Original Article

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Transplantation of neural stem cells encapsulated in hydrogels improve functional recovery in a cauda equina lesion model

Zhiyi Fu[§], Huidong Wang[§], Yujie Wu[*], Tong Zhu[*]

Shanghai Key Laboratory of Orthopaedic Implants, Department of Orthopaedic Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

SUMMARY This study explored the therapeutic effects of transplantation of neural stem cells (NSCs) encapsulated in hydrogels in a cauda equina lesion model. NSCs were isolated from neonatal dorsal root ganglion (nDRG) and cultured in three-dimensional porous hydrogel scaffolds. Immunohistochemistry, transmission electron microscopy and TUNEL assay were performed to detect the differentiation capability, ultrastructural and pathological changes, and apoptosis of NSCs. Furthermore, the functional recovery of sensorimotor reflexes was determined using the tail-flick test. NSCs derived from DRG were able to proliferate to form neurospheres and mainly differentiate into oligodendrocytes in the three-dimensional hydrogel culture system. After transplantation of NSCs encapsulated in hydrogels, NSCs differentiated into oligodendrocytes, neurons or astrocytes *in vivo*. Moreover, NSCs engrafted on the hydrogels decreased apoptosis and alleviated the ultrastructural and pathological changes of injured cauda equina. Behavioral analysis showed that transplanted hydrogel-encapsulated NSCs decreased the tail-flick latency and showed a neuroprotective role on injured cauda equina. Our results indicate transplantation of hydrogel-encapsulated NSCs promotes stem cell differentiation into oligodendrocytes, neurons or astrocytes and contributes to the functional recovery of injured cauda equina, suggesting that NSCs encapsulated in hydrogels may be applied for the treatment of cauda equina injury.

Keywords Cauda equina lesion, neural stem cells, neonatal dorsal root ganglion, Hydrogel

1. Introduction

Cauda equina syndrome (CES) is a neurological disease, which is usually caused by central lumbar disc herniation. The consequences of CES, such as neuropathic pain, lower extremity dysfunction, and sexual dysfunction adversely affect patients' life quality to various degrees, ranging from physical to mental conditions, and cause enormous economic loss to society. Although this disease has a low incidence in the population, ranging from 1:33,000 to 1:100,000 inhabitants, its sequelae still generate high public healthcare costs (*1*). Current treatment strategies include application of corticosteroid, surgical stabilization and decompression, although effective but with unsatisfied therapeutic efficacy (*2,3*). Surgery and neurotrophic drugs available for CES are limited because of the poor self-repair ability of nerve tissue, especially those in the central nervous system, the therapeutic effects of surgery and neurotrophic drugs on cauda equina injury-induced CES is limited.

Neural stem cells (NSCs) can make copies of themselves and generate different mature cell types. They are promising candidate cells for neural transplantation treatment of neurological disorders, such as brain trauma, spinal cord injury, and peripheral nerve injury (*4-6*). Many studies indicate that stem cells foster host axons to grow into the grafted spinal cord (*7-9*). In addition, induced pluripotent stem cells differentiate into astrocytes, neurons and oligodendrocytes and further improve functional recovery after spinal cord injury (*10*). Nevertheless, application of stem cell transplantation therapy is limited by poor cell survival at the injury site. Thus, development of novel approaches to maintain neural stem cell viability is important to achieve ideal therapeutic outcomes.

Recently, tissue engineering has been developed that could provide solutions to the problem of stem cell death during transplantation (*11*). Biopolymer hydrogels are designed to promote stem cell survival after cerebral transplantation, exhibiting a promising therapeutic role in central nervous system damage. One type of ionic hydrogel commonly used is made from polypeptide nanomaterials, which can be excited by Na^+ and K^+ to form solid or semi-solid (half-liquid) consolidated gel

products. Therefore, the gelation process of hydrogels can be initiated rapidly in the damaged part and used for damage repair. As previously reported by Singh *et al.* (*12*), neural stem cells derived from adult dorsal root ganglia not only retain multi-differentiation potential, but also tend to differentiate into sensory neurons after transplantation, supporting the premise that dorsal root ganglion neural stem cells (DRG NSCs) may be useful for repair of damaged cauda equina.

The nerve underneath lumbar 5-6 in rats (also called cauda equine nerve) is the sensorimotor nerve responsible for the tail of the rat. Therefore, in the present study, a rat CES model was established by application of compression to the site and NSCs were isolated from neonatal dorsal root ganglion (nDRG), encapsulated in three-dimensional porous hydrogel scaffolds, and used to repair damaged cauda equina in a rat model of CES.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats weighing 200-250 g and aged 6-8 weeks were purchased from the Animal Center of the Second Military Medical University. The surgical interventions for animal experiments were approved by the Ethical Committee of the Shanghai Jiao Tong University School of Medicine, and the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals after surgery. This study was conducted according to the guidelines laid down in the Declaration of Helsinki.

2.2. Culture of DRG-NSCs

DRG were dissected from postnatal day 2 rats, mechanically dissociated in Hank's balanced saline solution, pH 7.4, and seeded in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 2% B27, 10 ng/mL epidermal growth factor (EGF), and 10 ng/mL basic fibroblast growth factor (bFGF). NSCs were cultured in a 6-well culture plate at a density of 50-100 cells/μL with 5% CO_2 at 37°C. The medium was changed 2-3 times a week. The dissociated DRG cells formed clusters or neurospheres within 72 h. The neurospheres and culture medium in the whole culture plate were transferred to a 15 mL centrifuge tube, centrifuged at 200 g for 5 min, and the supernatants were discarded. 2 mL trypsin was added to the cell preparation. Pasteur pipettes were used to gently blow the neurospheres, place them at 37°C for 20 min, then centrifugation, and the supernatant was discarded. The preparation was suspended in a small amount of medium, gently beaten and mixed, counted, then laid in a 6-well plate, and each hole had about 200-300 neurospheres (*13*). After 3 generations of subcloning, the NSCs were derived from the neurospheres.

2.3. Transfection of NSCs with GFP

Neurospheres were digested into single cells with trypsin (#0458, Genebase, Shanghai, China) and inoculated into 2-well plates (200,000/250 μL). Lenti-virus-GFP was dissolved and diluted to a suitable MOI (final MOI = 100) with complete culture medium. A volume of 10 μg/mL of Polybrene (working concentration: 5 μg/mL) was added to promote virus infection. The virus solution (250 μL) was added to the plate and cultured in a 37 °C incubator for 24 h, followed by the cells being transferred into normal virus-free medium for further culture.

2.4. Hydrogel preparation

3D Cell Culture Hydrogels were purchased from Beaver Nano-Technologies Co., Ltd, (China). The original solution of the hydrogel was pre-treated in an ultrasonic water bath for 30 min at room temperature to reduce the viscosity. Half-liquid or solid hydrogels were formed according to manufacturer's protocol. The NSCs suspension was centrifuged at low speed to remove the supernatant. 5 mL of 10% sterile sucrose solution was added to the collected cells to resuspend them. The cell suspension was centrifuged again, and the supernatant was discarded to remove the remaining ionic components in the protocell preparation. Then, the cells were resuspended with 50 μL 10% sterile sucrose solution to prepare the salt ion free isotonic cell suspension. The 50 μL pretreatment hydrogel solution was mixed lightly with 50 μL NSCs suspension. A volume of 100 μL phosphate buffer saline was slightly added to the upper layer of the mixture. Then, the PBS layer and the hydrogel layer were mixed evenly with a pipette, and finally 200 μL hydrogel cell mixture was obtained. The final concentration of the hydrogel was 0.25%.

2.5. Model establishment and NSC transplantation

Forty-eight SD rats were randomly divided into 3 groups: Sham, CES model+Hydrogel and NSCs+Hydrogel, n = 16 in each group. For the latter two groups, animals were anesthetized with chloral hydrate before laminectomy was performed at lumbar 4. A silicone band (10 mm long, 1 mm wide, and 1 mm thick) was placed under the laminae of the L5-6 vertebra to produce the CES animal model (*14,15*). A sham operation was performed with a simple laminectomy but without contusion injury. The NSCs+Hydrogel group was subjected to transplantation of NSCs when the silicone band was removed 7 days after the compression injury, and the rats were intrathecally injected with 12 μL 0.25% hydrogels containing approximately 1,000,000 NSCs (transfected with lentivirus vectors carrying GFP) using a micropulled pipette connected to a Hamilton syringe (20 μL, Envta Technology, China).

The model+Hydrogel group was subjected to 12 μL 0.25% hydrogel containing no NSCs and injected into the subarachnoid space. GFP-transfected cells were observed under a microscope (Olympus, Japan).

2.6. Immunohistochemistry assay

The rats were perfused with 4% paraformaldehyde as the fixative. The cauda equina was then extracted, placed in EDTA solution, and heated in an oven for antigen retrieval. Then, 15 μm thick sections of the cauda equina around the lesion site were prepared longitudinally. The tissue sections were permeabilized with 0.2% Triton X-100 and blocked in blocking solution for 1 hour at room temperature. In order to identify the results of neural stem cell differentiation, the sections were incubated with primary antibodies against O4 (#MAB1326, R&D, USA), βIII-tubulin (#5568, CST, USA), glial fibrillary acidic protein (GFAP) (#12389, CST, USA), S100 (#ab52642, ABcam, Cambridge, UK) overnight at 4°C followed by incubation with secondary antibodies for 1 hour at 37°C after rinsing with PBS. The slices were stained with Hoechst for 10 min and images were photographed using inverted fluorescence Leica DMi8 microscopy (Germany). The staining of NSCs *in vitro* was the same as the above method.

2.7. Ultrastructural imaging

For transmission electron microscopic (TEM) studies, the sections were fractured with liquid nitrogen and quenched in hydrogen peroxide solution. After rinsing in PBS, the sections were prepared for ultra-thin sectioning. Tissue sections were fixed in osmium tetroxide, dehydrated in ethanol, and embedded in resin. All samples were observed under TEM (H-9500, Hitachi, Japan).

2.8. TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining

Apoptosis of cauda equina were measured using a TUNEL detection kit according to the manufacturer's instructions (Sigma, USA). In brief, paraffin-embedded tissue sections (4-mm-thick) were dewaxed, rehydrated, and incubated with reaction mixture of terminal deoxynucleotidyl transferase for 1 h. After rinsing in PBS, the sections were incubated with biotinylated antibody and ABC complex, and photographed using a light microscope (Zeiss, Germany) equipped with a digital camera.

2.9. Behavioral analysis

For the tail-flick test, the rats were immobilized for 20 mins before the test by using a cylinder tool provided together with a tail flick test instrument (SW-200, Techman Soft, China), and the tail was placed over a slit. A beam of light from a projection lamp (voltage of 18.5 V) was focused on the tail skin at the junction between the middle and distal 1/3 of the tail. The latency to respond was recorded with a maximal 15 s radiant heat stimulus (*13*).

2.10. Statistical analysis

Experimental data are presented as mean ± SD. One-Way ANOVA was used for comparison of different groups. Results were considered statistically significant when the *P* value was less than 0.05.

3. Results

3.1. Characterization of NSCs in hydrogel scaffolds

NSCs were isolated from the neonatal rat DRG and cultured in proliferation culture medium for different days. We observed that many cells floated in the medium and formed neurospheres (Figure 1A). Then, NSCs were successfully grown in 0.25% hydrogels, with neurospheres similarly observed (Figure 1B). After 7 days of differentiation in vitro, the neurospheres of neural progenitor cells (without GFP) attached to the hydrogel scaffolds, differentiated into different types of cells and were detected as previously described by Fu *et al.* (*13*). Consistent with their results, our results suggested most of the NSCs differentiated into oligodendrocytes (O4+), and only very few cells differentiated into Schwann cells (S100+), neurons (βIII-tubulin+) and astrocytes (GFAP+).

3.2. Transplantation of NSCs following in the injured cauda equina

To more easily track cells, we transfected NSCs with lentivirus vectors carrying green fluorescent protein (GFP) and cultured the cells on differentiation medium. Transfected NSCs displaying green fluorescence are shown in Figure 1C. Next, we successfully established the rat model of cauda equina injury as verified by the tail-flick test (Figure 2) and transplanted hydrogel-encapsulated NSCs (Figure 3A and 3B). To detect the viability of transplanted NSCs in the cauda equina, frozen sections were imaged by confocal microscopy after 7 days of transplantation. As expected, GFP-positive grafted NSCs were present in the injured cauda equina (Figure 3C).

3.3. Differentiation of NSCs in the injured cauda equina

To determine the differentiation status of NSCs *in vivo* after 7 days of transplantation, we further co-stained sagittal sections of the cauda equina with O4, S100, GFAP and βIII-tubulin respectively. The

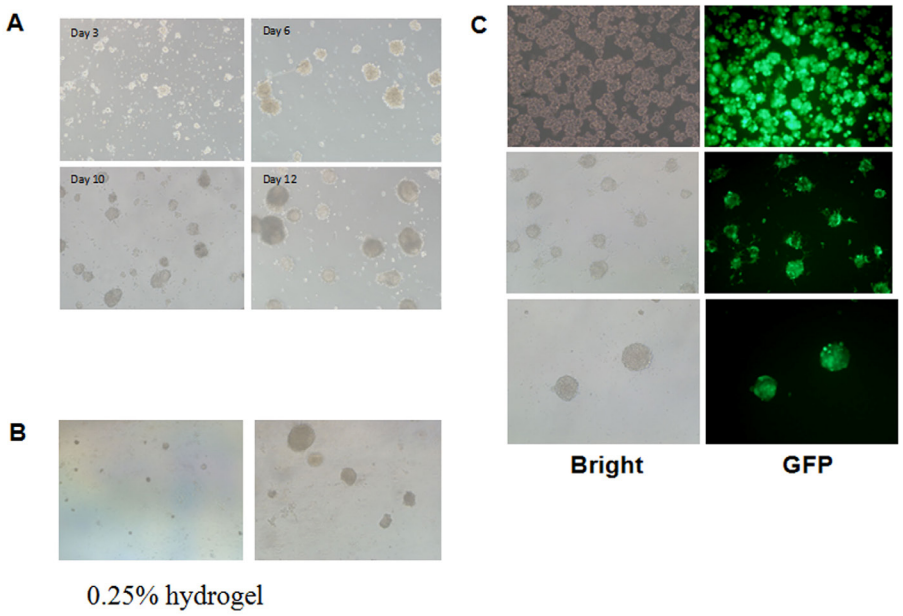

Figure 1. Characterization of NSCs in hydrogel scaffolds. (A) NSCs were isolated from the neonatal rat DRG and cultured on proliferation culture medium for different time points at 3, 6, 10 and 12 days. Magnification, ×100. **(B)** NSCs successfully grown on 0.25% hydrogels. Magnification, ×40, ×100. **(C)** NSCs transfected with lentivirus vectors carrying green fluorescent protein (GFP) and cultured on differentiation medium. Magnification, ×100.

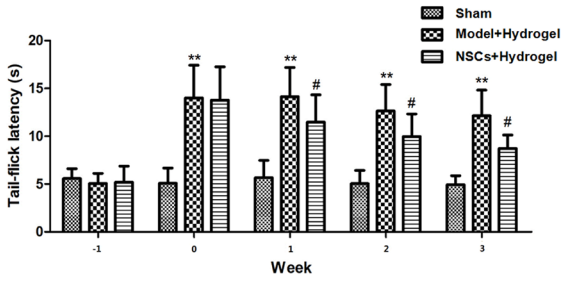

Figure 2. Functional recovery following transplantation of NSCs encapsulated in hydrogels. The functional recovery of sensorimotor reflexes in the sham, CES model+Hydrogel and NSCs+Hydrogel groups was determined using the tail-flick test. $^{**}p < 0.01$, compared *vs.* the sham group; $^{\#}p < 0.05$, *vs.* the CES model+Hydrogel group. Data are reported as means ± SD.

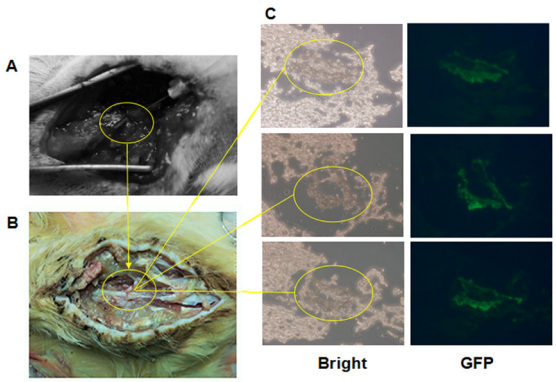

Figure 3. Transplantation of NSCs in the injured cauda equine. A rat model of cauda equina injury was established and transplanted with hydrogel-encapsulated NSCs **(A)**. After 7 days of transplantation, the frozen sections were imaged **(B)** and GFP-positive grafted NSCs in the injured cauda equina were observed in the bright and GFP channel respectively **(C)**.

outcomes demonstrated that NSCs differentiated into oligodendrocytes (O4+), neurons (βIII-tubulin+) or astrocytes (GFAP+) *in vivo* (Figure 4). However, there was no obvious detection of Schwann cells (S100+), which is an interesting result.

3.4. Functional recovery following NSC transplantation encapsulated in three-dimensional hydrogels

Fourteen days after transplantation, we investigated the regenerative effect of transplanted NSCs by examining cell apoptosis of DRG tissues and pathological morphology of cauda equina. Bilateral L5-6 DRG tissues were isolated from the rats and subjected to TUNEL staining. The results showed that the CES model+Hydrogel group led to a significant increase in apoptotic cells compared to the sham group (21.63% ± 2.08 *vs.* 1.41% ± 0.56, $p < 0.01$). By contrast, NSCs transplanted with three-dimensional hydrogels significanly decreased the apoptosis rate compared to the CES model+Hydrogel group (13.92% ± 3.67 *vs.* 21.63% ± 2.08, $p < 0.05$) (Figure 5A). Furthermore, TEM analysis showed in an organized state, normal axons, and intact myelin sheath of cauda equina nerve fibers. However, compression of cauda equina resulted in disorganized nerve fibers, swollen axons and myelin sheaths, and demyelination. These observations were alleviated after transplantation of NSCs encapsulated in hydrogels (Figure 5A). Additionally, G-ratio (inner

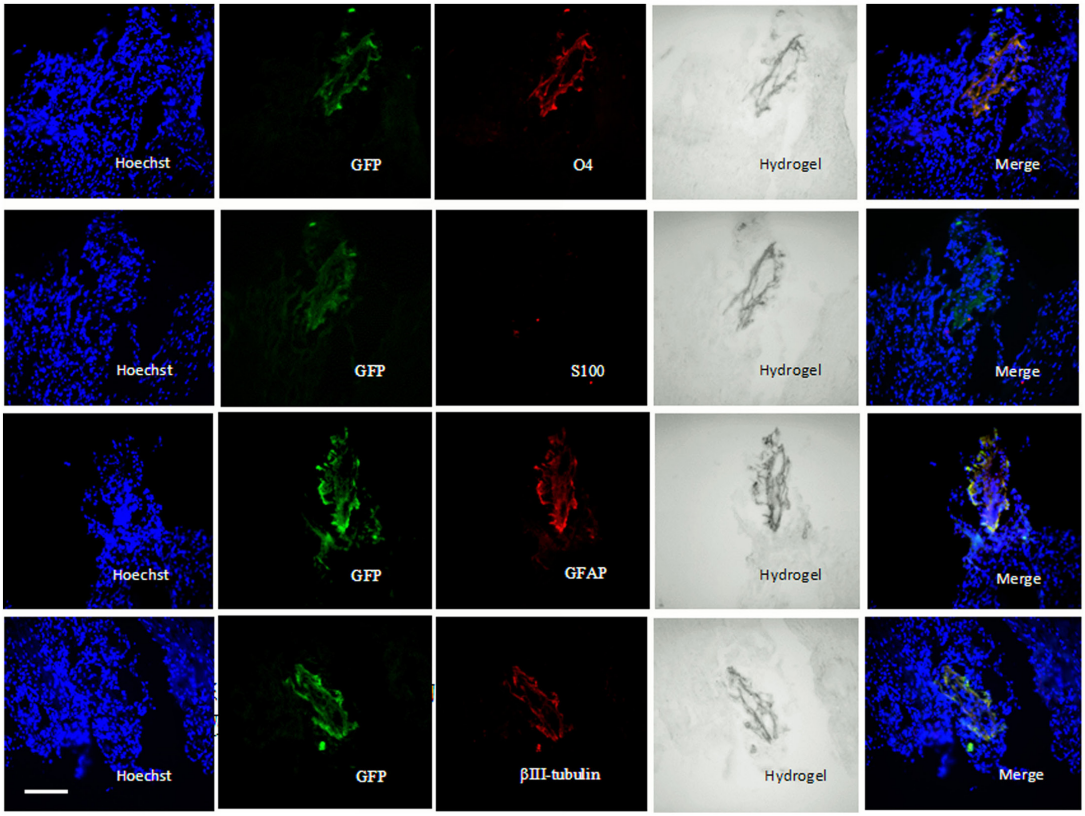

Figure 4. Differentiation of NSCs *in vivo*. After 7 days transplantation, the frozen sections were immunohistochemically stained with antibodies against O4, S100, βIII-tubulin, and GFAP, and co-stained with Hoechst for 10 min prior to images being taken using confocal microscopy. Magnification, ×200. Scale bar = 100μm.

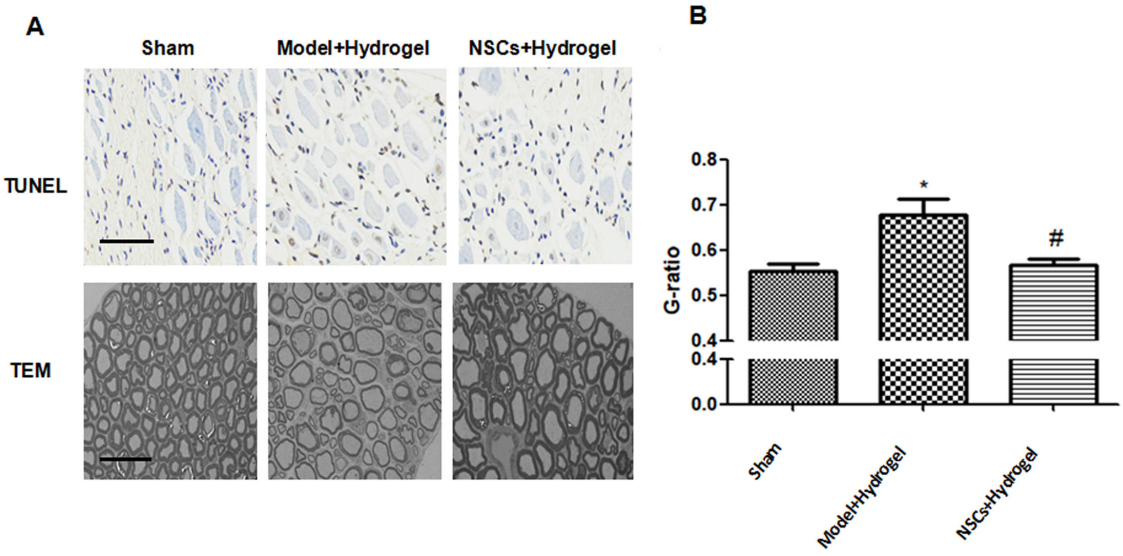

Figure 5. Ultrastructural and pathological changes of DRG after NSC transplantation. (A) The rat DRG tissues were subject to TUNEL and TEM. Magnification, ×200 (TUNEL), ×200 (TEM). **(B)** G-ratio (inner diameter/outer diameter of myelinated axons) was calculated. $^{*}p < 0.05$, *vs.* the sham group; $^{\#}p < 0.05$, *vs.* the CES model+Hydrogel group. Data are reported as means ± SD. Scale bar = 100 μm.

diameter/outer diameter of myelinated axons) was significantly higher in the model+Hydrogel group than the sham group, and then decreased in the grafted NSCs+Hydrogel animal group (Figure 5B).

We monitored the functional changes of sensorimotor reflexes of cauda equina using the tail-flick test before and after compression prior to transplantation, as well as on day 7, 14 and 21 of post-transplantation. After

compression for 7 days, we observed significantly higher tail flick latency (TFL) of the CES model+Hydrogel group than that of the sham group. This indicates the success of the CES model. This trend of the CES model+Hydrogel group was prolonged during the three weeks of post-transplantation, which corresponds with the phenotypes observed in TEM analysis fourteen days after transplantation (Figure 4A). Three weeks after transplantation, animals with cauda equina injury still exhibited a significant increase in tail-flick latency compared with those in the sham group. However, transplantation of hydrogel-encapsulated NSCs partly decreased tail-flick latency, exhibiting a neuroprotective activity on injured cauda equina (Figure 2).

4. Discussion

In the present study, we applied tissue engineering technology combined with NSCs transplantation, and explored the potential therapeutic effects on cauda equina injury. Consequently, our study demonstrated that transplantation of hydrogel-encapsulated NSCs can limitedly promote the differentiation of stem cells and improve the functional recovery of injured cauda equina.

CES is a rare neurological disorder characterized by low back pain, muscle weakness, and sensory disturbance (*16*). At the cellular level, DRG cells appear disorganized with some apoptotic bodies. In addition, CES will cause demyelination and swelling of myelin. Because of the poor self-repair ability of nerve tissue, especially those in the central nervous system, the therapeutic effects of surgery and neurotrophic drugs on cauda equina injury-induced CES is limited. Patients often have residual bladder and sexual dysfunction, and skin sensory disorder in the sella area. Although this disease has a low incidence in the population, ranging from 1: 33,000 to 1: 100,000 patients, its sequelae still generate high public healthcare costs (*1*). Thus, development of novel approaches to maintain neural stem cell viability is important to achieve ideal therapeutic outcomes. The functional recovery of CES is not satisfied because of the failure of axon regeneration and nerve damage.

Because the cauda equina is different from the spinal cord, it is impossible to transplant neural stem cells into the solid tissues. Our previous study showed that GFP-NSCs survived in the cerebrospinal fluid around the damaged cauda equina after intrathecal transplantation, but the surviving time was very short, *i.e.*, only one week (*13*). Based on the previous study, hydrogels were used to localize neural stem cells to the injured cauda equina to promote axon regeneration and remyelination of damaged cauda equina, eventually achieving the goal of neuron preservation and functional repair.

NSCs have shown promising and beneficial effects in the therapy of neurological disorders, such as spinal cord injury, brain trauma, and cauda equina lesion (*17*). Accumulating evidence demonstrates that transplanted NSCs successfully survive in the injured tissues and integrate into the host brain to achieve functional recovery (*18*). Moreover, the pluripotency of DRG has been reported by several research groups, including our group (*13,19,20*). The sensory branch in the cauda equina is composed of the central processes of DRG neurons. Thus, DRG-NSCs were used to repair the damaged cauda equina because of the homology of DRG-NSCs with cauda equina. The mechanisms by which NSCs exert their neuroprotective effects have begun to be elucidated. Increasing studies have shown that NSCs may synthesize a variety of neurotrophic cytokines stimulating nerve growth, such as vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (*21,22*). Previous studies showed that deficiency of endogenous neurotrophins is associated with poor neuronal survival and cell death (*23*). BDNF has very extensive neurotrophy and can maintain the survival of various kinds of neurons and directly promote their axon growth (*24*). Following a cervical spinal cord injury, administration of BDNF into the site of spinal cord injury promoted axonal regeneration and prevented axotomy-induced atrophy and/or death of rubrospinal neurons (*25,26*). Furthermore, cell transplantation may also enhance endogenous repair processes including neurogenesis, axonal sprouting, and angiogenesis (*27,28*). However, NSCs application is limited due to poor cell survival in host tissues. In our study, NSCs were successfully isolated and cultured in hydrogels. Moreover, we found the possibility of NSCs differentiating into oligodendrocytes, Schwann cells, neurons and astrocytes.

Tissue engineering may provide solutions to the challenges of stem cell death and damage associated with transplantation (*29*). Biopolymer hydrogels can promote stem cell survival, enhance stem cell engraftment, and minimize wound scar formation. Published studies have shown that hydrogels alter the survival and differentiation of stem cells both in vitro and *in vivo* (*30,31*). In the present study, we isolated NSCs from neonatal DRG to repair damaged cauda equina in a rat model of lumbar spinal canal stenosis. As a result, hydrogel-encapsulated NSCs presented high viability in the injured cauda equina and mainly differentiated to oligodendrocytes. Oligodendrocytes are known to be susceptible to spinal cord contusion and loss of oligodendrocytes may induce demyelination, disturb the functional recovery of damaged nerve tissues, and damage the conductive capacity of sensory nerves (*32*). Therefore, stem cell transplantation is helpful to improve myelination and enhance functional recovery after CNS injury (*33*). To evaluate the neuroprotective role of the hydrogel encapsulated NSCs, the tail-flick test was performed to measure the functional recovery of sensorimotor reflexes. As expected, NSCs engrafted on the hydrogels significantly decreased apoptosis of injured cauda equina tissue. Moreover, cauda equina nerve fibers

presented an organized state, normal axons, and intact myelin sheath. Additionally, transplanted hydrogel-encapsulated NSCs decreased the tail-flick latency and showed a neuroprotective role on injured cauda equina.

In summary, our study demonstrates that transplantation of hydrogel-encapsulated NSCs enhances the viability of transplanted cells, promotes stem cell differentiation into oligodendrocytes, thereby contributing to the functional recovery of injured cauda equina. These results implied that NSCs encapsulated in three-dimensional hydrogels may be used for the treatment of cauda equina disorder. Nevertheless, more related sensory and motor functions, time-dependence of the repair effect, or gender differences remain to be further investigated.

Our results indicate transplantation of hydrogel-encapsulated NSCs promotes stem cell differentiation into oligodendrocytes, neurons or astrocytes and contributes to the functional recovery of injured cauda equina, suggesting that NSCs encapsulated in hydrogels may be applied for the treatment of cauda equina injury.

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Conflict of Interest: The authors have no conflict of interest to disclose.

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[§]These authors contributed equally to this work.

**Address correspondence to:*
Yujie Wu and Tong Zhu, Shanghai Key Laboratory of Orthopaedic Implants, Department of Orthopaedic Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhizaoju Rd, Shanghai 200011, China.
E-mail: 611240@sh9hospital.org.cn (Wu YJ); alphaf@163.com (Zhu T)

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