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Repair and regeneration of lumbosacral nerve defects in rats with chitosan conduits containing bone marrow mesenchymal stem cells

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ABSTRACT

Objectives: Despite the great progress in surgical treatment of lumbosacral nerve injuries caused by high-energy trauma, functional recovery remains poor and insufficient. Bone marrow mesenchymal stem cells (BMSCs), which express neurotrophic factors and can also differentiate into nerve cells, have potential as an effective alternative therapy for lumbosacral nerve defects. The aim of the present study was to evaluate the functional recovery, nerve regeneration, motor neuron survival and apoptosis after lumbosacral nerve transection in rats receiving BMSC transplantation into the chitosan conduit.

Methods: The right L4–L6 nerve roots of rats were transected and bridged with three 1-cm-long chitosan conduits, which were further injected with the BMSCs (MSC-treated group) or culture medium (DMEM group). The nerve regeneration and motor function recovery were assessed by the sciatic functional index (SFI) and analysed electrophysiologically and morphologically.

Results: At 6 and 12 weeks after surgery, the SFI values in MSC-treated group were significantly higher than those in DMEM group ($P \leq 0.05$). The peak amplitude of CMAP (compound muscle action potential) and nerve conduction velocity in MSC-treated group were significantly higher than that in DMEM group ($P \leq 0.01$), while the latency of CMAP onset in MSC-treated group was significantly shorter than that in DMEM group ($P \leq 0.01$). The diameter of the myelinated fibres and thickness of the myelin sheath in MSC-treated group were significantly higher than those in DMEM group ($P \leq 0.05$). There was no difference in the number of motor neurons in the anterior horn of the spinal cord at 6 weeks post-operation ($P > 0.05$), while the number of motor neurons was significantly greater in MSC-treated group than that in DMEM group at 12 weeks post-operation ($P \leq 0.001$). The number of apoptotic cells was also significantly lower ($P \leq 0.01$).

Conclusions: The results of the present study showed that BMSCs treatment improved lumbosacral nerve regeneration and motor function. In addition, our data suggested that BMSCs inhibited motor neuron apoptosis, and improved motor neuron function and survival in the anterior horn of the spinal cord.

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Introduction

Lumbosacral nerve injury is rarely reported in the literature because of its low incidence and difficulty in reaching a precise diagnosis. However, for these reasons, the incidence of this peculiar trauma is probably much higher than believed, and surgical treatment should be taken into consideration more often [1]. Patients who have suffered lumbosacral nerve injuries may experience a very severe problem with deambulation because of the decreased strength of the lower extremities. Lumbosacral nerve injuries often pose a great challenge to surgeons due to the lack of anatomical, experimental and clinical Ref. [2]. Although some surgeons have attempted to develop surgical concepts such as direct anastomosis and contralateral nerve root transfer for the treatment of lumbosacral nerve injuries in patients and experimental animals in recent years, the functional recovery remains insufficient and unsatisfactory [3–6].

Autologous nerve grafting is the traditional gold standard for surgical repair of substantial peripheral nerve defects, knowing that it can act as an inert scaffold to provide necessary matrixes, neurotrophic growth factors and viable Schwann cells for axonal

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regeneration [7]. However, this technique has formidable disadvantages such as limited availability of the graft material, morbidity of the donor site, loss of sensation, neuroma formation and scarring [8], which urges researchers to develop new strategies to optimise the regenerative process [9].

To address these issues, tissue engineered nerve grafts have been developed as a promising alternative to autologous nerve grafts. A tissue engineered nerve graft is typically composed of a neural scaffold and a variety of biochemical cues [10]. Among a number of bioartificial scaffolds, chitosan, a natural biodegradable polysaccharide, has shown excellent neural biocompatibility with neural cells [11], as well as biodegradability and bioactive properties such as anti-microbial activity and scar reduction [12]. It has been known as a material for the preparation of nerve repair conduits [13]. Incorporation of neural cells into the tissue engineered nerve graft imitates the environment of native peripheral nerves and provides neurotrophic support for axonal regeneration [14]. We hypothesised whether chitosan conduits filled with bone marrow mesenchymal stem cells (BMSCs) that express neurotrophic factors and can also differentiate into nerve cells [15,16], has potential as an effective alternative therapy for lumbosacral nerve defects. The aim of the present study is to evaluate nerve regeneration and neuronal survival when transected lumbosacral nerves are treated with the chitosan conduit transplanted with BMSCs.

Materials and methods

Preparation of the chitosan conduit

Chitosan solution (2 wt.%, mean *MW* 800 kDa) was prepared, kept at room temperature for 24 h, vacuum filtered to remove impurities using filter paper, and allowed to freeze within the mould at $-80\,^\circ$C when it reached the top of the mould. The obtained sample was placed inside a high-vacuum pump to fully lose the humidity. Then, the conduit was neutralised with 1% (w/v) sodium hydroxide solution and washed with distilled water to remove the solvent and remaining dried alkali. Finally, the chitosan conduit with a wall thickness of 0.4 mm, an inner diameter of 2 mm and a length of 10 mm was produced and used for nerve-bridging.

BMSCs isolation, culture, and characterisation

For isolation of BMSCs, the tibia and femur were dissected from adult SD rat weighing 200–250 g (Department of Experimental Animal Centre of Fudan University, Shanghai, China). The experimental protocol was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Administration Committee of Experimental Animals of the Fudan university. All surgical procedures were performed under pentobarbital anaesthesia, and all efforts were made to minimise suffering of the animals. After the ends of the bones were cut, the marrow was rinsed and resuspended in DMEM solution, supplemented with 15% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin About 1×10^7 marrow cells were seeded to a 25 cm² plastic flask in L-DMEM and incubated at 37 °C with 5% humidified CO_2 for 48 h. Nonadherent cells were removed by replacing the medium. The medium was replaced every 3 days until cells grew to confluence. Confluent cells were harvested with 0.25% trypsin and 1 mmol/L EDTA for 5 minutes at 37 °C, replated on a 25 cm² plastic flask and cultured again.

Animals and surgical procedures

A total of 24 male SD rats weighing 200 to 220 g were equally randomised to two groups: those with BMSC grafts (*n* = 12 MSC-treated group) and those with acellular grafts (culture medium Dulbecco's Modified Eagle Medium, DMEM group, *n* = 12). The animals were anaesthetised by intraperitoneal injection of 1% 40 mg/kg pentobarbital. An incision was along the lumbar posterior midline of the L4-L6. Under a dissecting microscope, the right erector spine muscle was dissected along the spinous process to expose the upper and lower articular process and transverse process of L4-L6. Then the articular process and transverse process were bit with a needle holder to expose the right L4–L6 nerve roots (Fig. 1A). A segment of L4–L6 sacral nerve roots was excised respectively, leaving three 1-cm-long defects. The nerve defect was bridged with the chitosan conduit (Fig. 1B) and further injected with the BMSC suspension containing 10^7 cells/conduit (for the MSC-treated group) or with culture medium (for DMEM group). Both the proximal and distal stumps of the transected nerve roots were sutured to the conduit with two stitches each using 10/0 nylon suture. The incision was sutured and disinfected regularly after the operation until it healed. After a period of 6 and 12 weeks post surgery, the surviving rats were examined.

Behavioural analysis

Motor function was evaluated using the sciatic functional index (SFI) proposed by Medinaceli et al. [17] and modified by Hare et al. [18]. Walking track analysis was performed on rats (*n* = 6 in each group) at 6 and 12 weeks after surgery. The rat paw prints were registered with respect to the print length (PL), the distance from the heel to the third toe; the toe spread (TS), the distance from the first to fifth toe; and the intermediary toe spread (ITS), the distance between the second and the fourth toe. All measurements were taken from both the experimental sides (E) and the non-operated side (N). The SFI was calculated according to the equation:

$$SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS} + 13.3 \times \frac{EITS - NITS}{NITS} - 8.8$$

Haematoxylin-eosin (HE) and Terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) staining.

After transcardial perfusion, the L4–L6 spinal cord was fixed in 10% formaldehyde, paraffin embedded and cut into transverse sections. Some sections were subjected to HE staining after removal of the paraffin for quantification of neurons, and others were stored in 4% paraformaldehyde and stained by TUNEL using a commercial kit (Roche, USA). Proteins were digested in 100 μL 20 μg/mL proteinase K solution at room temperature for 15 min, washed twice in PBS, and then in 100 μL balance buffer and sealed for 10 min. The slide was opened and 50 μL terminal deoxynucleotidyl transferase enzyme reaction mixture was used to re-seal the slide which was incubated at 37 °C for 1 h and stopped by stop solution for 10 min. For HE and TUNEL staining, six slides were read in each group. The slides were observed under the 200× magnification view to count the number of motor neurons and apoptotic cells in the ventral horn of the spinal cord. For each slide, the number was measured by photographs taken from three random fields and analysed with FW4000 image analysis system (Leica, Germany).

Morphometric analysis

The distal segment of the regenerated nerve roots was harvested, immersed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.3) for 1 h at room temperature, dehydrated and resin embedded according to the standard protocol. Ultrathin (50 nm) sections were obtained with an ultramicrotome (Leica, Germany), stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEM-1200EX,
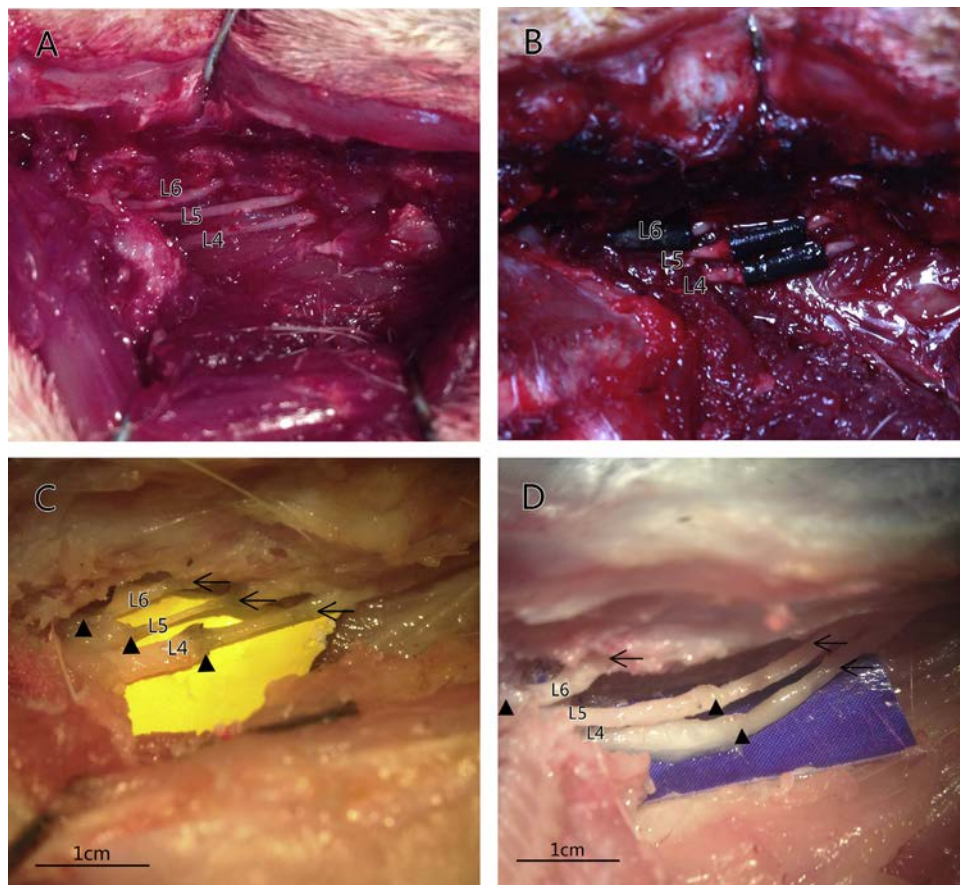
Fig. 1. *Gross views of the sacral nerve roots.* (A) The right L4–L6 sacral nerve roots were exposed. (B) A segment of L4–L6 sacral nerve roots was excised and the nerve defect was bridged by a chitosan conduit. (C) The regenerated nerve-like tissue at 12 weeks after surgery in DMEM group, and (D) MSC-treated group. Scale bar, 1 cm. The arrowhead and arrow mark the distal and proximal coaptation, respectively.

JOEL, Japan). Photographs of the ultrathin sections were digitalised into FW4000 image analysis system (Leica, Germany). The thickness of myelin sheaths and the diameter of myelinated nerve fibres were calculated.

Electrophysiological assessment

Electrophysiological analysis was performed on rats 6 weeks and 12 weeks after surgery. After anaesthesia as mentioned above, the right sciatic nerve was exposed. A bipolar stimulating electrode was attached to the middle of the sciatic nerve. The compound muscle action potential (CMAP) was recorded on the belly of the gastrocnemius muscle on the ipsilateral side. The peak amplitude of CMAP and the latency of CMAP onset were obtained. The nerve conduction velocity was calculated from the CMAP amplitude and the distance between the stimulated and the recorded sites.

Statistical analysis

All data were expressed as the mean ± SD. The data were analysed using a Student's t-test for comparisons between two groups with the Predictive Analytics Software (PASW) 18.0 (SPSS Inc., Chicago, USA). A P-value of 0.05 was considered statistically significant.

Results

#### Gross observation after nerve grafting

No animal developed sacral nerve injury-related complications such as self mutilation and ulcers in hind limbs. At 6 and 12 weeks after surgery, the grafts were completely replaced by nerve-like tissues. No complications such as rejection, local inflammation, swelling, abscess or incision infection were observed except a certain degree of adhesion of the conduit with the surrounding tissue, indicating that the conduit possesses good biocompatibility (Fig. 1C and D).

BMSC treatment improves functional recovery

The motor function recovery was assessed by the walking track analysis. The SFI value varied from −100 to 0, where −100 indicates complete loss of function and 0 represents normal function. The SFI values in the MSC-treated group (−75.67 ± 6.68 at 6 weeks, −62.83 ± 7.96 at 12 weeks) were significantly higher than those in the DMEM group (Fig. 2; P = 0.015, −85.5 ± 4.8 at 6 weeks; P = 0.001, −81.67 ± 5.12 at 12 weeks).

CMAP was recorded from the belly of the gastrocnemius muscle upon stimulation of the sciatic nerve. Function recovery in MSC-treated group rats was better than that in DMEM group rats (Fig. 3). The peak amplitude of CMAP in MSC-treated group (33.90 ± 2.76 mV at 6 weeks, 50.63 ± 1.91 mV at 12 weeks) was significantly higher than that in DMEM group (Fig. 3B; P = 0.005, 29.32 ± 1.55 mV at 6 weeks; P = 0.0000002, 38.23 ± 1.57 mV at 12 weeks). The latency of CMAP onset in MSC-treated group (0.74 ± 0.12 ms at 6 weeks, 0.55 ± 0.07 ms at 12 weeks) was significantly shorter than that in DMEM group (Fig. 3C; P = 0.014, 0.90 ± 0.06 ms at 6 weeks; P = 0.005, 0.72 ± 0.10 ms at 12 weeks). The nerve conduction velocity in MSC-treated group (16.07 ± 1.68 m/s at 6 weeks, 21.9 ± 1.17 m/s at 12 weeks) was significantly higher than that in DMEM group (Fig. 3D; P = 0.00008, 10.83 ± 1.12 m/s at 6 weeks; P = 0.002, 19.12 ± 1.23 m/s at 12 weeks).
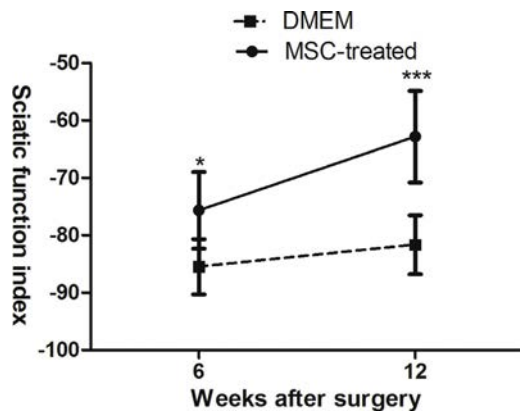
Fig. 2. *The SFI of rats in DMEM and MSC-treated groups at 6 weeks and 12 weeks after surgery.* The motor function in MSC-treated group rats was significantly better than that in DMEM group rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

BMSCs treatment enhances the thickness of myelin sheaths and diameter of myelinated fibres

Transmission electron microscopy of regenerated nerves showed that the thickness of myelin sheath in MSC-treated group was (0.29 ± 0.13 μm at 6 weeks, 0.59 ± 0.13 μm at 12 weeks) significantly higher than that in DMEM group (Fig. 4E; $P = 0.007$, 0.20 ± 0.08 μm at 6 weeks; $P = 0.00000005$, 0.31 ± 0.13 μm at 12 weeks). The diameter of myelinated fibres in MSC-treated group (2.73 ± 0.10 μm at 6 weeks, 3.90 ± 0.94 μm at 12 weeks) was

significantly higher than that in DMEM group (Fig. 4F; $P = 0.04$, 2.19 ± 0.67 μm at 6 weeks; $P = 0.007$, 2.96 ± 1.24 μm at 12 weeks). These results showed that the rats receiving the chitosan conduit containing BMSCs achieved better levels of nerve regeneration than the rats receiving the chitosan conduit without BMSCs, indicating that the chitosan conduit containing BMSCs is capable of promoting remyelination during the process of nerve regeneration.

BMSCs treatment improves motor neurons survival and inhibits motor neuron apoptosis

The number of motor neurons in the anterior horn of the experimental side in MSC-treated group was similar to that in the DMEM group ($P = 0.38$) at 6 weeks post-operation. However, 12 weeks after surgery, the number of motor neurons in MSC-treated group (9.11 ± 1.64) was significantly higher than that in DMEM group (Fig. 5E; $P = 0.0002$, 6.67 ± 1.89).

For apoptosis detection by TUNEL, the brown spots indicate apoptotic cells. By counting the number of brown spots in different periods, we could see that the number of apoptotic cells in the anterior horn of the experimental side in DMEM group (34.33 ± 6.88 at 6 weeks, 40.39 ± 5.99 at 12 weeks) was significantly greater than that in MSC-treated group (Fig. 6E; $P = 0.00000001$, 10.44 ± 5.24 at 6 weeks; $P = 0.00000002$, 13.33 ± 5.84 at 12 weeks).

Discussion

As it is impossible to use autologous grafting in the treatment of patients with multiple lumbosacral nerve lesions because of limited nerve sources, various techniques such as tissue
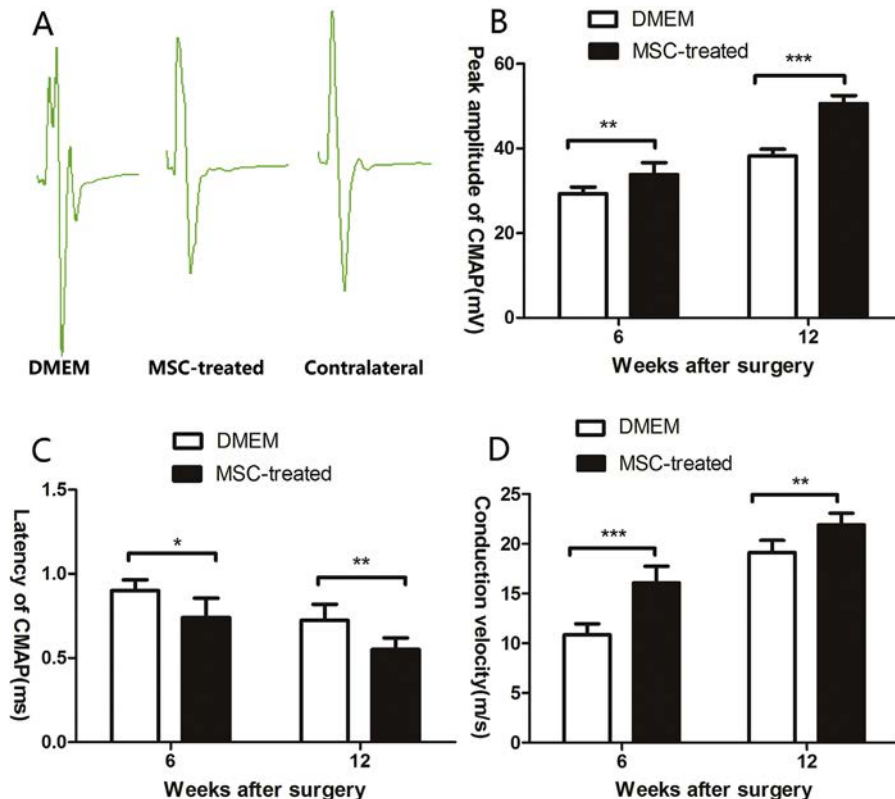
Fig. 3. *Electrophysiological study was performed at 6 and 12 weeks after surgery.* (A) Representative recordings on the operated side in DMEM groups, MSC-treated groups or on the contralateral, uninjured side 12 weeks after operation. Histograms respectively showing the amplitude of CMAP (B), latency of CMAP (C) and nerve conduction velocity (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
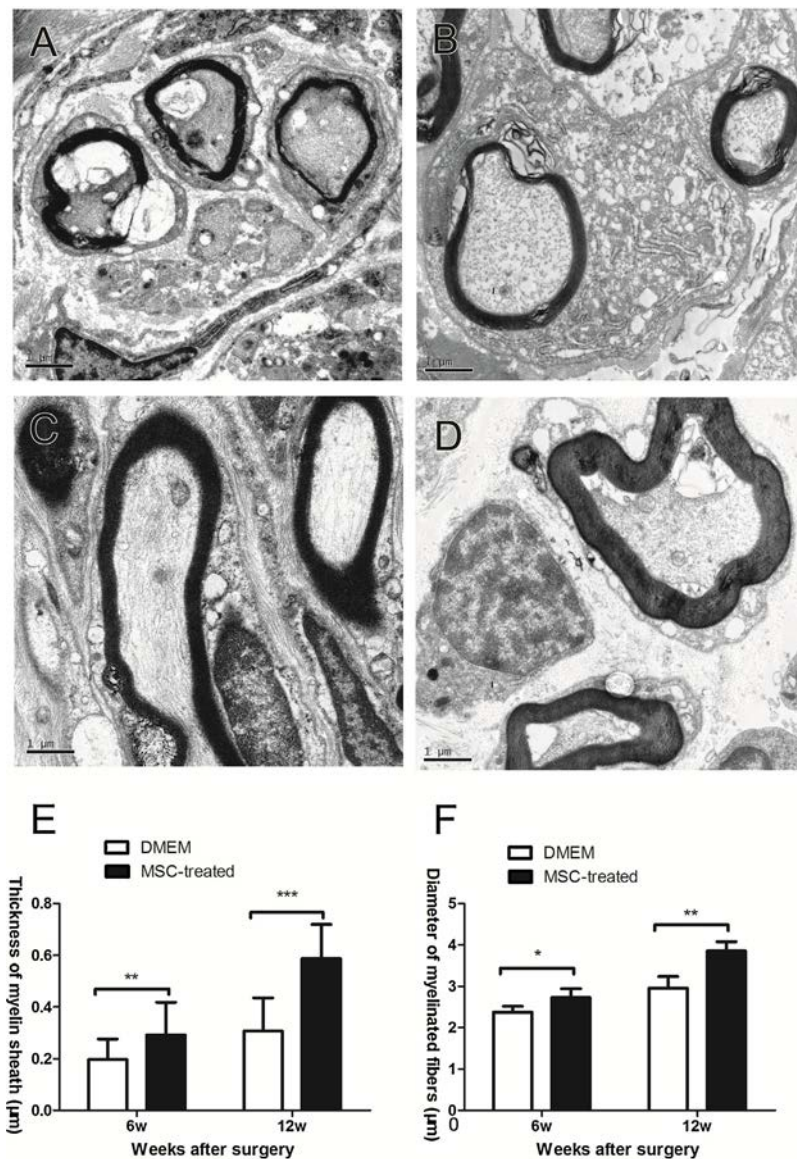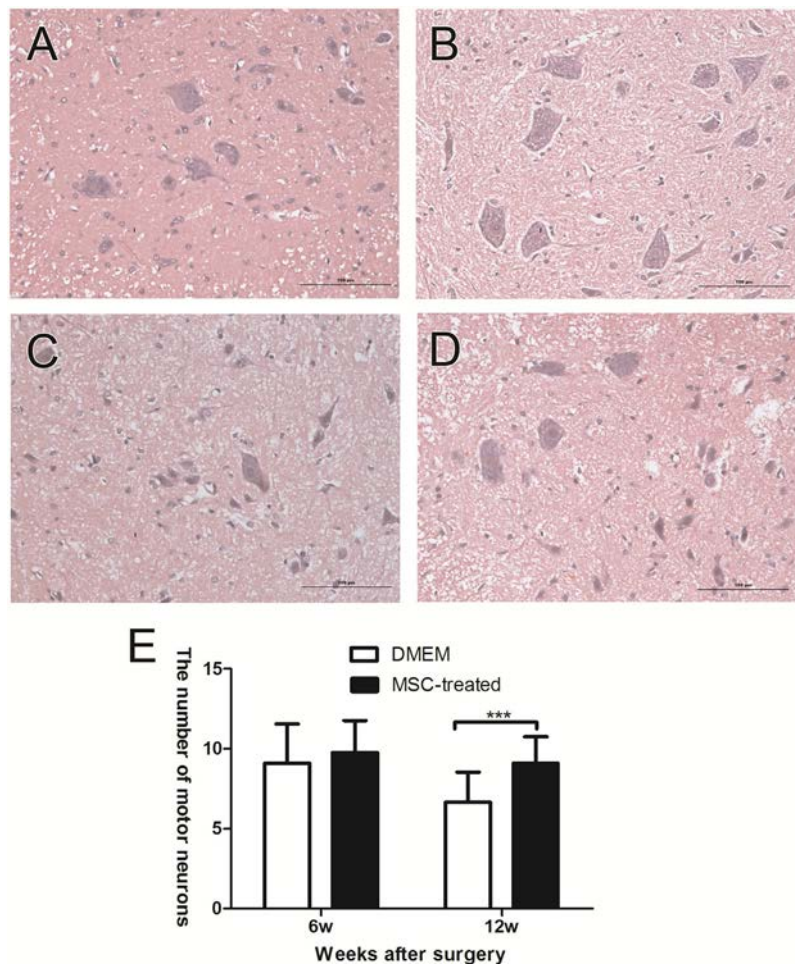
Fig. 4. *Transmission electron micrographs of transverse sections taken from the regenerated L4 nerve.* The sections were obtained from DMEM (A and C) and MSC-treated (B and D) groups, at 6 weeks (A and B) and 12 weeks (C and D) post surgery respectively. Scale bar,1 μm. Histograms comparing the thickness of regenerated myelin sheath (E) and diameter of myelinated fibres (F) respectively, in four different groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

engineered nerve grafts have been suggested to complement the gold standard treatment. In the present study, we investigated the outcome of repairing 1-cm-long lumbosacral nerve defects in rats with chitosan conduit containing BMSCs. On one hand, the chitosan conduit provided the microenvironment for nerve regeneration, devoid of invasion of the surrounding tissue into nerve defects. On the other hand, the use of the conduit could avoid the drawbacks of autologous nerve grafting such as neuroma formation and scarring on the donor side. The micro environment provided by chitosan conduit can help mesenchymal stem cells to promote nerve regeneration. A number of published studies on tissue engineered nerve grafts have attempted to improve the regeneration and remyelination process [19,20].

With increased incidences of high-energy trauma in recent years, lumbosacral plexus injuries caused by pelvic and sacral fractures are on the rise. As the functional outcome after surgical treatment of lumbosacral nerve defects and lumbosacral plexus avulsion remains poor, conservative treatment has been advocated

[21]. The peripheral nerve injury model in our study is similar to human lumbosacral nerve in terms of anatomy and function. As a result, our study may provide a potential strategy for the treatment of lumbosacral nerve injuries.

The advantages of BMSCs for nerve regeneration including multipotency, proliferation ability and release of neurotrophic factors have been reported in recent studies [22–25]. Some evidence has shown that Schwann cells induced from BMSCs could promote peripheral nerve regeneration [26,27], while other studies suggest that BMSCs are capable of differentiating into Schwann-like cells and secreting neurotrophic factors in vivo [28,29], thus providing therapeutic benefits for peripheral nerve repair. Hong et al. [30] reported that implantation of untransdifferentiated human umbilical mesenchymal stem cells could markedly improve the cognitive function and tissue morphology in the rat model of traumatic brain injury, as compared with implantation of transdifferentiated human umbilical mesenchymal stem cells. These findings indicate that
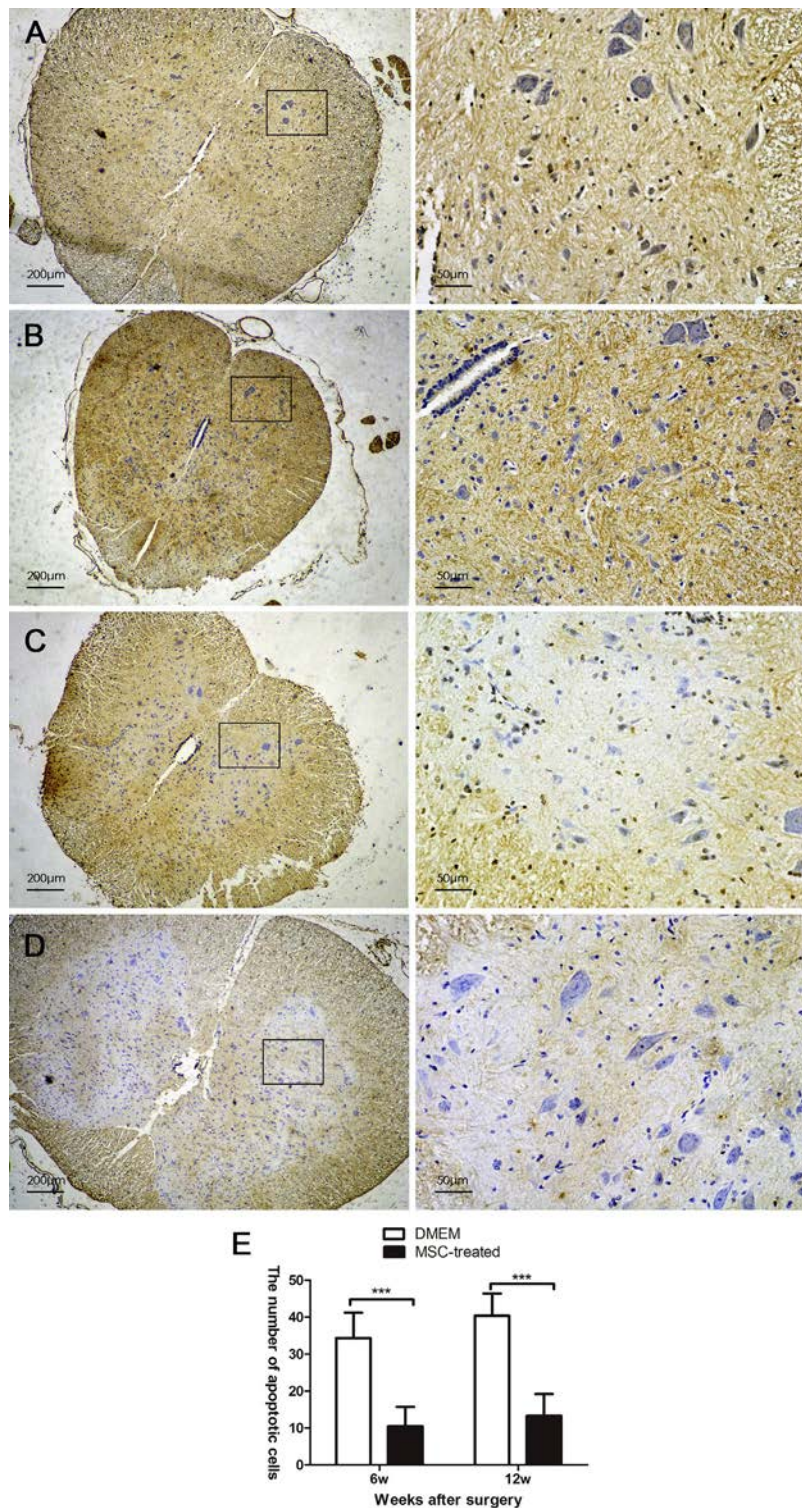
Fig. 5. *HE staining of transverse sections taken from the anterior horn of the experimental side.* The sections were obtained from DMEM (A and C) and MSC-treated (B and D) groups at 6 weeks (A and B) and 12 weeks (C and D) post surgery respectively. Scale bar, 100 μm. A histogram comparing the number of motor neurons in four different groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

untransdifferentiated mesenchymal stem cells are more appropriate for transplantation and their therapeutic benefits may result from neuroprotection rather than cell replacement. Therefore, we used mesenchymal stem cells without induced transdifferentiation in our study. The results showed that it increased remyelination, nerve fibre regeneration and functional recovery. Therefore, BMSCs hold great promise for therapeutic application of sacral plexus injury.

Our morphological analysis of regenerated lumbosacral nerves indicated that the regenerated nerves in MSC-treated group had thicker myelin sheaths and larger mean diameter, as compared with DMEM group. These improvements contribute to the higher conduction velocity of the sciatic nerve as shown by the electrophysiological study in MSC-treated group rats. Our electrophysiological evaluation also showed significantly higher amplitude of CMAP in MSC-treated group, indicating the existence of more nerve connections with the target muscle [31]. Walking track analysis is a useful tool in the evaluation of functional peripheral nerve recovery in rats [32]. The mean SFI value in MSC-treated group was significantly lower than that in DMEM group, indicating better functional recovery in MSC-treated group. All these results showed that chitosan conduit containing BMSCs can significantly promote the regeneration of lumbosacral nerves.

In addition, HE and TUNEL staining in our study showed that motor neurons in the anterior horn of the spinal cord underwent apoptosis after lumbosacral nerve transection. At six weeks after surgery, no statistical difference was observed in the number of motor neurons on the experimental side between BMSC and DMEM groups. However, 12 weeks after surgery, the number of motor neurons in the anterior horn of spinal cord of MSC-treated group was significantly larger than that in DMEM group. Similarly, the number of apoptotic cells was smaller in MSC-treated group as compared with that in DMEM group. More surviving motor neurons contributed to better functional recovery in MSC-treated group rats. Peripheral nerves regenerate spontaneously after injury due to a permissive environment and activation of the intrinsic growth capacity of neurons [33]. However, it takes several weeks for the spinal motor neurons to regenerate their axons into the defects and reinnervate the target muscle. The distance between the neuron body and injury site will cause death of the motor neurons in the anterior horn of the spinal cord after lumbosacral nerve injury, which might be caused by lack of neurotrophic factors secreted by Schwann cells and target muscle [34,35]. Therefore, the neurotrophic factors secreted by BMSCs and chitosan conduit provide an appropriate micro environment for axotomised neurons to reach the target muscle.

Fig. 6. *TUNEL staining of transverse sections taken from the anterior horn of the experimental side.* The sections were obtained from DMEM (A and C) and MSC-treated (B and D) groups at 6 weeks (A and B) and 12 weeks (C and D) post surgery respectively. Scale bar, 200 μm and 50 μm. A histogram comparing the number of apoptotic cells in the anterior horn of spinal cord in four different groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Conclusions

In the present study, we evaluated the effect of the chitosan conduit containing BMSCs on the repair of sacral nerve defects in rats. It was found that BMSC treatment improved sacral nerve regeneration and motor function 6 and 12 weeks after surgery, and that mesenchymal stem cells inhibited apoptosis and prevented the death of motor neurons in the anterior horn of the spinal cord, thus improving the motor function in MSC-treated group rats. These results suggest that the use of chitosan conduits containing BMSCs may prove to be a potential strategy for repairing lumbosacral nerve defects.

Conflict of interest statement

We have no conflict of interest concerning the materials or methods used in this study or the findings specified in this article. The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article. Dr.zhu has full access to all the data in the study and claims responsibility for accuracy of these data.

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