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Original Article

A chitosan scaffold infused with neurotrophin-3 and human umbilical cord mesenchymal stem cells suppresses inflammation and promotes functional recovery after spinal cord injury in mice

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Abstract: Despite the severe consequences of spinal cord injuries (SCI), effective treatments are lacking. In this study, the ability of chitosan/neurotrophin-3 (NT3) scaffolds seeded with human umbilical cord mesenchymal stem cells (hUC-MSCs) to treat SCI was investigated. The structure of chitosan/NT3 scaffolds and their effects on proliferation were detected by scanning electron microscopy and MTT assays. The scaffold was implanted into the injured site of SCI mouse models to evaluate its treatment ability. After 8 weeks, locomotor functional recovery was evaluated based on Basso mouse scale (BMS) assessment scores and a CatWalk automated quantitative gait analysis. The density and shape of nissl's body were measured by Nissl staining. The expression of ionized calcium-binding adapter molecules 1 (Iba1) and inflammatory cytokine levels were analyzed. Isolated hUC-MSCs exhibited abundant expression of mesenchymal stem cell surface markers (CD73, CD90, and CD105). The chitosan/NT3 scaffold had a dense shell and a porous core, which did not affect hUC-MSC proliferation. The chitosan/NT-3+hUC-MSCs treatment significantly improved indicators of locomotion ability, and promoted neuron regeneration. The chitosan/NT3+hUC-MSCs treatment inhibited Iba-1 expression, suppressed microglia activation, reduced MIP-1β, IL-6, IL-17, MCP-1, and MIP-1α levels, and increased IL-10 levels. The ability of chitosan/NT3+hUC-MSCs to promote SCI recovery was stronger than those of chitosan/NT3 and chitosan+hUC-MSCs. Chitosan/NT3+hUC-MSCs treatment could reduce inflammation, silence microglia, promote neuron regeneration, and enhance the recovery of neurological function after SCI. Thus, these scaffolds open new and promising avenues for SCI treatment.

Keywords: Spinal cord injury, chitosan, neurotrophin-3, human umbilical cord mesenchymal stem cells, inflammation

Introduction

Spinal cord injury (SCI) from vehicular accidents may cause paraplegia and even death [1]. At present, there are no effective treatments. Human umbilical cord mesenchymal stem cells (hUC-MSCs) can be induced to differentiate into nerve cells for SCI repair, but recovery is nevertheless limited [2], mainly because glial scarring at the lesion site prevents hUC-MSC adhesion and differentiation.

However, novel tissue engineering techniques that combine seed cells and scaffold materials have brought new hope for recovery [3]. One such scaffold is based on chitosan, a compound that is compatible with seed cells, such as bone marrow mesenchymal stem cells, neural stem cells, and hUC-MSCs, which promote injury repair by inducing cell adhesion and differentiation, accelerating the regeneration of axons, and inhibiting fibroblast growth [4-6]. Neurotrophin3 (NT3) is a key regulator of spinal cord development, neuronal development and differentiation, and axonal regeneration [7]. Notably, a chitosan scaffold that slowly releasesneurotrophin-3 (NT3) has been used to support long-term neural stem cell adhesion, growth, migration, and proliferation as well as to enhance neurogenesis [8, 9]. Based on these

findings, we predicted that a chitosan scaffold infused with NT3 and hUC-MSCs more effectively promotes the repair of SCI than does a chitosan scaffold infused with only MSCs.

In this work, hUC-MSCs were transplanted into a chitosan scaffold infused with NT3 and injected into the lesion site. We observed that the chitosan scaffold infused with NT3 and hUC-MSCs suppresses inflammation and promotes functional recovery after SCI in mice.

Materials and methods

Experimental animals and materials

A total of 120 healthy adult C57BL/6 male mice weighing 20 ± 2 g were provided by Guangdong Medical Lab Animal Center. Chitosan scaffolds with NT3 and chitosan scaffolds were fabricated by the College of Science and Engineering, Jinan University. Umbilical cords were obtained with consent from healthy women who delivered full-term babies by Caesarean section at the Department of Obstetrics of the First Affiliated Hospital of Jinan University. The study was approved by the hospital ethics committee.

Isolation and identification of hUC-MSCs

hUC-MSCs were isolated from sterile umbilical cord tissue (about 10 cm and close to the fetal side) from healthy full-term births (according to the hospital regulations and with the consent of their families) by enzymatic digestion following previously described methods [10]. hUC-MSCs were analyzed for surface markers after three generations. Briefly, cells were digested with 0.25% trypsin, washed with PBS three times, and resuspended at 1×10^6/mL. Aliquots (0.1 mL) were separately labeled for 30 minat room temperature in the dark with 20 μL of mouse antibodies against HLA-DR, CD105, CD90, CD73, CD54, CD45, CD44, CD40, and CD29 (1:1000; BD Pharmingen, San Diego, CA, USA). The antibodies were fluorescently labeled with FITC (HLA-DR, CD54, CD40, and CD29), PE (CD105, CD73, and CD44), or PC5 (CD90 and CD45). Mouse IgG-FITC and IgG-PE were used as negative controls. Finally, cells were fixed with 40 g/L paraformaldehyde and analyzed by flow cytometry (Coulter-Elite, Brea, CA, USA).

Morphology and biocompatibility of chitosan/NT-3 scaffolds

Chitosan/NT-3 scaffolds were coated with gold and images were obtained using a scanning electron microscope (HITACHI TM3030; Tokyo, Japan). To test biocompatibility, scaffold materials deposited on aluminum foil were cut into 5 mm × 5 mm pieces, irradiated with UV for 1 h, carefully peeled from the foil, washed in a Petri dish containing PBS, placed flat in a 96-well cell culture plate, and seeded with 200 μL of cell suspension. Seeds were prepared by digesting cultured hUC-MSCs with 0.25% trypsin and 0.05% EDTA, and resuspended at 1.5×10^4/mL. For comparison, cells were also seeded into wells without scaffolds. Cells were then cultured at 37°C in a humidified incubator with 5% CO_2. Media was replaced every 3 days, and a plate was tested by an MTT assay every other day for 9 days. Data were collected from triplicate samples.

Establishment of the SCI model and treatment

Animal experiments were performed with the approval of the Ethics Committee at Jinan University. Thirty mice were completely anesthetized with 13 μL/g tribromoethanol mixture. Under an operation microscope (Leica DM651; Wetzlar, Germany), laminectomy was performed at the level from the seventh to tenth thoracic vertebrae, followed by a right lateral incision at the T9 level to excise a segment of spinal cord tissue of 4.0 mm in length and 2.0 mm in width. Micro-bending forceps were used to dissect the spinal cord segment through the ventralroots, and approximately 2 mm of spinal cord was eventually excised. Tails wagged during transection, and the procedure resulted in paralysis in the lower extremities. The aponeurotic fascia, subcutaneous tissue, and skin were sutured, and mice were returned to cages and routinely examined. Mice were fed standard chow, and urine squeezing was performed two times per day until the micturition reflex was regained. After the operation, all mice were randomly divided into five groups. Mice were implanted with nothing (control), chitosan scaffold, chitosan/NT3 scaffold, scaffold seeded with hUC-MSCs (scaffold+hUC-MSCs), and chitosan/NT3 scaffold seeded with hUC-MSCs (chitosan/NT3+hUC-MSCs).

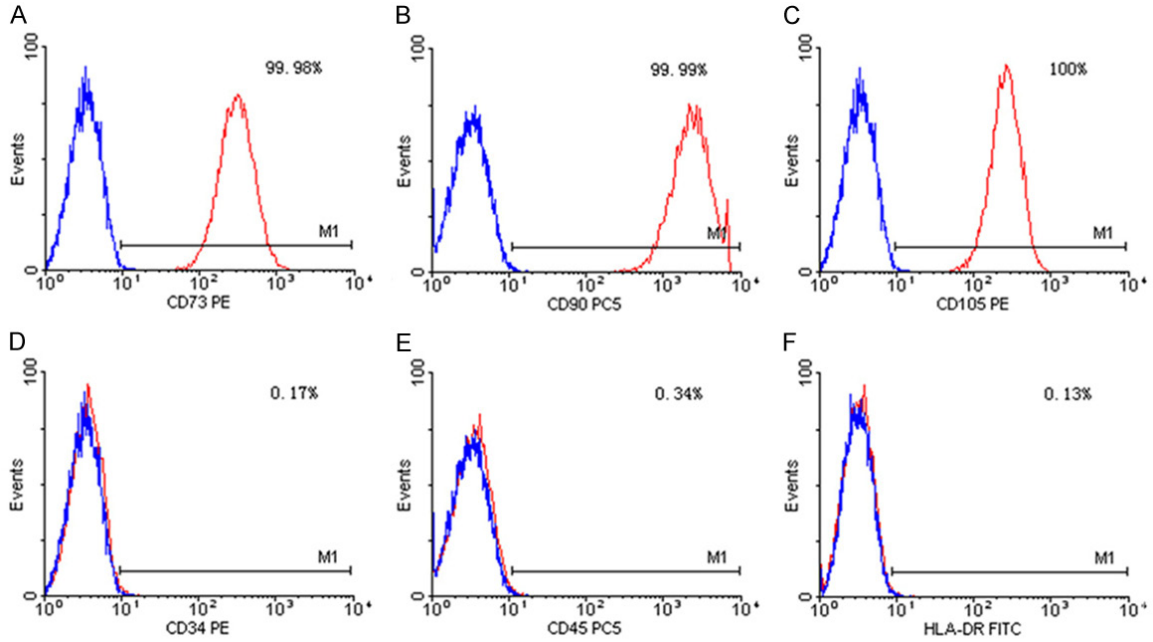

Figure 1. Expression of the surface markers CD73, CD90, CD105, CD34, CD45, and HLA-DR (A-F) in hUC-MSCs.

Behavioral assessment

Recovery after SCI was evaluated using Basso mouse scale (BMS) assessment scores [11]. Two blinded expert observers evaluated the mice on the day of surgery, and at 3 d, 5 d, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, and 8 weeks thereafter. Scores from the two observers were averaged. In addition, recovery was assessed according to published methods using the CatWalkXT 9.0 automatic quantitative gait analysis system (NoldusCompany, Wageningen, Netherlands) [12]. Briefly, mice were trained five times a day for 1 week before surgery to walk in a single direction the entire length of a darkened CatWalk chamber placed in a quiet room. Eight weeks after surgery, mice were assessed five times using the same system. The gaitregularity index and hind MaxContact area were recorded.

Motor-evoked potentials

To evaluate SCI recovery, the motor evoked potentials (MEP) were assayed by MYTO electromyography (Esaote, Florence, Italy) at 8 weeks after treatment following previously described methods [13]. First, the mice were anesthetized using a compound anesthetic (3.0 mL/kg). Then, a stimulation electrode was applied to the rostral ends of the surgical spinal cord. The recording electrode was placed in the gastrocnemius and the reference electrode was placed in paravertebral muscles in the middle of the stimulation point and recording point. The ground electrode was placed on the tail. A single square wave stimulus of 8.0 mA, 0.1 ms in duration, 2-ms time delay, and 4 Hz was used. The latency period was measured as the length of time from the stimulus to the onset of the first response wave. The amplitude was measured from the initiation point of the first response wave to its highest point. All potentials were amplified and obtained using a digital oscilloscope (Tektronix 450S; Beaverton, OR, USA).

Nissl staining and immunofluorescence

Fifty-six days after surgery, six mice were selected from each group, anesthetized, and fixed by perfusion with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The T9 vertebra was exposed as described, and 1-cm spinal cord specimens around the transected site were collected. Injured sites, anterior and posterior ends, and dorsal and ventral sides were marked. Specimens were fixed for 24 h with 4% neutral paraformaldehyde, embedded, and sectioned using a cryostat with a sagittal plane thickness of 20 μm. To measure nissl's body density, and shape, frozen sections were stained with Nissl reagent (Genmed, Laval, Canada) strictly according to the manufacturer's instructions. Frozen sections were also probed with rabbit

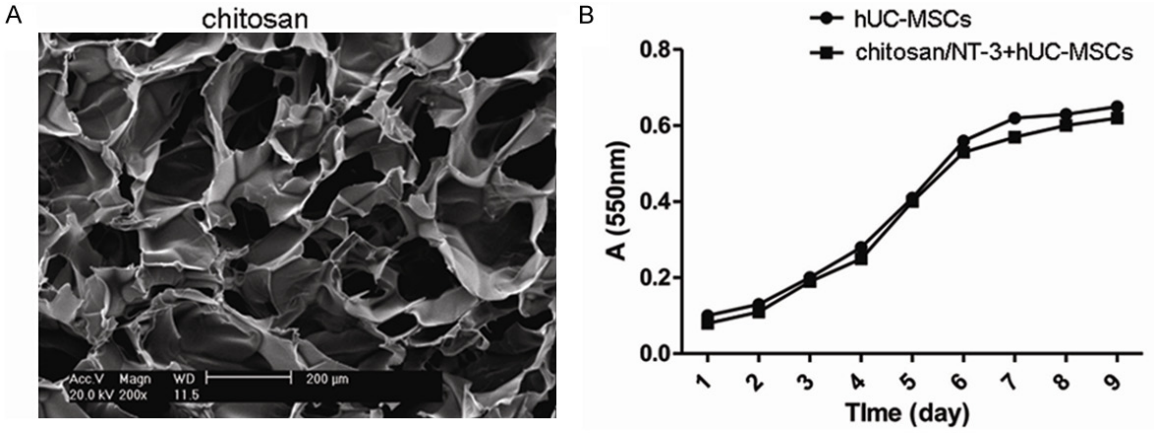

Figure 2. Scanning electron micrograph of chitosan/neurotrophin-3 scaffolds (A) and the growth of hUC-MSCs with or without the chitosan/neurotrophin-3 scaffold (B).

antibodies againstionized calcium-binding adaptor molecule 1 (Iba-1, diluted 1:1000; Abcam, Cambridge, UK), labeled with AlexaFluor488-conjugatedgoat anti-rabbit IgG (diluted 1:500; Abcam), and images were obtained using a fluorescence inverted microscope (Leica DM1000). Fluorescence intensity was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Inflammatory cytokine analysis

At 8 weeks after treatment, the levels of MIP-1β, IL-6, IL-17, MCP-1, MIP-1α, and IL-10 in mouse spinal cords were analyzed using the Bio-Plex system (Bio-Rad, Hercules, CA, USA) using a 23-Plex Cytokine Array Kit (Catalog No. M60009RDPD).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA). Data are represented as means ± SD. Groups were compared using one-way analysis of variance (ANOVA) followed by a post-hoc LSD test. *P*-values of < 0.05 were considered statistically significant.

Results

Expression of surface markers in the hUC-MSCs

HUC-MSCs exhibited abundant expression of mesenchymal stem cell surface markers (CD73, CD90, and CD105), but did not express hematopoietic stem cell markers (CD34 and CD45) or human leukocyte antigen markers (HLA-DR). Flow cytometry results are shown in **Figure 1**.

Structure and biocompatibility of chitosan/NT3 scaffolds

The scaffold had a dense shell, which may prevent fibroblasts from migrating to the damaged area and forming a scar. The scaffold also had a porous core, which may facilitate seed cell adhesion, migration, proliferation, and differentiation (**Figure 2A**). In addition, hUC-MSC proliferation rates with or without the chitosan/NT3 scaffolds did not differ significantly ($P > 0.05$, **Figure 2B**), suggesting that the scaffold did not affect cell proliferation.

Chitosan/NT3 scaffold seeded with hUC-MSCs promotes locomotor functional recovery after SCI

All mice presented complete paraplegia with a BMS score of 0 after surgery. The BMS score, regularity index, and Hind MaxContact area were significantly higher in the treatment groups than in the control group after 5d of treatment (**Figure 3A-C**). We also observed that the chitosan/NT3+hUC-MSCs treatment significantly recovered locomotor function; the BMS score, regularity index, and Hind MaxContact area were higher in this group than in the other four groups. In addition, the MEPs of all groups were measured by electrical stimulation (**Figure 3D** and **3E**). The latency periods of MEP were significantly higher in the four treatment groups than in the control group ($P < 0.05$). As expected, the amplitude of MEP in the chito-

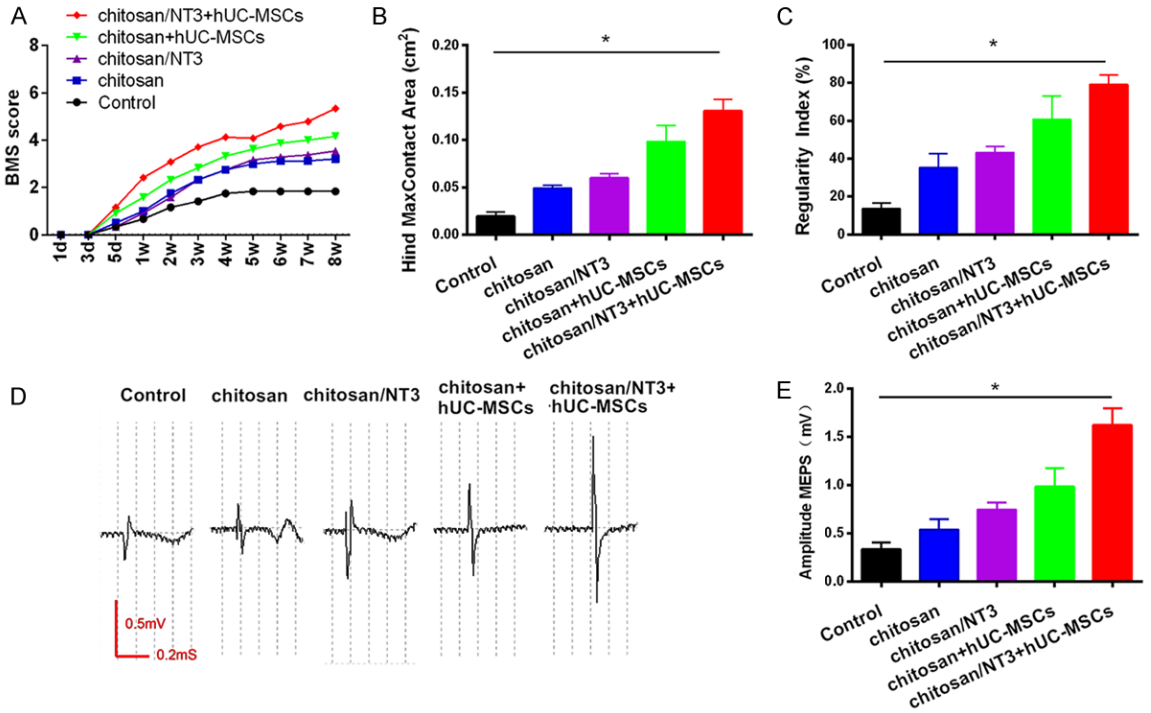

Figure 3. After treatment for 8 weeks, recovery from spinal cord injury was evaluated based on BMS assessment scores, regularity index, and Hind MaxContact area analyses as well as a motor-evoked potentials analysis. A-C: BMS assessment scores, regularity index, and Hind MaxContact area analysis, respectively. D and E: Motor-evoked potentials analysis.

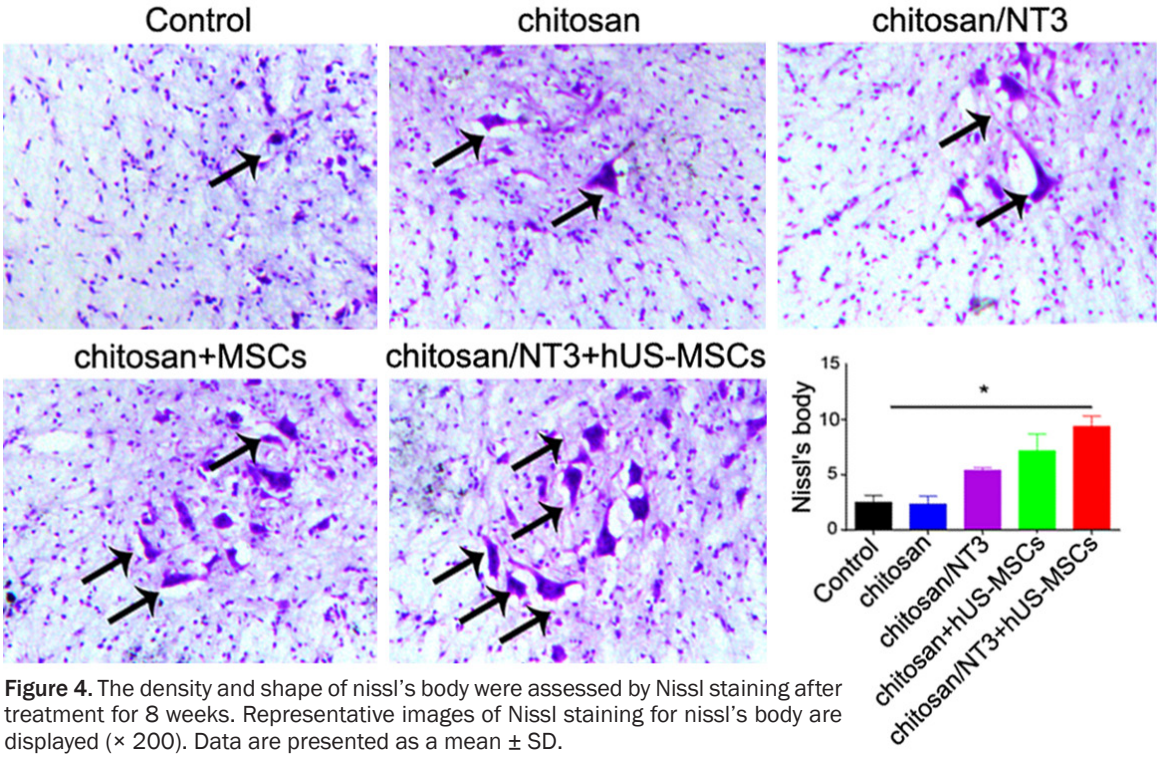

Figure 4. The density and shape of nissl's body were assessed by Nissl staining after treatment for 8 weeks. Representative images of Nissl staining for nissl's body are displayed (× 200). Data are presented as a mean ± SD.

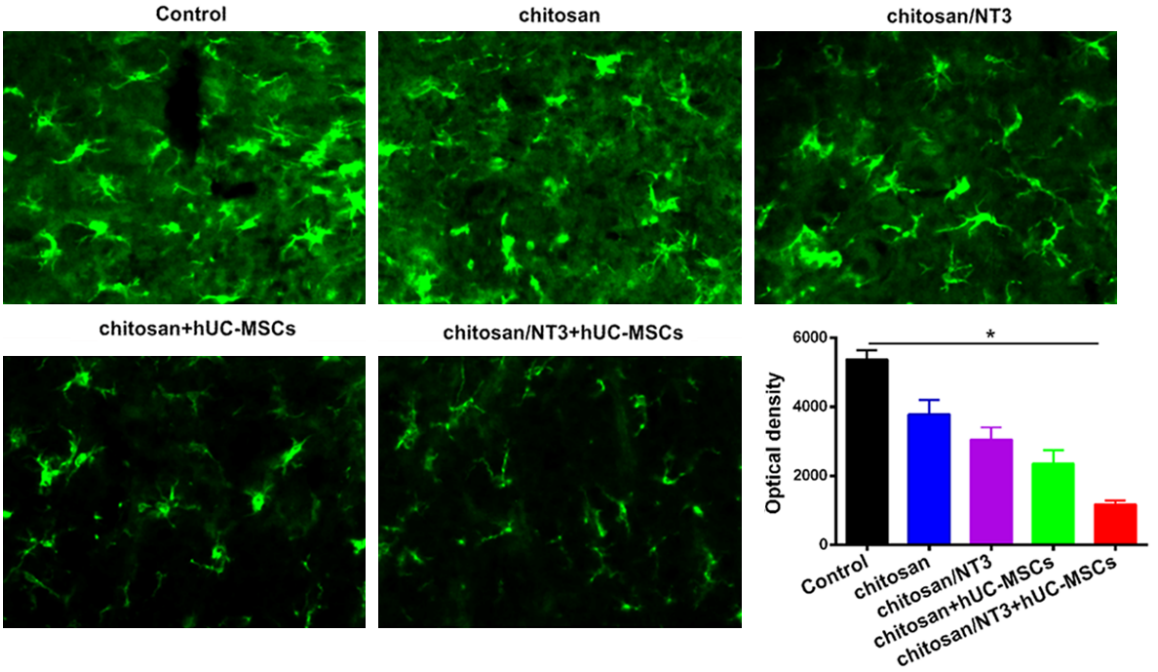

Figure 5. The expression of ionized calcium-binding adapter molecules 1 (Iba1) was measured by immunofluorescence after treatment for 8 weeks. Representative images of immunofluorescence for Iba1 are displayed (× 200). Data are presented as a mean ± SD.

san/NT3+hUC-MSCs group was evidently higher than those of the other four groups ($P < 0.05$).

Chitosan/NT3 scaffold seeded with hUC-MSCs promotes neuron regeneration after SCI

Nissl staining was performed to evaluate neurons in each section after treatment for 8 weeks. The numbers of nissl's body were significantly higher in the four treatment groups than the control group ($P < 0.05$; **Figure 4**). In addition, compared to other four groups, the numbers of nissl's body were significantly higher in the chitosan/NT3+hUC-MSCs group than the other four groups.

Chitosan/NT3 scaffold seeded with hUC-MSCs inhibited the expression of Iba1

Iba1 is a sensitive marker associated with activated microglia [14]. Representative immunofluorescence images for Iba1 detection are displayed in **Figure 5**. Iba1 expression levels were significantly lower in the four treatment groups than in the control group ($P < 0.05$). In addition, the expression of Iba1 in was significantly lower in the chitosan/NT3+hUC-MSCs group than in the other four groups ($P < 0.05$).

Chitosan/NT3 scaffold seeded with hUC-MSCs affects inflammatory cytokine levels

The levels of the inflammatory markers of IL-6, IL-17, MCP-1, MIP-1β, and MIP-1α (M1 cytokines) and IL-10 (M2 cytokine) were evaluated after SCI treatment for 8 weeks (**Figure 6**). The levels of MIP-1β, IL-6, IL-17, MCP-1, and MIP-1α were significantly lower in the four treatment groups than in the control group, and the reductions were most highly significantly in the Chitosan/NT3+hUC-MSCs group. In contrast, the IL-10 levels were significantly higher in the four treatment groups than in the control group, and the increase was most highly significant in the Chitosan/NT3+hUC-MSCs group.

Discussion

In this study, we investigated the ability of a chitosan/NT3 scaffold seeded with hUC-MSCs to promote recovery after SCI. This scaffold had a porous core with the potential to promote transplanted cell adhesion, growth, and differentiation. Furthermore, the scaffold did not affect the proliferation of hUC-MSCs, indicating good biocompatibility.

To assess the therapeutic benefits for spinal cord regeneration, the chitosan/NT3 scaffold

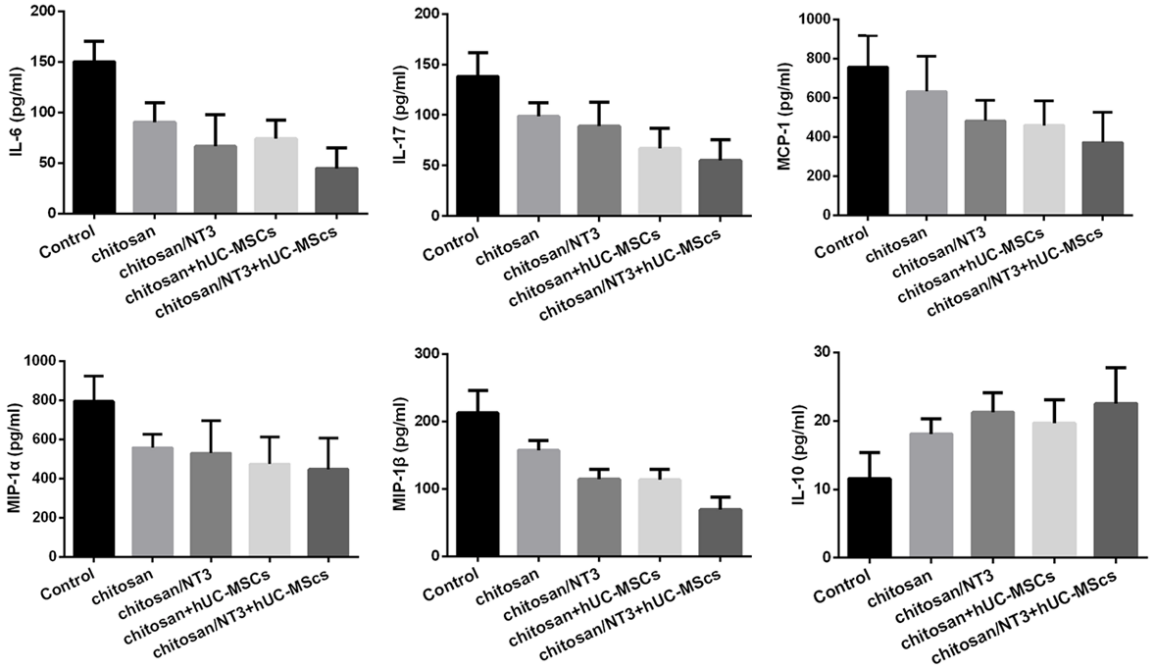

Figure 6. Levels of IL-6, IL-17, MCP-1, MIP-1α, MIP-1β, and IL-10 were detected after 8 weeks of treatment.

seeded with hUC-MSCs was implanted into the injured site of SCI mice. The chitosan/NT3+hUC-MSC treatment significantly improved the BMS score, regularity index, Hind MaxContact area, and MEP, promoted neuron regeneration, and recovered locomotor function. These results demonstrated that implanted chitosan/NT3+hUC-MSCs could promote neuronal functional recovery after SCI.

The immune-inflammatory response after SCI, including innate and adaptive immunity, is a critical pathological process facilitated by various pro-inflammatory cytokines and chemokines [15]. Microglia, the major cell type in the nervous system with immune functions, are both neuroprotective and neurotoxic. Activated microglia produce proinflammatory cytokines that induce a reactive process of secondary cell death surrounding the injury site [16]. Indeed, the inhibition of microglial activation alleviates inflammation and is a potential strategy to treat spinal cord injuries [17]. In this study, the chitosan/NT3+hUC-MSCs treatment significantly decreased the expression of Iba1, inhibited MIP-1β, IL-6, IL-17, MCP-1, and MIP-1α expression, and increased IL-10 expression. These results indicated that chitosan/NT3+hUC-MSC scaffolds inhibit microglial activation, reduce inflammation, and thereby protect nerve cells.

According to previous studies, chitosan, chitosan/NT3, and chitosan+hUC-MSCs could promote recovery from SCI [8, 18]. Consistent with these previous studies, we also found that chitosan, chitosan/NT3, and chitosan+hUC-MSCs promote recovery after SCI, to different degrees. Our results also showed that chitosan/NT3+hUC-MSCs, in particular, could enhance the effect of chitosan/NT3 with respect to recovery after SCI. In addition, we found that the ability of chitosan/NT3+hUC-MSCs to promote repair after SCI was stronger than that of the chitosan+hUC-MSCs scaffold. Accordingly, a chitosan scaffold that slowly releases NT3 is suitable to promote the function of hUC-MSCs in SCI repair.

Conclusions

Taken together, the chitosan/NT3+hUC-MSC scaffold could reduce inflammation and micrroglial activation, promote nerve cell differentiation, and enhance the recovery of neurological function after SCI in mice. Thus, these scaffolds open new and promising avenues for the treatment of SCI.

Acknowledgements

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Disclosure of conflict of interest

None.

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