point is shown. Bars represent the mean  $\pm$  SEM (n=6 per group) of 12 mice from 2 independent experiments. Significance analyzed by an independent Student's test. \* $P < 0.05$ .

## Results

### Characterization of EVs

To confirmed that the isolated pellets were genuine EVs, the harvested pellet was captured by transmission electron microscope (TEM) and Scanning electron microscope (SEM). By TEM, EVs displayed to be mainly round and oval in shape, ranged in size from 40-300 nm, aggregated in clusters (**Figure 1A**). As shown in **Figure 1B**, in situ EVs revealed the unique membrane structure characteristics by SEM, and their morphology and dimensions were similar to **Figure 1A**.

### Rat sciatic nerve injury and nerve regeneration assessment

To examine the effects of MSC-EVs in rat nerve injury repair, we constructed a rat sciatic nerve injury model by surgery. As shown in **Figure 2A** and **2B**, sciatic nerve 3 mm crush injury point and EVs injection point. SFI values are showed in **Figure 3**. The SFI score in all groups is near

zero before surgery. After sciatic nerve crush, the mean SFI decreased to -100 as a result of the complete loss of sciatic nerve function in all rats. In one week after the injury, no significant differences were observed between EVs group and control group ( $P > 0.05$ ). From the second week to the fourth week, SFI improved significantly in the EVs groups compared with that of the control group ( $P < 0.05$ ) (**Figure 2C**). These results indicate that MSC-EVs can protect rat from severe sciatic nerve injury.

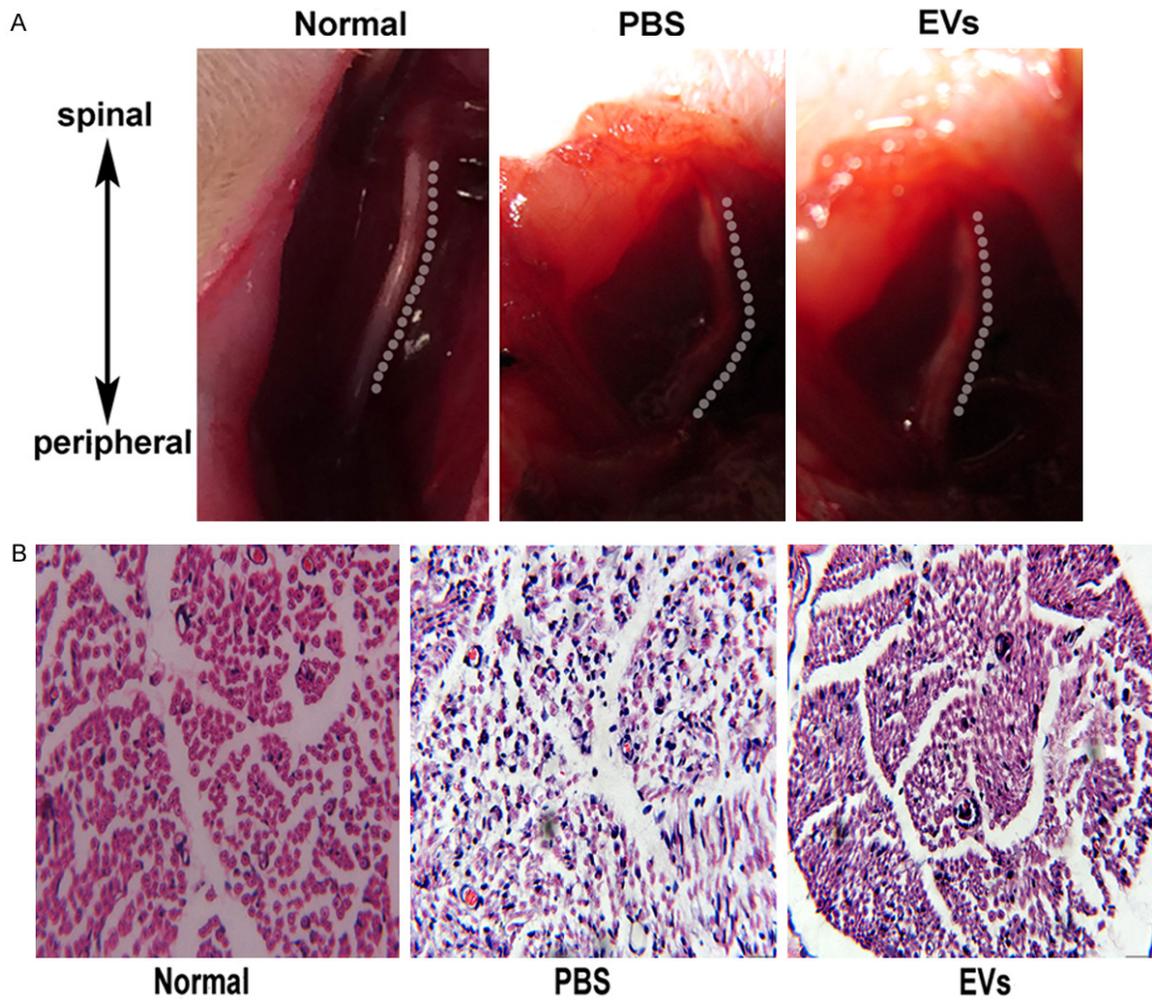
### Morphologic analysis of nerve regeneration

To evaluate the effect of EVs, neurological morphology after sciatic nerve crush in fourth week was observed. As shown in **Figure 3A**, the anatomical morphology of the injured nerves treated with EVs is closely resembled to normal rat,

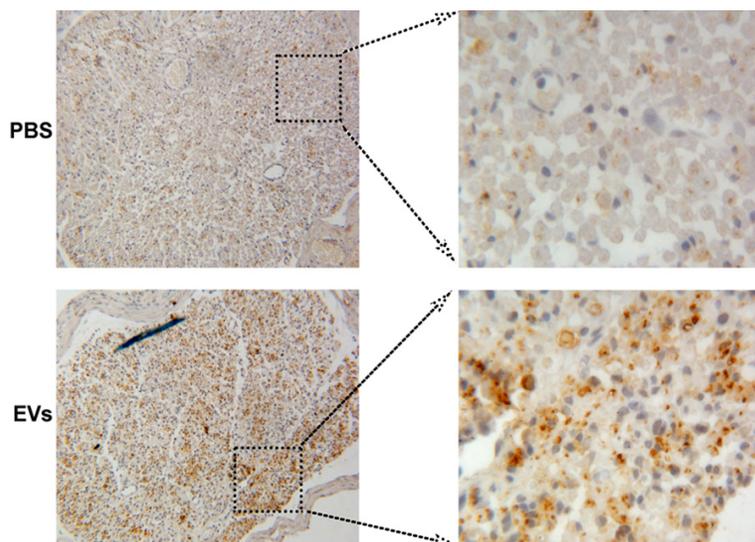
while PBS-treated rat still remain injury morphology. By H&E staining, injection of EVs regenerated nerve myelin, which showed incomplete shape and thickness, but was very similar to normal nerves myelin. However, the regenerated nerves myelin from PBS-injected rat was irregular and thin (**Figure 3B**). The results suggest that MSC-EVs can really repair sciatic nerve injury.

### Immunohistochemical analysis

GAP-43 is the major protein kinase C component of growth cones and developing nerve terminals, which is considered a good marker for evaluating the extent of axonal regeneration [23]. Immunostaining for the GAP-43 protein was measured in the cross-sections of crushed sciatic nerves from rats treated with EVs or PBS at fourth week after the surgery. As shown in **Figure 4**, EVs treatment increased the brown of GAP-43 expression compared with PBS-treated rat. The results indicate that MSC-EVs may promote axonal regeneration, which was involved in nerve injury repair.



**Figure 3.** At week 4 after surgery, the rats were killed and their injured nerves were analyzed for histology. A. The anatomical morphology of the injured nerves for each group is shown. B. The histology (hematoxylin-eosin staining) for each group is shown (magnification, 400 × ).



**Figure 4.** Rat BMSC-EVs increased GAP-43 expression. GAP-43 expression of the injured nerves for each group is shown at week 4 after surgery. The brown particles used as positive staining of the GAP-43 (magnification, 400 × ).

### Discussion

After peripheral nerve injury, the peripheral nervous system is endowed with an intrinsic regeneration potential, which can lead to an almost complete recovery. However, nerve regeneration often fails in

cases of proximal nerve injury, diffuse axonal loss, or chronic insults [21]. Clinically, surgical repair is usually the primary treatment for peripheral nerve injury, but patients can't restore normal motor control and fine sensory function [24]. Mesenchymal stromal cells represent a promising therapeutic approach for organ repair, and their biological characteristics have been recognized, such as repair, immunological, transmission, secretion properties and so on [25]. Several studies demonstrated that the administration of bone marrow-derived MSCs in peripheral nerve injury could improve nerve regeneration, with axonal regrowth and myelin formation [26-30]. At present, no strong evidence basis is displayed that transplanted MSCs truly trans-differentiate into cell populations specific to the injured organ. Therefore, the paracrine mechanism has been proposed to be one of the underlying mechanisms of transplanted stem cells with therapeutic effect [31].

EVs are small vesicles released by diverse cell types, including platelets, dendritic cells, endothelial cells, and MSCs. EVs exert their effects on fundamental biological processes in a pleiotropic manner, such as directly activating cell surface receptors via protein and bioactive lipid ligands, merging their membrane contents into the recipient cell plasma membrane, delivering proteins, mRNA, miRNA, bioactive lipids and even whole organelles into target cells [15, 32-35]. A recent study showed that the administration of BMSC-EVs can promote functional recovery and neurovascular plasticity in a stroke model [17]. Later, the research group further confirmed that the protective mechanism of BMSC-EVs was involved in transferring microRNA-133b to astrocytes and neurons [36]. Additionally, BMSC-EVs have been shown to protect optic nerve against injury through promoting survival of retinal ganglion cells (RGC) and regeneration of their axons, while partially preventing RGC axonal loss and RGC dysfunction [37].

In this study, rat BMSC-EVs have been successfully extracted and displayed a spherical shape of the membrane structure under TEM and SEM, their size and shape are consistent with the previous publication [38]. According to recent advances for extracellular vesicles (EVs), the terms 'exosome' and 'microvesicle' have been used interchangeably in many published

studies. Exosomes and microvesicle are two major types of extracellular vesicles that can be differentiated by their size and content [39]. However, there are studies found that microvesicle exhibit CD63, a typical marker of exosomes, as determined by NTA [40]. Because of an as yet incomplete understanding of extracellular vesicle biogenesis, inconsistencies in extracellular vesicle purification protocols and a lack of thorough vesicle characterization, therefore, we use the term 'extracellular vesicle' to refer to both of these vesicle types [41]. In this study, we found that rat BMSC-EVs promote the function recovery of sciatic nerve damage and myelination. Rat BMSC-EVs treatment significantly improved motor performance, repaired the anatomical morphology of the injured nerves, and promoted the myelin and axon regeneration by H&E staining and GAP-43 expression. These data are supported with a previous study showing that the local implantation of BMSCs can promote nerve regeneration and the recovery of nerve function in sciatic nerve crush injury model [42]. Consistent with our data, Raisi *et al.* [43] demonstrated that omental adipose MSC-EVs drastically improved the process of myelination and the structural recovery of regenerated nerve fibers in a rat sciatic nerve transection model.

### Conclusions

The results of the present study have demonstrated that BMSC-EVs could restore nerve function and promote nerve regeneration in sciatic nerve crush injury model. However, the detailed repair mechanism of BMSC-EVs needs further research.

### Acknowledgements

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## Disclosure of conflict of interest

None.

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