Collagen/heparin sulfate scaffold combined with mesenchymal stem cells treatment for canines with spinal cord injury: A pilot feasibility study

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Abstract

Background: Due to endogenous neuronal deficiency and glial scar formation, spinal cord injury (SCI) often leads to irreversible neurological loss. Accumulating evidence has shown that a suitable scaffold has important value for promoting nerve regeneration after SCI. Collagen/heparin sulfate scaffold (CHSS) has shown effect for guiding axonal regeneration and decreasing glial scar deposition after SCI. The current research aimed to evaluate the utility of the CHSSs adsorbed with mesenchymal stem cells (MSCs) on nerve regeneration, and functional recovery after acute complete SCI.

Methods: CHSSs were prepared, and evaluated for biocompatibility. The CHSSs adsorbed with MSCs were transplanted into these canines with complete SCI.

Results: We observed that MSCs had good biocompatibility with CHSSs. In complete transverse SCI models, the implantation of CHSS co-cultured with MSCs exhibited significant improvement in locomotion, motor evoked potential, magnetic resonance imaging, diffusion tensor imaging, and urodynamic parameters. Meanwhile, nerve fibers were markedly improved in the CHSS adsorbed with MSCs group. Moreover, we observed that the implantation of CHSS combined with MSCs modulated inflammatory cytokine levels.

Conclusions: The results preliminarily demonstrated that the transplantation of MSCs on a CHSS could improve the recovery of motor function after SCI. Thus, implanting the MSCs-laden CHSS is a promising combinatorial therapy for treatment in acute SCI.

Keywords
canine, collagen/heparin sulfate scaffold, human mesenchymal stem cells, nerve regeneration, spinal cord injury

Introduction

Spinal cord injury (SCI) results in high mortality rates, deficiency of autonomic, and sensory-motor functions.1 SCI brings about huge impact on property and mentality to patients, and seriously restricts social stability.2 Although current interventions including the administration of anti-inflammatory agents, and surgery of the vertebral column can extend the lifespan of patients, no effective strategies that substantially promote recovery of functional loss have been established.3 Thus, one potential therapy is to rebuild the loss of spinal cord tissue, and provide a supportive substrate to guide neurons. Implantation of...
biological scaffolds combined with stem cell may be an optimal alternative for SCI treatment.

Due to the complex microenvironment in the spine cord and its limited ability of nerve regeneration in human, there is currently no effective strategy to treat SCI. Experimental strategies utilizing different types of cells are being studied extensively. Adult mesenchymal stem cell (MSCs) can secrete cytokines, and restrain inflammation response. However, stem cells implantation alone do not show satisfactory results in clinical trials. Therefore, the implantation of MSCs combined with a biological scaffold will facilitate the functional restoration after SCI. It is necessary to assess these approaches on larger animals whose anatomical structures and immune responses are closer to humans, and which allow us to have a long follow-up period.

At present, collagen scaffolds were suitable biomaterial for SCI treatment in terms of preparation cost, degradation products and mechanical properties. Collagen possesses low antigenicity, and excellent biocompatibility and biodegradability. However, many studies have shown that it is not ideal to use collagen scaffold alone to improve neurological function, which might be related to the lack of strategies to alleviate secondary injury, such as inflammation. Furthermore, transplanting collagen scaffolds alone without any functional modification into the lesion sites could significantly inhibit formation of glial scar within the lesion center.

Heparin sulfate is a glycosaminoglycan that consist of neuronal basement membrane, which plays an important role in neural regeneration. Previous researches have showed that in the fabricating process of the biomaterial scaffold, a cross-linking reaction between collagen and heparin sulfate could significantly increase the mechanical strength of the scaffold. Newly researches reported that the CHSSs have good biocompatibility with olfactory ensheathing cells and bone marrow mesenchymal stem cells. In recent years, therapeutic strategy for SCI has been developed from simple stem cell implantation to a combined therapy based on biomaterial combining various stem cells, and has been obtained certain effect. Interestingly, whether CHSSs modulate MSCs growth by regulating the above or another potential signaling pathway in acute SCI is attractive.

In this study, our objective was to develop a functionalized scaffold using a conceptually new technology with synergistic incorporation of collagen and heparin sulfate. The experiments were to assess the effects of transplanting CHSSs seeded with MSCs in canines after SCI.

### Materials and methods

In this study, we mainly aimed to assess the therapeutic effects of CHSSs functionalized with MSCs on promoting axonal regeneration, and functional recovery after SCI. In total, 12 Beagle dogs that underwent complete spinal cord transection were randomly divided into two groups: control group and CHSS functionalized with MSCs group. The CHSS alone was not utilized as a separate group, as CHSS transplantation alone without any functional modification into the lesion sites in rats and dogs has already been repeatedly validated to notably restrain deposition of chondroitin sulfate proteoglycans within the lesion center. Furthermore, the scaffold alone showed very limited effect on promoting nerve regeneration. Therefore, CHSS is a proper scaffold only to guide axonal regeneration and reduce glial scar formation.

### Ethics statement

All experimental procedures were performed according to the care and use of 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 (approval numbers 02688049).

### CHSS preparation

The fresh bovine aponeuroses were obtained from a slaughterhouse (Wanlifa, Beijing, China) and repeatedly rinsed with cold distilled water, the fat tissue was removed, and the tendon was crushed and soaked in 0.05 M Tris buffer for 24 hours to remove soluble impurities. After centrifugation, the precipitate was added to an acetic acid solution containing pepsin. After full swelling, the supernatant was collected. The salting out precipitate was collected after centrifugation and dialyzed in deionized water for 5 days to obtain a purified collagen gel. Previous study had reported that a mass ratio of 20:1 (collagen/heparin sulfate) could bring the best biocompatibility and mechanical properties for collagen-heparin sulfate composite scaffold. Therefore, the collagen and heparin sulfate were dissolved into 0.05 M acetic acid solution according to the above proportion, and then the solution was uniformly mixed using magnetic stirrer.

The mixed solution was irradiated to ultraviolet lamp for 10 min (365 nm, 18 W cm$^{-2}$) to induce a cross-linking reaction. The mixture was freeze-dried with a vacuum freeze dryer for 2 days, and immersed in 1% NaOH solution for 12 h. Finally, the composite material of collagen and heparin sulfate was doused in deionized water repeatedly. The samples were sterilized by Co$^{60}$. The product was a standard CHSS.

### Isolation, culture, and identification of human mesenchymal stem cells

Human MSCs were derived from umbilical cord according to the protocols reported before. MSCs were cultured in low-sugar Dulbecco’s Modified Eagle medium containing 10% Fetal bovine serum. After 24 h, nonadherent cells were removed. MSCs were identified by a CytoFLEX flow cytometer (BD Biosciences, San Jose, CA) of Cambridge, Abcam, U.K.). The third passage MSCs were adsorb into the CHSSs in vitro.
**Scaffold biocompatibility**

The experiment was divided into co-culture group and control group without CHSSs. The CHSS was cut into 5-mm × 5-mm specimens for co-culture. For cell seeding, 100 µl of MSCs suspension (1 × 10^6 cells/ml) was seeded onto CHSSs, and co-cultured 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 CHSSs co-cultured with MSCs were coated with gold, the MSCs growth were observed under a SEM. The CHSSs without MSCs were also analyzed by 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 MSCs co-cultured with CHSSs. The experiment was divided into co-culture group and control group without CHSSs. Five replicate wells were prepared in each group. Briefly, according to manufacturer’s instruction, 30 µl serum-free medium containing 10% CCK-8 reagent were added to each well, and then the samples were incubated for 4 h. Finally, the solution (100 µl) was transferred into a 96-well plate. The optical density (OD) at 450 nm were measured by a multi-mode microplate reader (Bio Tek, Synergy2, USA).

CHSS co-cultured with MSCs and the control group without CHSS were incubated for 1 week in neural differentiation medium containing 100 ng/ml Dulbecco’s modified eagle medium (DMEM/F12) (Gibco, New York, USA), 2 ng/ml EGF (Amresco, St. Louis, Washington, USA), 10% fetal bovine serum (MRC Biotechnology Co., Ltd.), 2%N2/B27 (Invitrogen, New York, USA), and 100 µ/ml penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.). Cells differentiation were identified by immunofluorescence. The cells were incubated with primary antibodies against NSE (1:200, Chemicon, USA) and GFAP (1:200, Chemicon, USA) at 4°C overnight. The slides were washed with PBS (three times, 5 min each), followed by incubation with a secondary antibody against rabbit IgG conjugated to FITC (1:200, Life Technologies, USA) at room temperature for 1 hour in the dark. The samples were analyzed under an OLYMPUS inverted fluorescent microscope. Four samples from each group were performed quantitative analyses, six random areas of each sample were chosen to record the positive NSE and GFAP at 200× magnification. The percentage of positive NSE and GFAP (positive staining area/total area) was analyzed by Image-Pro Plus software (Media Cybernetics).

**Spinal cord injury and transplantation**

Twelve healthy canines (beagle, female, 1 year old, weighing 11–14 kg) were supplied from Beijing Fang Yuan Yuan Animal Center (animal license number SCXK 2019-0012, animal batch number No.12415315715834). The animal raising conditions were as follows: animals were fed in single cage with appropriate temperature and relative humidity (T = 22–23°C, H = 50–60% RH). The canines were randomly divided into control group (undergoing spinal cord transection without MSCs/CHSS implantation, n = 6), and CHSS + MSCs group (undergoing spinal cord transection followed by implantation of the CHSS loaded with MSCs, n = 6).

The Beagle canines were received complete spinal cord transection as previous reported. A 4–5 mm long section of the spinal cord at T8–11 levels was completely transected. After complete hemostasis, the CHSS co-cultured with 1 × 10^7 MSCs was transplanted for the CHSS + MSCs group. The dura mater was sutured tightly, and the paraspinal muscles and skin were sutured separately. The beagles were kept warm by using a blanket and an electric heater until the beagles recovered completely from anesthesia. The bladder was squeezed six times a day. Beagles with dysuria underwent indwelling catheterization until spontaneous urination was restored. The beagles were intramuscularly injected with gentamicin (80000 units/day) once a day for 7 days after SCI to prevent infection. To prevent complications such as hypothermia and hypotension, the beagles were injected intravenously with ringer’s lactate solution (200 ml/day) once a day for 3 days after surgery.

**Assessment of neurological function**

In canines, the Olby scoring system was used to evaluate the movements of the hindlimbs before implantation and at 0.5, 1, 3, and 6 months after implantation. Two researchers blinded to the experimental groups observed the videos to give each canine a corresponding score.

At 6 months after SCI, the motor evoked potential (MEP) was measured in each canine as described previously. The MEP signals of left and right hind limbs were recorded using Nicolet Viking Quest evoked potential equipment (Nicolet Biomedical Inc., San Carlos, CA, USA).

At 6 months after SCI, a 3.0T Magnetic resonance imaging (MRI) scanner (Magnetom Verio, Siemens, Germany) was performed in each canine. The workstation (Advantage Windows, version 4.2; GE Healthcare, Waukesha, Wisconsin, USA) was used to obtain diffusion tensor imaging (DTI).

**Urodynamics assessment**

Urodynamics assessment (ELLIPSE CHAIR, ANDRO-MEDA, Germany) was performed in all groups at 6 months postoperatively. The urinary meatus in female beagles located at the distal end of the cervical opening of the ventral wall (4–6 cm away from the vaginal outdoor). After general anesthesia, vaginal outdoor was propped up ventrally (4–6 cm away from the vaginal outdoor). A 7F bladder manometer catheter was inserted into the bladder. Residual urine volume was emptied with a syringe. A 9F rectal manometer balloon catheter was placed into the rectum and filled the balloon with saline (37°C). 0 kPa was defined as the level of pubic symphysis at atmospheric pressure, then vented the gas in the catheter.
Urethral pressure profile. The 7F bladder manometer catheter was connected via a three-way stopcock to a pressure transducer and a micro-infusion pump, then the saline was infused uniformly into the bladder (3 ml/min) through 7F bladder manometer catheter with the micro-infusion pump. Urine appeared at the urinary meatus as a beginning of voiding. Bladder threshold volume, detrusor pressure (Pdet) and bladder compliance (BC) were recorded subsequently.

Urethral function study. The manometer catheter was connected to an infusion pump and a pressure transducer, respectively, then the saline was infused (3 ml/min) uniformly and the catheter was pulled out (2 mm/s) uniformly to measure urethral pressure (UP) simultaneously.

Measurement of cytokines level

The levels of inflammatory cytokines were measured to estimate the effects of CHSS combined with MSCs transplantation on inflammation in the cerebrospinal fluid at 7, 14, and 28 days after SCI according to previously reported method. Under general anesthesia with isoflurane inhalation (volume concentration 2.0%, oxygen flow 2 l/min), the cerebrospinal fluid was drawn from the cisterna magna in the canines. The levels of TNFα, IL-10, IL-1β, and TGF-β1 were determined according to the instructions of ELISA kit (Xinfan Biotechnology Co., LTD, Shanghai, China).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Data were analyzed using the SPSS 15.0 package (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was analyzed for multiple-group comparisons. Statistically significant difference in two groups comparison was determined using Mann-Whitney U test Fisher’s exact test, or t-test. P values less than 0.05 were statistically significant.
**Results**

**Structure and biocompatibility of the CHSS**

The CHSS was fabricated using freeze drying technology (Figure 1(a)). The SEM images revealed that the CHSS had a three-dimensional porous structure and that the pores were connected (Figure 1(b)). MSCs were mostly fusiform under a phase-contrast microscopy at 3 days after culture (Figure 1(c)). After the MSCs and scaffolds were co-cultured at 7 days, SEM results showed that MSCs adhered firmly on the surface of the CHSS, and the cells grew inside the pores (Figure 1(d)). The special biomarker CD90 and CD105 of MSCs were detected by immunofluorescence staining (Figure 1(e)–(h)).

![Figure 2. Positive cell ratio of GFAP, and NSE in differentiated MSCs. Compare with the control group, CHSS co-cultured with MSCs group significantly increased expression of NSE and inhibited expression of GFAP. *P < 0.05, **P < 0.01 vs control group. Scale bars = 50 μm in panels (a, b, c, d) and Positive cell ratio of GFAP, and NSE in control group and CHSS co-cultured with MSCs group (e). Data are shown as the mean ± sd; SD: standard deviation.](image-url)
values showed no significant differences between the two groups at 1–7 days ($P > 0.05$) (Figure 1(i)).

Compare with the control group, CHSS co-cultured with MSCs group significantly promoted expression of NSE and inhibited expression of GFAP (Figure 2(a)–(e)).

**Recovery of motor and electrophysiological function in canines**

At 1, 3, and 6 months after transplantation, the Olby scores of the CHSS + MSCs group were significantly better than those in the control group ($P < 0.05$ or $P < 0.01$; Figure 3). After 6 months, the hind limbs of the canines in the CHSS + MSCs group showed some standing and ambulating abilities, while the control group displayed no voluntary movement (video. S1–2). In addition, compared with the control group, the amplitude and latency of MEP in the CHSS + MSCs group were noticeably improved (Figure 4(a)–(c)).

**Nerve fiber regeneration in canines**

At 6 months after surgery, T2WI in the CHSS + MSCs group showed continuity between the rostral and caudal stumps of the transected spinal cord (Figure 5(b1), (b2)). However, broken ends of the spinal cord in the control group were far apart (Figure 5(a1), (a2)). Compared with the control group, the DTI images in the CHSS + MSCs group showed more nerve fibers at the sites of SCI (Figure 5(a3), (a4), (b3), and (b4)). Furthermore, transplanting CHSS co-cultured with MSCs resulted in significant increase in FA value both in 150 μm rostral and caudal away from epicenter lesion (Figure 5(c)).

**Improvement of bladder in canines**

Table 1 showed the results in urodynamic parameters. Compared with the control group, a significant increase in maximum cystometric capacity (MCC), bladder compliance (BC), functional profile length (FPL), and control area (CA) and a striking decrease in detrusor pressure (Pdet), urethral pressure (Pura) and maximum urethral closure pressure (MUCP) were found in the CHSS + MSCs group.

At 6 months postoperatively, the beagles in control group showed a low BC, MCC was decreased to less than 52 ml and...
the profile increased rapidly during the bladder-filling phase (Figure 6(a)). What’s more, the detrusor pressure (Pdet), urethral pressure (Pura) increased significantly in the control group (Figure 6(a)). However, the BC and MCC in the CHSS + MSCs group increased significantly but the Pdet, intra-vesicle pressure (Pves) and Pura showed a downward trend (Figure 6(b)).

Implantation of the MSCs-laden CHSS may reduce inflammatory cytokines

Compared with the control group, the levels of IL-1β and TNF-α in the CHSS adsorbed with MSCs group decreased greatly at 7, and 14 days post-SCI (Figure 7(a) and (b)).

Table 1. Comparing the urodynamic parameters in all groups at 6 months.

| Parameter          | Control      | CHSS + MSCs  |
|--------------------|--------------|--------------|
| BC/ml cm H₂O      | 3.25 ± 1.06  | 6.15 ± 1.45* |
| MCC/mL            | 71.32 ± 6.43 | 109.38 ± 13.32** |
| Pdet/cm H₂O      | 40.18 ± 6.24 | 27.53 ± 3.18* |
| Pura/cm H₂O      | 63.91 ± 6.37 | 48.23 ± 4.86** |
| MUCP/cm H₂O      | 66.25 ± 7.17 | 54.03 ± 4.65* |
| CA/cm H₂O*mm     | 119.69 ± 15.30 | 191.74 ± 24.23** |
| FPL/mm           | 20.05 ± 3.21 | 37.86 ± 5.48** |

BC: bladder compliance; MCC: maximum cystometric capacity; Pdet: detrusor pressure at max; Pura: urethral pressure; MUCP: Maximum urethral closure pressure; CA: Control area; FPL: functional profile length;*P < 0.05,**P < 0.01 vs Control group.

Figure 5. Magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) in canines at 6 months after surgery. ((a1–a4), (b1–a4)) T2WI images and DTI in the control group (a1, a2, a3, a4), CHSS + MSCs group (b1, b2, b3, b4), (a2, a4, b2, b4) Amplified images of the yellow boxes in (a1, a3, b1, b3), respectively. (c) Apparent fractional anisotropy (FA) value was measured. All red arrows indicate segments of SCI at T10, *P < 0.05, **P < 0.01 vs Control group. n = number of animals under each condition (n is expressed as dots in the bars). In a1–a4, b1–b4 and C, n = 6. Data are shown as the mean ± sd; SD: standard deviation.
The levels of IL-10 and TGF-β1 in the CHSS combined with MSCs group were significantly higher than those in the control group at 7 and 14 days after SCI (Figure 7(c) and (d)). At 28 days after SCI, the cytokine levels were not significantly different between the control group and CHSS combined with MSCs group, and a decreasing trend of TNF-α, IL-10, IL-1β, and TGF-β1 were detected (Figure 7(a)–(d)).

These results indicated that the implantation of CHSS combined with MSCs reduced inflammatory cytokine levels, that CHSS did not increase the inflammatory response.
Discussion

Recently, although many organizations have observed inspiring results in researches, there are few effective strategies to induce neuronal regeneration after SCI. Different methods have been developed to promote the recovery of SCI, such as stem cell technology, immunomodulation and biomaterial-based treatments. In the research, we designed a functionalized scaffold and investigated the effects of CHSSs combined with MSCs on nerve regeneration after SCI. When the CHSSs adsorbed with MSCs were transplanted into the injured site, the composite scaffold provided an ideal microstructure for MSCs proliferation and migration.

In our study, after the transplantation of CHSS combined with MSCs, the canines with SCI showed improvement of neurological function. The Olby score analyses also showed the recovery of some standing and ambulating abilities in the CHSS+MSCs group. As expected, compared with control group, the CHSS + MSCs group showed better recovery in the locomotor function, and MEP, which indicated that the implantation of CHSS loaded with MSCs could further improve the pathophysiological process and provide an effective molecular delivery platform for neurogenesis after SCI. Moreover, the MRI and DTI results indicated that the implantation promoted tissue repair and axonal regeneration.

The improvements in motor and sensory function are considered the primary results in assessing the therapeutic roles of SCI. However, urination is an essential outcome in the assessment of neurological recovery. There is no effective approach for urination dysfunction, which respond poorly to conventional treatment. We observed that compared with control group, the urodynamic parameters in the CHSS loaded with MSCs group were significantly improved. Similarly, Dai et al. reported mild improvement in residual urine volume after MSCs transplantation. The recovery of urinary function is a satisfactory result because it is an important indicator for assessing therapeutic effect after SCI.

After SCI, the extracellular matrix and local microenvironment in spinal cord was destroyed, so the ideal biological scaffold should provide mechanical support for the axonal regeneration and regulate the local microenvironment to facilitate its regeneration in vivo. Collagen and heparin sulfate have excellent mechanical properties and without cytotoxicity. Moreover, CHSSs showed favorable effects on MSCs adhesion, growth and proliferation. In the study, the CHSSs possess a porous spongy structure, which is very suitable for cell adhesion and proliferation. Similarly, many literatures have shown that collagen could guide axonal growth along its orderly fibers, rebuild the injured defect, and serve on a carrier for cells.

The aim of tissue engineering is to restore damaged tissue by combining biological scaffolds and stem cell.
Human MSCs in the host spinal cord tissue were not observed at either 4 or 24 weeks post-implantation, illustrating that the human MSCs died and vanished within 4 weeks post-implantation. Despite the limited survival time, MSCs implantation improved the microenvironment in the injured spinal cord, such as alterations in the levels of pro-inflammatory cytokines, gene expression, and trophic factors. Moreover, compared with control group, levels of IL-1β and TNF-α remained suppressed after 7 and 14 days in the CHSS loaded with MSCs group, while levels of IL-10 and TGF-β1 significantly increased, indicating that the transplantation can up-regulate the expression of anti-inflammatory factors and down-regulate the expression of pro-inflammatory factors. The recovery of neurological function in the CHSS + MSCs group could be interpreted as those MSCs provide a neurotrophic environment for axonal regeneration, regulate inflammation response, and differentiate into neurons in a specific microenvironment in the spinal cord. These results again demonstrated that suitable support and guidance for nerve regeneration should be important in facilitating regeneration.

Our study has some limitations. First, this animal model created complete spinal cord transection is different from normal SCI. However, the complete spinal cord transection is the most serious injuries in vertebral fracture. It may be possible to impact the treatment of SCI for using this animal model. Second, further studies are required to discuss the mechanisms of nerve regeneration. Finally, the histopathology of nerve regeneration is not fully elucidated in this study. Additional histopathological evaluation will be helpful to increase the persuasiveness of experimental results.

Conclusion

In this study, we report that the CHSSs combined with MSCs can promote axonal regeneration, electrophysiological response, and locomotion recovery in canines with acute complete spinal cord transection. Taken together, the transplantation of CHSS loaded with MSCs has shown therapeutic effects for larger animals with severe SCI, and the combinatorial therapy may have great prospects for clinical application.

Declaration of conflicting interests

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Supplemental material

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References

1. Singh A, Tetreault L, Kalsi-Ryan S, et al. Global prevalence and incidence of traumatic spinal cord injury. *Clin Epidemiol* 2014; 6: 309–331.
2. Rao JS, Zhao C, Zhang A, et al. NT3-chitosan enables de novo regeneration and functional recovery in monkeys after spinal cord injury. *Proc Natl Acad Sci USA* 2018; 115(24): E5559–E5604.
3. Garcia-Altés A, Pérez K, Novoa A, et al. Spinal cord injury and traumatic brain injury: a cost-of-illness study. *Neuropediatrics* 2012; 39(2): 103–108.
4. Cizkova D, Le Marrec-Croq F, Franck J, et al. Alterations of protein composition along the rostro-caudal axis after spinal cord injury: proteomic, in vitro and in vivo analyses. *Front Cell Neurosci* 2014; 8: 105.
5. Cizkova D, Cubinkova V, Smolek T, et al. Localized intrathecal delivery of mesenchymal stromal cells conditioned medium improves functional recovery in a rat model of spinal cord injury. *Int J Mol Sci* 2018; 19(3): 870.
6. Cizkova D, Murgoci AN, Cubinkova V, et al. Spinal cord injury: animal models, imaging tools and the treatment strategies. *Neurochem Res* 2020; 45(1): 134–143.
7. Ruff CA, Wilcox JT and Fehlings MG. Cell-based transplantation strategies to promote plasticity following spinal cord injury. *Exp Neurol* 2012; 235(1): 78–90.
8. Ritfeld GI, Patel A, Chou A, et al. The role of brain-derived neurotrophic factor in bone marrow stromal cell-mediated spinal cord repair. *Cell Transplant* 2015; 24(11): 2209–2220.
9. Noh MY, Lim SM, Oh KW, et al. Mesenchymal stem cells modulate the functional properties of microglia via TGF-β secretion. *Stem Cells Transl Med* 2016; 5(11): 1538–1549.
10. Li X, Yang C, Li L, et al. A therapeutic strategy for spinal cord defect: human dental follicle cells combined with aligned PCL/PLGA electrospun material. *Biomater Res Int* 2015; 2015: 197183.
11. Han S, Xiao Z, Li X, et al. Human placenta-derived mesenchymal stem cells loaded on linear ordered collagen scaffold improves functional recovery after completely transected spinal cord injury in canine. *Sci China Life Sci* 2018; 61(1): 2–13.
12. Fan C, Li X, Xiao Z, et al. A modified collagen scaffold facilitates endogenous neurogenesis for acute spinal cord injury repair. *Acta Biomater* 2017; 51: 304–316.
13. Han Q, Jin W, Xiao Z, et al. The promotion of neural regeneration in an extreme rat spinal cord injury model using a collagen scaffold containing a collagen binding neuroprotective protein and an EGFR neutralizing antibody. *Biomaterials* 2010; 31(35): 9212–9220.
14. Li X, Han J, Zhao Y, et al. Functionalized collagen scaffold neutralizing the myelin-inhibitory molecules promoted neurites outgrowth in vitro and facilitated spinal cord regeneration.
in vivo. ACS Appl Mater Interfaces 2015; 7(25): 13960–13971.

15. Li X, Zhao Y, Cheng S, et al. Cetuximab modified collagen scaffold directs neurogenesis of injury-activated endogenous neural stem cells for acute spinal cord injury repair. Biomaterials 2017; 137: 73–86.

16. Zhao S, Wang Z, Chen J, et al. Preparation of heparan sulfate-like polysaccharide and application in stem cell chondrogenic differentiation. Carbohydr Res 2015; 401: 32–38.

17. Farrag M and Leipzig ND. Subcutaneous maturation of neural stem cell-loaded hydrogels forms region-specific neuroepithelium. Cells 2018; 7(10): 173.

18. Kanekiyo K, Wakabayashi T, Nakano N, et al. Effects of intrathecal injection of the conditioned medium from bone marrow stromal cells on spinal cord injury in rats. J Neurotrauma 2018; 35(3): 521–532.

19. You K, Chang H, Zhang F, et al. Cell-seeded porous silk fibroin scaffolds promotes axonal regeneration and myelination in spinal cord injury rats. Biochem Biophys Res Commun 2019; 514(1): 273–279.

20. Li G, Che MT, Zeng X, et al. Neurotrophin-3 released from implant of tissue-engineered fibroin scaffolds inhibits inflammation, enhances nerve fiber regeneration, and improves motor function in canine spinal cord injury. J Biomed Mater Res A 2018; 106(8): 2158–2170.

21. Zhong J, Xu J, Lu S, et al. A prevascularization strategy using novel fibrous porous silk scaffolds for tissue regeneration in mice with spinal cord injury. Stem Cells Dev 2020; 29(9): 615–624.

22. Chen C, Zhao ML, Zhang RK, et al. Collagen/heparin sulfate scaffolds fabricated by a 3D bioprinter improved mechanical properties and neurological function after spinal cord injury in rats. J Biomed Mater Res A 2017; 105(5): 1324–1332.

23. Carwardine D, Prager J, Neeves J, et al. Transplantation of canine olfactory ensheathing cells producing chondroitinase ABC promotes chondroitin sulphate proteoglycan digestion and axonal sprouting following spinal cord injury. PLoS One 2017; 12(12): e0188967.

24. Li X, Tan J, Xiao Z, et al. Transplantation of hUC-MSCs seeded collagen scaffolds reduces scar formation and promotes functional recovery in canines with chronic spinal cord injury. Sci Rep 2017; 7: 43559.

25. Cullen WC, Fletcher TF and Bradley WE. Histology of the canine urethra. I. Morphometry of the female urethra. Anat Rec 1981; 199(2): 177–186.

26. McCafferty GP, Coatney RW, Laping NJ, et al. Urodynamic measurements by radiotelemetry in conscious, freely moving beagle dogs. J Urol 2009; 181(3): 1444–1451.

27. Machova Urdzikova L, Karova K, Ruzicka J, et al. The anti-inflammatory compound curcumin enhances locomotor and sensory recovery after spinal cord injury in rats by immunomodulation. Int J Mol Sci 2015; 17(1): 49.

28. Wang Q, Zhang H, Xu H, et al. Novel multi-drug delivery hydrogel using scar-homing liposomes improves spinal cord injury repair. Theranostics 2018; 8(16): 4429–4446.

29. Liu H, Schwarze WM and Xie C. Dual differentiation-exogenous mesenchymal stem cell therapy for traumatic spinal cord injury repair in a murine hemisection model. Stem Cells Int 2013; 2013: 928982.

30. McCall J, Weidner N and Blesch A. Neurotrophic factors in combinatorial approaches for spinal cord regeneration. Cell Tissue Res 2012; 349(1): 27–37.

31. Dai G, Liu X, Zhang Z, et al. Transplantation of autologous bone marrow mesenchymal stem cells in the treatment of complete and chronic cervical spinal cord injury. Brain Res 2013; 1533: 73–79.

32. Nguyen TP, Nguyen QV, Nguyen VH, et al. Silk fibroin-based biomaterials for biomedical applications: a review. Polymers (Basel) 2019; 11(12): 1933.

33. Shi Q, Gao W, Han X, et al. Collagen scaffolds modified with collagen-binding bFGF promotes the neural regeneration in a rat hemisection spinal cord injury model. Sci China Life Sci 2014; 57(2): 232–240.

34. Fan J, Xiao Z, Zhang H, et al. Linear ordered collagen scaffolds loaded with collagen-binding neurotrophin-3 promote axonal regeneration and partial functional recovery after complete spinal cord transection. J Neurotrauma 2010; 27(9): 1671–1683.

35. Madigan NN, McMahon S, O’Brien T, et al. Current tissue engineering and novel therapeutic approaches to axonal regeneration following spinal cord injury using polymer scaffolds. Respir Physiol Neurobiol 2009; 169(2): 183–199.

36. Liu J, Chen J, Liu B, et al. Acellular spinal cord scaffold seeded with mesenchymal stem cells promotes long-distance axon regeneration and functional recovery in spinal cord injured rats. J Neurosci 2013; 35(12): 127–136.

37. Wang N, Xiao Z, Zhao Y, et al. Collagen scaffold combined with human umbilical cord-derived mesenchymal stem cells promote functional recovery after scar resection in rats with chronic spinal cord injury. J Tissue Eng Regen Med 2018; 12(2): e1154–e1163.

38. Wang GH, Liu Y, Wu XB, et al. Neuroprotective effects of human umbilical cord-derived mesenchymal stromal cells combined with nimodipine against radiation-induced brain injury through inhibition of apoptosis. Cytotherapy 2016; 18(1): 53–64.

39. Zhou X, Gu J, Gu Y, et al. Human umbilical cord-derived mesenchymal stem cells improve learning and memory function in hypoxic-ischemic brain-damaged rats via an IL-8-mediated secretion mechanism rather than differentiation pattern induction. Cell Physiol Biochem 2015; 35(6): 2383–2401.