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Recovery of motor function in rats with complete spinal cord injury following implantation of collagen/silk fibroin scaffold combined with human umbilical cord-mesenchymal stem cells

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SUMMARY

OBJECTIVE: This study aimed to assess the effect of the collagen/silk fibroin scaffolds seeded with human umbilical cord-mesenchymal stem cells on functional recovery after acute complete spinal cord injury.

METHODS: The fibroin and collagen were mixed (mass ratio, 3:7), and the composite scaffolds were produced. Forty rats were randomly divided into the Sham group (without spinal cord injury), spinal cord injury group (spinal cord transection without any implantation), collagen/silk fibroin scaffolds group (spinal cord transection with implantation of the collagen/silk fibroin scaffolds), and collagen/silk fibroin scaffolds + human umbilical cord-mesenchymal stem cells group (spinal cord transection with the implantation of the collagen/silk fibroin scaffolds co-cultured with human umbilical cord-mesenchymal stem cells). Motor evoked potential, Basso-Beattie-Bresnahan scale, modified Bielschowsky's silver staining, and immunofluorescence staining were performed.

RESULTS: The BBB scores in the collagen/silk fibroin scaffolds + human umbilical cord-mesenchymal stem cells group were significantly higher than those in the spinal cord injury and collagen/silk fibroin scaffolds groups ($p<0.05$ or $p<0.01$). The amplitude and latency were markedly improved in the collagen/silk fibroin scaffolds + human umbilical cord-mesenchymal stem cells group compared with the spinal cord injury and collagen/silk fibroin scaffolds groups ($p<0.05$ or $p<0.01$). Meanwhile, compared to the spinal cord injury and collagen/silk fibroin scaffolds groups, more neurofilament positive nerve fiber ensheathed by myelin basic protein positive structure at the injury site were observed in the collagen/silk fibroin scaffolds + human umbilical cord-mesenchymal stem cells group ($p<0.01$, $p<0.05$). The results of Bielschowsky's silver staining indicated more nerve fibers was observed at the lesion site in the collagen/silk fibroin scaffolds + human umbilical cord-mesenchymal stem cells group compared with the spinal cord injury and collagen/silk fibroin scaffolds groups ($p<0.01$, $p< 0.05$).

CONCLUSION: The results demonstrated that the transplantation of human umbilical cord-mesenchymal stem cells on a collagen/silk fibroin scaffolds could promote nerve regeneration, and recovery of neurological function after acute spinal cord injury.

KEYWORDS: Rats. Collagen. Silk fibroin. human mesenchymal stem cells. Nerve regeneration. Spinal cord injuries.

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INTRODUCTION

Spinal cord injury (SCI) that results in high mortality and disability rates is a major global health problem[1]. SCI brings about huge impact on property and mental to patients, and also seriously restricts social stability[2]. Thus, development of functional scaffold will become a new strategy for SCI treatment.

Experimental strategies utilizing different types of cells are being studied extensively[3]. Adult mesenchymal stem cell can modulate immune response, secrete cytokines, and inhibit inflammation and apoptosis[4]. However, stem cell implantation alone does not achieve satisfactory results in acute SCI repair[5], which might be related to the lack of structural basis. Therefore, combined with a bionic organization scaffold, will augment hUC-MSCs growth, and promote the recovery of SCI.

The ideal biomaterials for spinal cord repair should possess excellent biocompatibility, nontoxic degradation, and suitable mechanical properties. Collagen is the most important extracellular matrix component in the body. Due to its abundance, excellent biocompatibility and low antigenicity, collagen has been widely used in various tissue engineering applications[6]. However, the weaknesses of collagen scaffolds are poor mechanical strength and rapid biodegradability[7].Silk fibroin is a unique natural protein with high mechanical strength, remarkable elasticity and environmental stability[8]. Incorporation of silk fibroin could compensate for the drawbacks of utilizing a collagen scaffold alone[9]. Our goal was to evaluate the effect of the collagen/silk fibroin scaffolds (CSFSs) seeded with human umbilical cord-mesenchymal stem cells (hUC-MSCs) on functional recovery after acute complete SCI. The implantation of CSFSs combined with hUC-MSCs may be candidates for SCI treatment[10].

METHODS

Ethics statement

All experimental procedures were performed in accordance with laboratory animals form US National Institute of Health (NIH), and approved by the Ethics Committee of Characteristic Medical Center of Chinese People's Armed Police Force (CPAPF).

Fabrication of CSFS

The CSFS were obtained as previously reported[11]. Briefly, the fibroin and collagen were mixed (mass ratio, 3:7), and the composite scaffolds were produced.

Isolation, culture, and identification of hUC-MSCs

The harvest of the human umbilical cord was approved by the Characteristic Medical Center of CPAPF (approval N°. PJLEC2019), and consent was obtained from the donator. hUC-MSCs were isolated, cultured, and identified as described previously[12]. hUC-MSCs were identified by flow cytometry and immunofluorescence.

Scaffold biocompatibility

For cell seeding, 100 µl of MSCs suspension (1×10^5 cells/mL) was seeded onto CSFSs followed by incubated at a 37°C, 5% CO_2 incubator for 7 days. Then, the growth of the MSCs were observed under an inverted phase-contrast microscope and a scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). Finally, the CSFSs co-cultured with MSCs were coated with gold, the MSCs growth were observed under a SEM. At 1, 3, 5 and 7 days after seeding MSCs, Cell Counting Kit-8 (CCK-8, Solarbio Science & Technology Co., Ltd.) was performed to assess the proliferation of hUC-MSCs co-cultured with CSFSs.

Spinal cord injury and transplantation

The adult female specific-pathogen-free Sprague-Dawley rats (n=40, 260±20 g) (animal batch N°. 2019-0025) were randomly divided into the Sham group (without SCI, n=10), SCI group (spinal cord transection without any implantation, n=10), CSFS group (spinal cord transection with implantation of the CSFS, n=10), and CSFS + hUC-MSCs group (spinal cord transection with the implantation of the CSFS co-cultured with hUC-MSCs, n=10).

The surgery procedure was slightly modified according to previous report[13]. Immediately after the SCI, a 2-mm-diameter CSFS was transplanted into the completely transected gap of the CSFS group, and the CSFS co-cultured with 1×10^6 hUC-MSCs was implanted into the gap of the CSFS + hUC-MSCs group.

Assessment of neurological function

Before surgery and 1, 2, 3, 4, 6, and 8 weeks after surgery, the rats were individually rated on the 21-point Basso-Beattie-Bresnahan (BBB) locomotor rating scale (n=10 for each group). The motor evoked potential (MEP) was measured in each rat as described previously[14] 8 weeks after the SCI (n=10 for each group).

Histological analysis

At eight weeks after modelling, the samples were incubated with the primary antibodies overnight at 4°C: a mouse anti-neurofilament (NF, 1:200, Abcam), a rabbit anti-myelin basic protein monoclonal (MBP, 1:200, polyclonal, Millipore). The sections were incubated in Alexa Fluor 568-conjugated (1:1000, Invitrogen, Carlsbad, CA, USA) or Oregon Green 488-conjugated secondary antibodies (1:1000, Invitrogen, Carlsbad, CA, USA) for 1 h at RT.

Modified Bielschowsky's silver staining was used to observe nerve fibers. Then, ammonium silver alcohol solution was added to spinal cords sections (200 µl/section) for 5 min. Finally, the samples were directly reduced in 10% formaldehyde until they became dark brown and then rinsed 3 times.

Statistical analysis

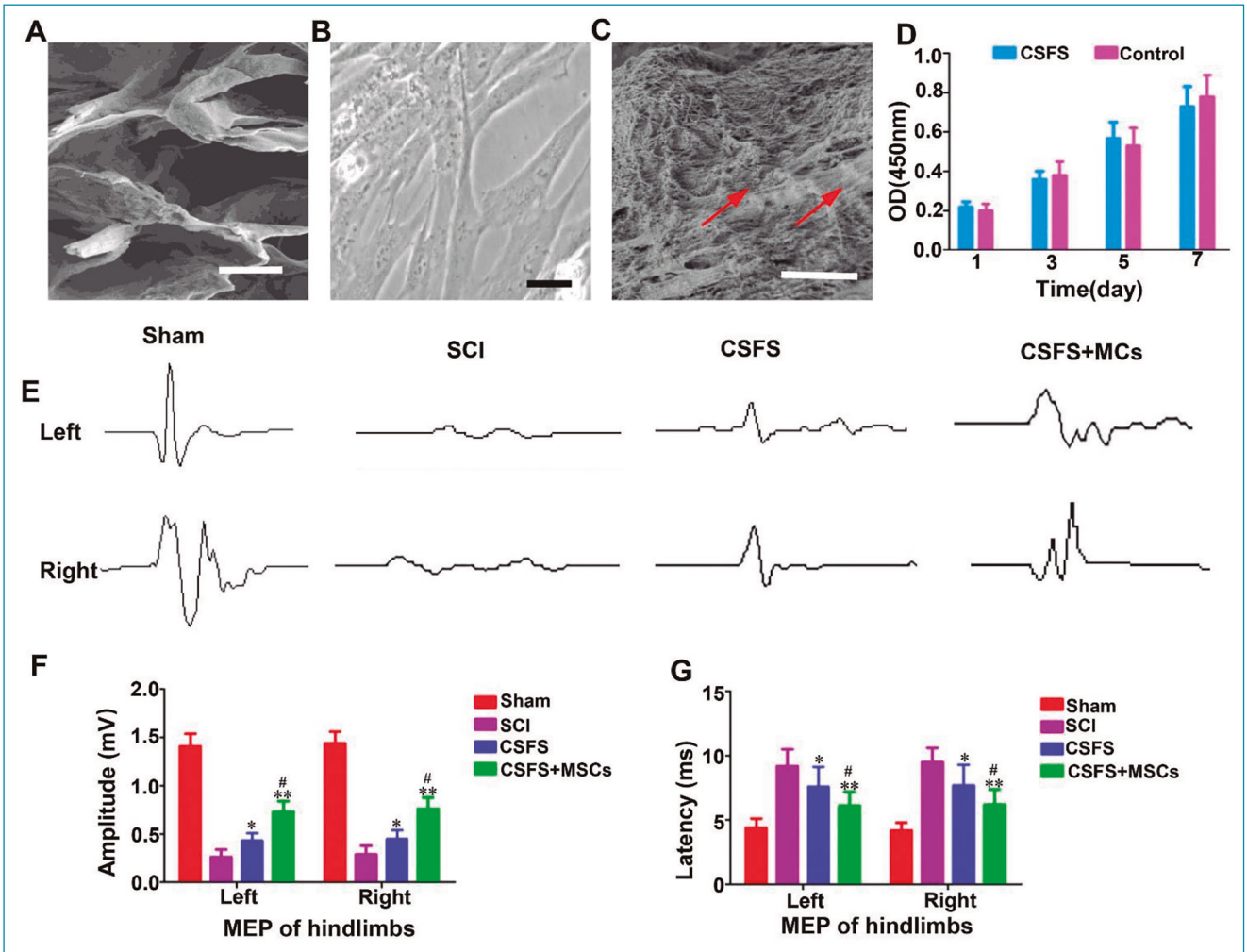
Data are presented as the mean ± standard deviation (SD). Data were analyzed by using the SPSS 15.0 package (SPSS, Chicago, IL, USA). One-way analysis of variance was used for multiple-group comparisons. Statistically significant difference in 2 parameters was performed by 2-tailed Student's t-tests. P values less than 0.05 were statistically significant.

RESULTS

Structure and biocompatibility of the CSFS

SEM images showed that the CSFS had a three-dimensional porous structure and that the pores were interconnected (Figure 1A). Phase-contrast microscopy images after 3 days of incubation with hUC-MSCs revealed that the cells were mostly fusiform (Figure 1B). After the CSFSs were incubated with the hUC-MSCs for 7 days, SEM images showed that the hUC-MSCs adhered firmly to the surface of the CSFS and were growing well inside the pores (Figure 1C).

There was no statistically significant difference between the OD values of the two groups at any time point (p>0.05; Figure 1D).



*p<0.05, **p<0.01 *versus* SCI group; #p<0.05, ##p<0.01, *versus* CSFS group. CSFS: collagen/silk fibroin scaffold; MSCs: mesenchymal stem cells; MEP: motor evoked potential; SCI: spinal cord injury. Scale bars: 5 µm in C; 10 µm in A; 100 µm in B. OD: optical density.

Figure 1. Morphology and characterization of human umbilical cord-mesenchymal stem cells (hUC-MSCs), the collagen/silk fibroin scaffold (CSFS), and electrophysiological results for all groups. (A) Scanning electron microscope (SEM) images of the CSFS. (B) hUC-MSCs morphology. (C) Morphology of the CSFS co-cultured with hUC-MSCs: the red arrows indicated hUC-MSCs. (D) Cell Counting Kit-8 assay of the hUC-MSCs cultured with the CSFS. (E) MEP traces of rats. (F, G) Amplitude (F) and latency (G) of the MEP.

Neurological function in rats

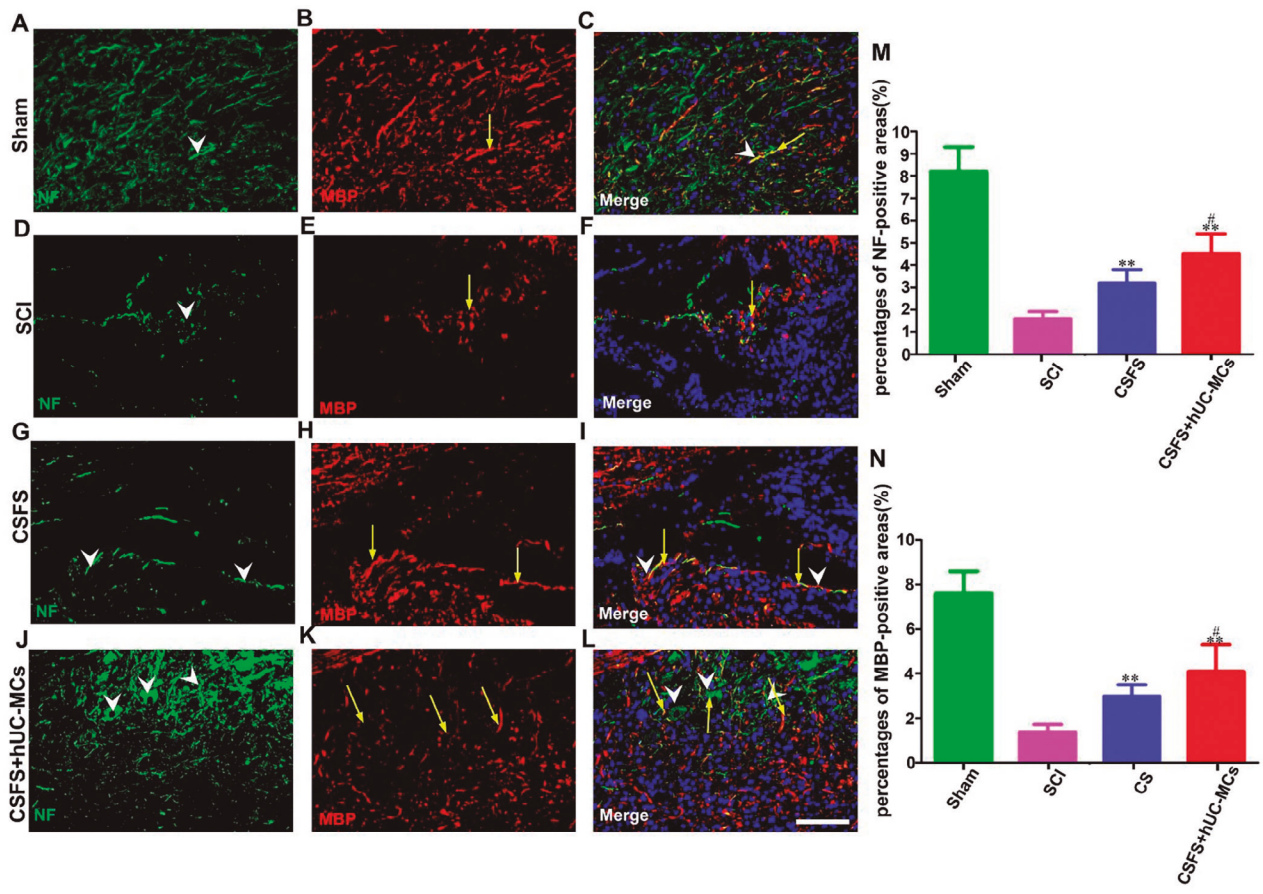
At 3, 4, 6, and 8 weeks after injury, the BBB scores in the CSFS + hUC-MSCs group were significantly higher than those in the SCI and CSFS groups ($p<0.05$ or $p<0.01$; . The amplitude and latency were markedly improved in the CSFS + hUC-MSCs group compared with the SCI and CSFS groups (Figures 1E–G).

Regeneration of nerve fiber and myelin sheaths in rats

The results indicated plentiful NF-positive fiber in lesion areas in the CSFS and CSFS+hUC-MSCs groups (Figures 2G and 2J). The relative density of NF-positive staining (equivalent to nerve fiber number) in the lesions were higher in the CSFS+hUC-MSCs group than in the CSFS and SCI groups ($p<0.05$ or $p<0.01$, Figure 2M).

For percentages of MBP-positive myelin sheaths in lesion areas, the CSFS+hUC-MSCs group exhibited higher area than the SCI and CSFS groups ($p<0.01$, $p<0.05$, Figures 2E, 2H, 2K, 2N). NF and MBP double immunofluorescence staining results demonstrated that, compared to the SCI group and the CSFS group, more NF positive nerve fiber ensheathed by MBP positive structure at the injury site were observed in the CSFS+hUC-MSCs group (Figures 2F, 2I, 2L).

The spinal cord in the Sham group was intact and nerve fibers were neatly arranged (Figure 3A). Compared with the SCI group and CSFS group, more nerve fibers was observed at the lesion site in the CSFS+hUC-MSCs group (Figure 3B-D). The axonal number in the CSFS+hUC-MSCs group dramatically increased compared to CSFS group ($p<0.05$ or $p<0.01$, Figure 3E).



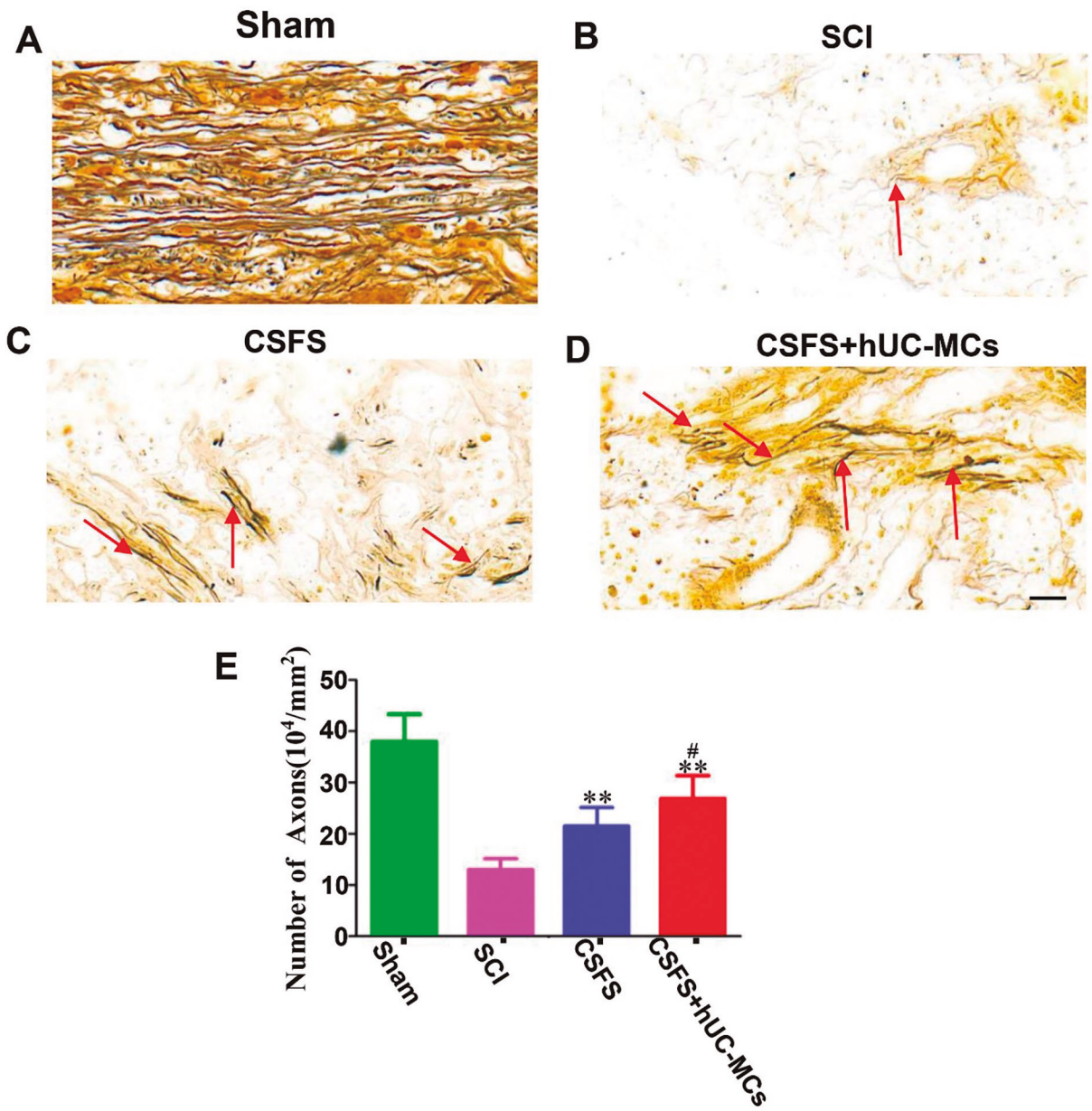
p<0.01 vs the SCI group. #p<0.05 ***versus* CSFS group. Scale bars=50 μm in (A–L). n= number of animals under each condition (n is expressed as dots in the bars). In A–N, n=10. NF: neurofilament; MBP: myelin basic protein.

Figure 2. Regeneration of NF and myelin sheaths in the lesions. (A, D, G, J) Staining for NF in the lesions of each group. (B, E, H, K) MBP-positive myelin sheaths in the lesions of each group. (C, F, I, L) Immunofluorescence staining exhibiting MBP-positive myelin sheaths (B, E, H, K, yellow arrow) surrounding positive nerve fiber (A, D, G, J, white arrow) in the graft site. (M, N) Statistical analyses of percentages of NF-(M) and MBP-positive myelin sheaths positive areas (N) in the lesions.

DISCUSSION

Restoration of neurological function after SCI is still a huge challenge for clinicians. The microenvironment at the injury site prevents axon regeneration[15]. Excellent biocompatible materials can bridge axons through glial scar tissue and play an important role in regulating the microenvironment and improving axonal regeneration[16].In this study, we reported that a CSFS combined with hUC-MSCs could promote axonal regeneration, myelination, and locomotion recovery in rats with acute complete spinal cord transection.

Motor functional recovery is one of the indicators for assessing therapeutic effect of SCI. The locomotor recovery was the best in the CSFS + hUC-MSCs group, indicating that the CSFS contributed to the reconstruction of motor functions



*p<0.05, **p<0.01 ***versus*** the SCI group. #p<0.05 ***versus*** CSFS group. Scale bars=50 μm in (A–D). n= number of animals under each condition (n is expressed as dots in the bars). In A–D, n=10.

Figure 3. Bielschowsky's silver staining of the spinal cord tissues in rats. The spinal cord tissues on the Sham group (A), the SCI group (B), the CSFS group (C) and the CSFS+hUC-MSCs group (D). All red arrows indicated the nerve fibers.

after SCI. Furthermore, the improvement of MEP in the CSFSs adsorbed with hUC-MSCs group was significantly better than other groups. The results in MEP further demonstrated that this strategy facilitated reestablishment of new reticular circuitry after SCI.

Mesenchymal stem cells are considered to be a promising therapy for SCI[17]. However, several controversies exist about the method of mesenchymal stem cell transplantation in SCI model. MSCs injection can result in cell migration to non-targeted organs[18]. Moreover, the complex injury milieu may affect the survival and differentiation of directly implanted MSCs[19]. After the MSCs and CSFSs were co-cultured, SEM results showed that MSCs attached firmly on the surface of the CSFSs, and the cells grew inside the pores. The results have demonstrated that CSFSs can provide a favorable environment to support MSCs survival. Furthermore, previous studies reported cells could grew along collagen nanofibers in many tiny channels, and had no adverse impacts on the expression of proteins and cell neurotropic factor[20]. In current studies, the CSFSs presented a porous structure, which is beneficial to cell adhesion and sufficient exchange of nutrients and oxygen[11].

The pathophysiological changes provide strong evidence for functional recovery. Many literatures have shown that collagen could reduce MSCs migration, fill the injured gap, and act as a carrier for transplanted cells or endogenous cells[21]. In this study, the myelination of axons and NF in the CSFS + hUC-MSCs group were significantly superior to other groups. MSCs could also facilitate myelination by differentiating into oligodendrocytes[22]. The cytokines secreted by the stem cell could promote the expression of MBP and NF[23]. These results demonstrated that the axon regeneration and myelination could have a rapid and effective conduction of nerve impulses and improve the recovery of neurological function. The improvement of neurological function in the CSFS+ hUC-MSCs group might partly be due to the ability of CSFS that guided the orderly regeneration of neural fibers, reduced the formation of glial scar and contributed to the reconstruction and regeneration of synapses[24,25].

CONCLUSIONS

Following completely transected SCI, the implantation of the hUC-MSCs-laden CSFS has shown obvious therapeutic effects for SCI repair, and the combinatorial therapy used in this study may have very great prospects for clinical application.

AUTHOR'S CONTRIBUTIONS

WD: Conceptualization, Data Curation, Formal Analysis, Writing – Original Draft. **XYL:** Conceptualization, Data Curation. **KM:** Conceptualization, Formal Analysis. **BL:** Conceptualization. **YFL:** Data Curation. **RJW:** Data Curation. **XYC:** Resources. SZ: Resources.

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