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Collagen scaffolds modified with collagen-binding bFGF promotes the neural regeneration in a rat hemisected spinal cord injury model

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Nerve conduit is one of strategies for spine cord injury (SCI) treatment. Recently, studies showed that biomaterials could guide the neurite growth and promote axon regeneration at the injury site. However, the scaffold by itself was difficult to meet the need of SCI functional recovery. The basic fibroblast growth factor (bFGF) administration significantly promotes functional recovery after organ injuries. Here, using a rat model of T9 hemisected SCI, we aimed at assessing the repair capacity of implantation of collagen scaffold (CS) modified by collagen binding bFGF (CBD-bFGF). The results showed that CS combined with CBD-bFGF treatment improved survival rates after the lateral hemisection SCI. The CS/CBD-bFGF group showed more significant improvements in motor than the simply CS-implanted and untreated control group, when evaluated by the 21-point Basso-Beattie-Bresnahan (BBB) score and footprint analysis. Both hematoxylin and eosin (H&E) and immunohistochemical staining of neurofilament (NF) and glial fibrillary acidic protein (GFAP) demonstrated that fibers were guided to grow through the implants. These findings indicated that administration of CS modified with CBD-bFGF could promote spinal cord regeneration and functional recovery.

collagen scaffold, basic fibroblast growth factor, spinal cord injury, nerve regeneration

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After spinal cord injury (SCI), axons in the central nervous system are damaged and show limited regeneration because of an adverse environment such as inflammation, ischemia, lipid peroxidation, or glial scarring [1,2]. In order to create desirable environment for axons regeneration, people have focused on studies bridging and guiding the axon growth to the correct direction with natural or synthetic scaffold, aiming to reduce various secondary injury and glial scar formation. In addition, growing evidence showed beneficial effects of neurotrophic factors on spinal cord regeneration [3,4].

Collagen material has already been applied in clinic because of its biodegradability, biocompatibility, low toxicity and antigenicity [5,6]. It has also been used widely in tissue engineering and regeneration medicine due to its nano-level

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linear structure similar to extracellular matrix [7–9]. As a novel nerve guidance material, collagen scaffold (CS) can guide the orientated growth of axons after SCI in rabbits [10]. It also binds to neurotrophic factors as a drug delivery system to support axonal growth along its fibers to form correct neural connection, thus enhancing functional recovery in rat SCI models [7,11].

Basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor family and highly expressed in neuronal tissues. In rodents, a significant increase of bFGF expression was found after many central nervous system diseases including SCI. It has been shown that bFGF plays an important role in neuronal axon regeneration and repair after spinal cord injury, especially in the early stages of injury [12]. There also existed more bFGF positive cells in rat spinal cord after contusion injury than those in normal spinal cord, indicating that bFGF might be beneficial for spinal cord repair [13]. Recent studies have also shown that administration of bFGF following SCI is neuroprotective and significantly promotes functional recovery [14,15]. But because of its short half-life time and rapid diffusion in body [16], avoidance of the rapid diffusion became necessary. In order to limit its diffusion, a collagen binding domain (CBD) was designed to fuse bFGF (CBD-bFGF) with collagen in extrahepatic bile duct regeneration [17] and uterine horn regeneration [16].

In this study, the lateral 3 mm hemisection SCI of rat model was made, and CS fused with CBD-bFGF (CS/bFGF) was implanted into the hemi-transected gap to investigate the effects of the scaffolds combined with growth factors on the neural recovery after SCI.

1 Materials and methods

The guideline for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH) were followed during all the experimental procedures, and the experimental protocol was approved by the Animal Research Committee of Soochow University.

1.1 Production of CS and CBD-bFGF

CS was prepared from bovine aponeurosis as previously described [14]. Briefly, fresh bovine aponeurosis was separated from muscles, and then was cut into proper size. The residual muscles, connective tissues and fats were further removed from the aponeurosis. Subsequently, the cellular components and soluble proteins were extracted, freeze-dried and cut into the 2 mm×2 mm×3 mm bundles.

CBD-bFGF protein was prepared [18]. The expression vectors of CBD-bFGF were transformed into BL_{21} (DE_3) strain of *E. coli*, induced with isopropyl β-D-thiogalactopyranoside (IPTG). Proteins of CBD-bFGF containing six histidine tags were purified, concentrated and weighted.

1.2 SCI modeling and grouping

Healthy male adult Sprague-Dawley (SD) rats with a body weight of 240–260 g were used for surgical procedures. Rats were anesthetized with sodium pentobarbital (50 mg kg^{-1} body weight) and the model of a T9 half-cut spinal cord column was made. After shaved and cleaned with a betadine solution, the skin was incised and the muscle tissue was dissected to expose the T9 vertebral body. Laminectomy was performed at T9 to expose the dura-covered spinal cord. After a longitudinal incision was made in the dura to expose the posterior median sulcus, the left half of the spinal cord was cut at T9, followed by the removal of a 3 mm hemicord segment, and the extent of the lesion was defined by a 3 mm width scissor-cut scrip (Figure 1A–C). All rats were randomly divided into four groups: (i) sham-operated group: laminectomy without SCI; (ii) control group: half-cut spinal cord column was made without treatment; (iii) CS-treated group: a 2 mm×2 mm×3 mm bundle of CS scaffold was implanted into hemi-transected gap; (iv) CS/bFGF group: a bundle of 2 mm×2 mm×3 mm. CS fused with CBD-bFGF (2 μg/10 μL/bundle) was implanted into hemi-transected gap. Then, the muscle layers and the skin were sutured individually. After surgical procedures, animal post-operative care was required including prevention of autophagia and infection, proper bladder and bowel care. In 30 d after SCI, the survival curve was made according to the time of death.

1.3 Exclusion criteria

Spinal cord section in rats would result in a little sparing of ventral white matter, which was enough for the rats to spontaneously recover hindlimb locomotion [19]. As previously described, rats using the ipsilateral hindlimb to bear weight and walk at 1 d postoperation were excluded from the study [20].

1.4 Basso-Beattie-Bresnahan (BBB) test

Hindlimb locomotor function was assessed with the 21-point (BBB) scale [21]. The tests were performed before the operation and at 1 week intervals after SCI for 8 weeks. Animals were placed individually on open field and allowed to move freely for 5 min. Two experienced observers who were blinded to the groups evaluated the performance according to the BBB scale, ranging from 0 (no limb movement or weight support) to 21 (normal locomotion).

1.5 Footprint analysis

Four and eight weeks after operation, footprint analysis was performed in order to assess body weight support and limb coordination, respectively. The fore- and hind-paws of the rats were inked with red and blue, respectively, and the

footprints were recorded on white paper during walking on a straight runway of 1 m length and 7 cm width. The base of support was determined by measuring perpendicular distance between the centers of left and right hind limbs. Stride length was defined by distance between the footprint centers of ipsilateral adjacent footprints, through which interlimb coordination also can be assessed. The angle of hindpaw rotation was measured as the angle formed between the line which joins the print of the third digit and the central pad and the walking direction line [19,22]. The average of five sequential steps was measured for every animal. Two observers were blinded to all groups.

1.6 Histology and immunohistochemistry

The histological evaluation was performed at four and eight weeks post injury. After the rats were euthanized, pieces of spinal cord from T7-T11 were dissected and fixed in 4% paraformaldehyde. Longitudinal or cross-sectional spinal cord sections were cut and stained with hematoxylin and eosin (H&E). For immunohistochemistry, the spinal cord sections were incubated with antibodies against neurofilament(NF) and glial fibrillary acidic protein(GFAP) (mouse anti-NF and mouse anti-GFAP, Santa Cruz Biotechnology, USA). Using a digital microscope camera (Zeiss, German), the sections of spinal cord were imaged at 50× and 200× magnification.

1.7 Statistical analysis

All the data were presented as mean±SEM. Single comparisons were analyzed using unpaired two-tailed student's *t*-test. Data from more than two groups was compared by one-way analysis of variance (ANOVA). Statistical analysis was performed using GraphPad Prism 5 for Windows.

2 Results

2.1 SCI and survival analysis

After the left hemicord was completely transected at T9 level in this experiment, the function of hindlimb ipsilateral to the injury was impaired severely, with milder effects on the function of contralateral hindlimb, as described in a previous study [18]. Figure 1D shows rat dragged the hindlimb ipsilateral to the injury at the first-week postinjury (seven animals with incomplete left hemisection were excluded from the experiment: two from control group, three from CS group, and two from CS/bFGF group).

At the 30 d after SCI, the rate of death was high in both control group and CS-treated groups. The survival curve showed that CS/bFGF group had a significantly higher survival rate than that of control and CS-treated groups, with the control group had the lowest survival rate. There was no death in the sham group (Figure 2).

2.2 BBB score

Hindlimb movements were evaluated using the 21-point BBB rating Scale once a week after SCI for eight weeks. For the sham group, the BBB score was around 21 (n=11), indicating that there was no harm to the spinal cord. Although the SCI animals showed a gradual recovery in hindlimb locomotor function during the eight-week period, BBB score increased more rapidly in the first four weeks and then reached a plateau in each SCI group. In addition, the left hindlimb function in CS/bFGF group recovered faster and better than that of the control group and CS-treated groups. At the end of the eighth week, the BBB scores in both the CS/bFGF (15.67±0.83, n=9) and CS (13.00±0.71, n=8) groups were significantly higher than that in the control group (10.89±0.42, n=9) (P<0.05 for CS/bFGF group vs. CS group, P<0.05 for CS/bFGF group vs. control group, and P<0.05 for CS group vs. control group, Figure 3A). There was no significant difference in BBB scores of the right hindlimb between the SCI groups in the whole experiment processes (Figure 3B), suggesting a good uniformity for the SCI models.

2.3 Footprint analysis

Footprint analysis of the left ipsilateral hindlimb of SCI groups was summarized in Figure 4. At four and eight weeks after SCI, the sham-operated group showed the same interlimb coordination in each stride length between fore-

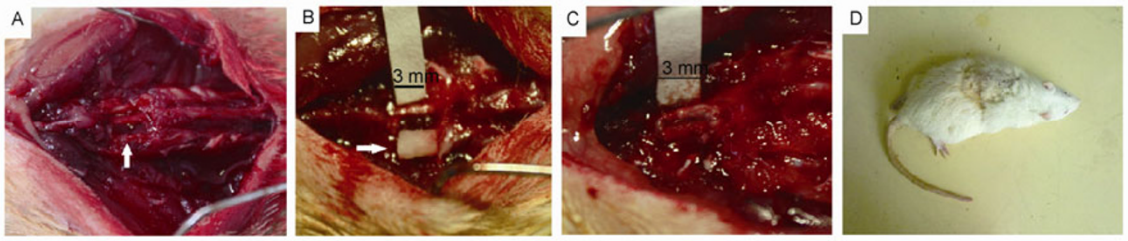

Figure 1 Surgical procedures of hemisected SCI model at T9. A, The white arrow indicates the hemisection site. B, The white arrow indicates the CS, and the extent of the lesion was defined by a 3 mm scissor-cut scrip. C, The hemisection space that was filled with the CS. D, A rat dragged the hindlimb ipsilateral to the injury at the first-week postinjury.

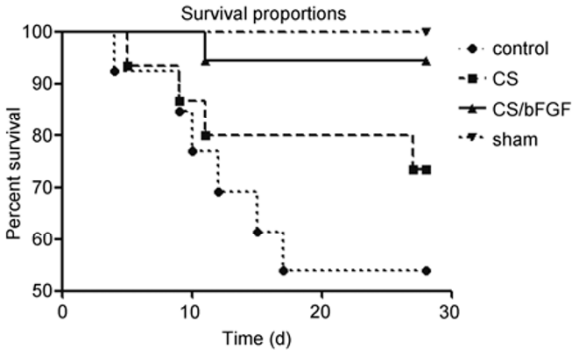

Figure 2 The survival curve of the surgical groups 30 d after the operation. Sham group (n=25); control group (n=25); CS group (n=23); CS/bFGF (n=19).

and hindlimb. The base of support (3.4±0.06 cm) and angle of rotation (1.8±0.19°) were all minimum in the sham-operated group (Figure 4A). At four and eight weeks, the base of support of the CS/bFGF group was obviously reduced compared to that of the CS group and control groups (Figure 4B, P<0.05), and it was even approximate to that of the sham-operated group at eight weeks after SCI (Figure 4B, P>0.05). In addition, toe dragging was more serious in the control and CS-treated groups than that in the CS/bFGF group (Figure 4C, P<0.01 vs. control, P<0.05 vs. CS). The differences of the stride lengths of fore- and hindlimb in the SCI groups revealed that interlimb coordination was significantly improved in the CS/bFGF group compared to that in the CS-treated and control groups at four and eight weeks, respectively (Figure 4D and 4E, P<0.05 vs. control, P<0.05 vs. CS). Relative to the control and CS-treated groups, the CS/bFGF group showed smaller angle of rotation (Figure 4F and 4G, P<0.05 vs. control, P<0.05 vs. CS).

Although consistent improvement could be seen in stride length, base of support and angle of rotation in the right contralateral hindlimb all the time, no significant differences were found between the SCI groups, indicating the good uniformity of the SCI models.

2.4 Morphological and immunohistochemical study

For macroscopical evaluation, a large coloboma was formed in the spinal cord in the control group. In contrast, the coloboma was completely filled with the scaffold in the CS-treated and CS/bFGF group. Moreover, in the CS-treated group, there were more scar tissues at the hemisection site than those of the CS/bFGF group (Figure 5).

At four and eight weeks, the density of linear fibrous tissues and cell infiltration were significantly increased in and around the scaffold of CS/ bFGF group compared to those of the control and CS-treated groups (Figure 6). The H&E staining also showed that CS degraded gradually with the time went on.

The CS/bFGF group showed higher NF-positive neural fiber density than that of the control and CS group. Besides, the NF-positive neural fibers could extend into the scaffold, and grew along with the direction of CS. The NF-positive fibers were hardly observed in and around SCI sites in the control and CS-treated group (Figure 7).

$GFAP^+$ astrocytes were present around the hemi-transected site in all SCI rats. At four and eight weeks after the surgery, the number of $GFAP^+$ cells was lower in the CS/bFGF group compared to that of the control and CS-treated groups, with the highest number present in the control group (Figure 8).

3 Discussion

SCI often results in irreversible dysfunction and disability. Treatment for SCI still remains limited to promote the axonal regeneration. Recently, there have been different approaches proposed to improve SCI repair such as biomolecular, cellular, and biomaterial-based therapies [23,24]. However, it is unlikely that a single strategy will be adequate to treat SCI. Nerve conduit is one of strategies for SCI treatment. It is hoped that some combination strategies would promote spinal cord axons to stretch through the glial scar and recreate connections. In this study, we attempted to use a combined implantation of scaffolds fused with the growth factor to repair SCI.

Among various biomaterials, CS has shown great development potential to repair the SCI because of its good biocompatibility and low antigenicity [6]. Currently, the re-

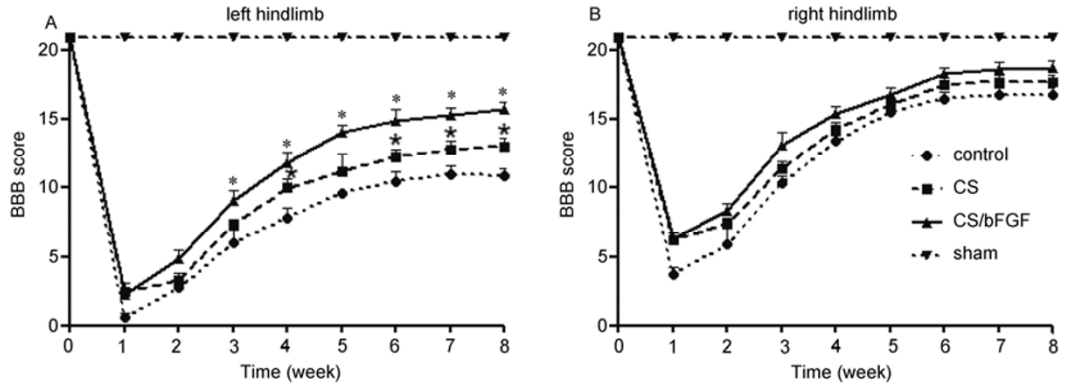

Figure 3 Hindlimb movements were evaluated using the 21-point BBB rating scale after SCI for eight weeks. A, The BBB score of left hindlimb. B, The BBB score of the right hindlimb. All the data were presented as mean±SEM. *, P<0.05.

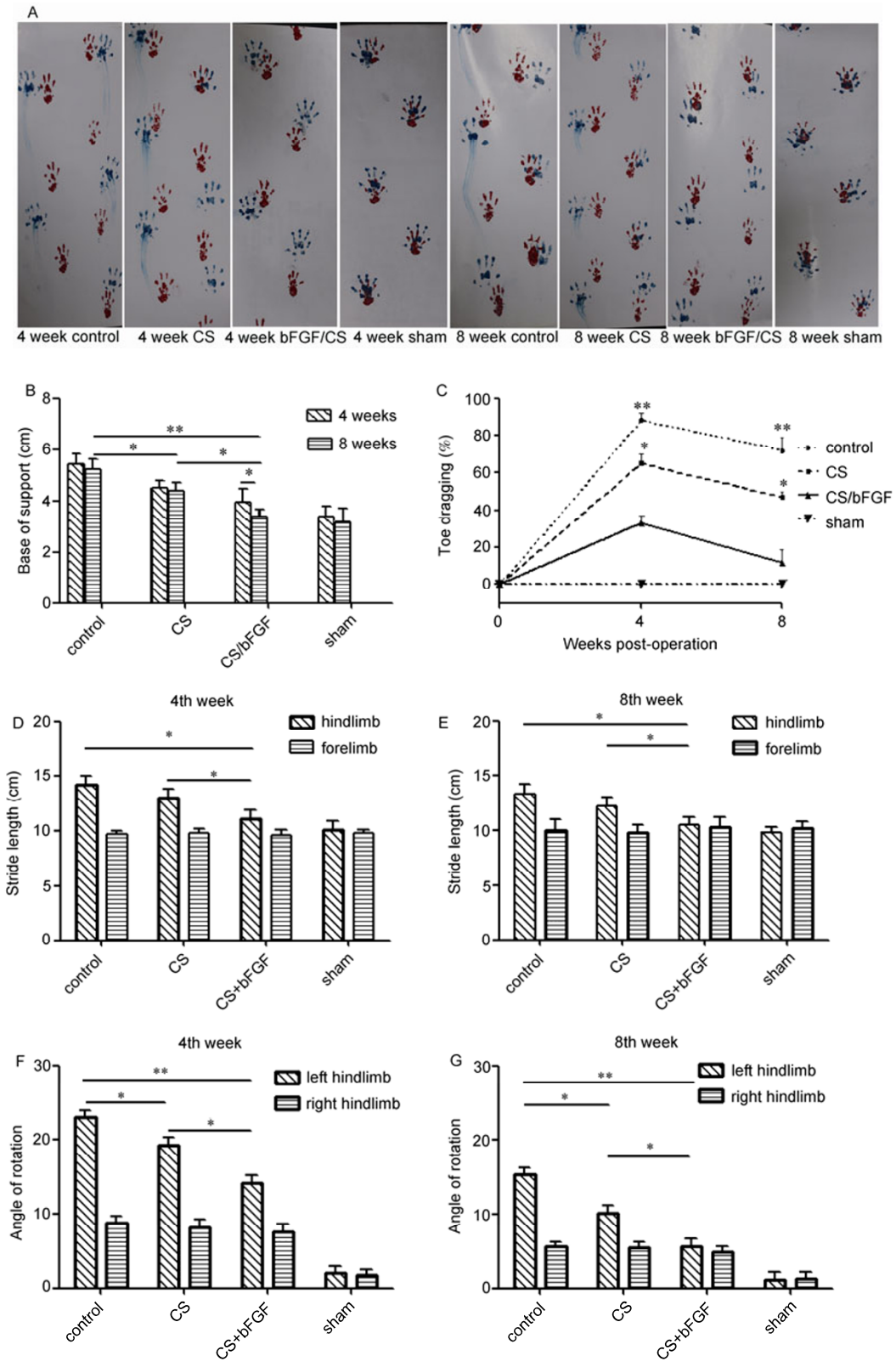

Figure 4 Footprint analysis at four and eight weeks after SCI. A, Photoes of rat fore-(inked in red) and hindlimb (inked in blue) coordination and toe spreading. B, Base of support scored by centremeters. C, The frequency of toe dragging. D and E, The stride length of fore- and hindlimb at four and eight weeks, respectively. F and G, The angle of rotations at four and eight weeks, respectively. Sham group (n=10); control group (n=9); CS group (n=8); CS/bFGF group (n=9). *, P<0.05; **, P<0.01.

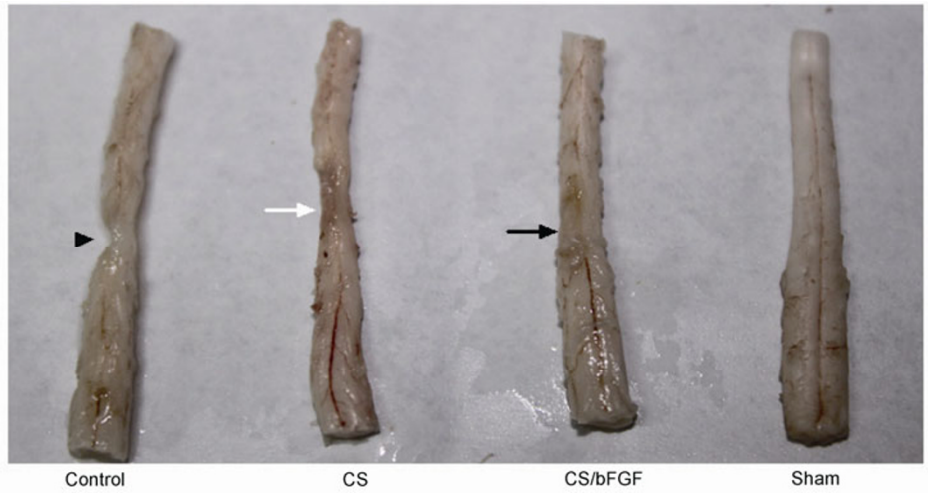

Figure 5 Dorsal view of the rat spinal cords at eight weeks after surgery. Triangular arrow indicated a large coloboma in the control group, white and black arrow indicated the coloboma filled with the scaffold in the CS and CS/bFGF group, respectively.

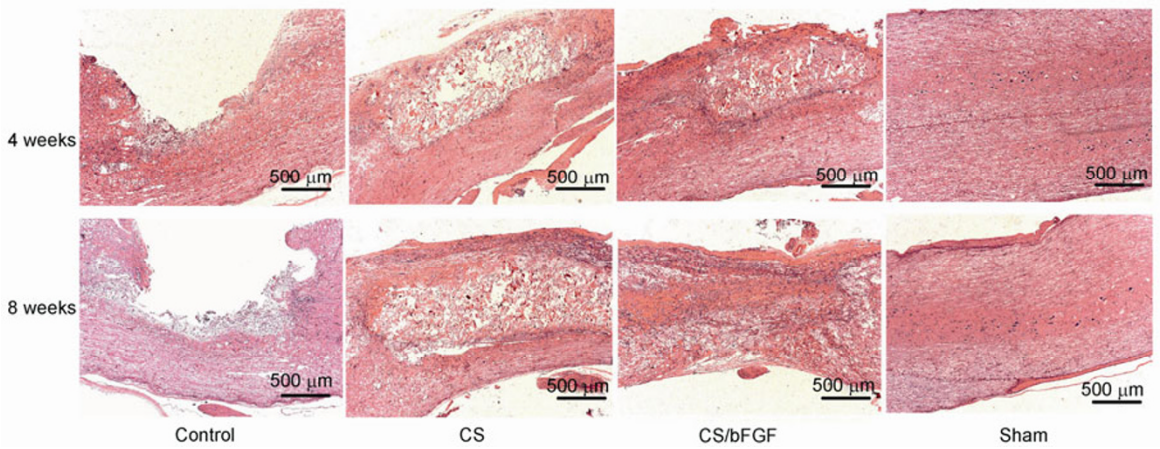

Figure 6 H&E staining for injury lesions of spinal cords at four and eight weeks after operation. Scale bar, 500 μm.

generated axons often lengthen blindly and can not reach at a proper position. The lack of contact guidance in transected axons has become one of the main problems. The CS may solve this problem because of its structure and good cell-adhesion [10]. It can provide not only a physical support for axon extension, but also a delivery system for cytokines and growth factors [18].

bFGF plays an important role in functional recovery after SCI [14]. Besides the role in promoting axon regeneration, administration of bFGF has been reported to have neuroprotective and improved behavioral effects *in vivo* after SCI [25–27]. Recent experiments showed that bFGF could inhibit apoptosis and increase the survival of neurons in injury site of the spinal cord [28,29]. However, the bioactivity of bFGF could rapidly be cleared from the tissue [18]. bFGF fused on CS through collagen binding domain may offer a strategy to retain high-enough concentration in localized lesions and work on the generation of neurons after SCI [16,17].

In order to test the effect of the implantation system, we established the lateral hemisection SCI rat model in this study. After the removal of a 3 mm hemicord segment, the damages were serious and it was impossible for the rostral nerve fibers to stretch across the injury gap due to the lack of support. According to open field BBB analyses of the ipsilateral hindlimb, the function of hindlimb ipsilateral to the injury was impaired severely after the operation; meanwhile the contralateral hindlimb also was affected inevitably. At the early stage of post-operation, especially in the first three weeks, the rate of death was high in the control group. These animals might die from number of causes, such as blood loss, neurogenic pulmonary oedema, lung infection, urinary infection and retention, or systemic inflammatory response syndrome (SIRS) [30]. The outcome was better in the CS- and CS/bFGF-treated groups than in the control group. Moreover, the rate of the death and the neuronal function after SCI in the CS/bFGF-treated group was significantly improved compared to those of the CS-treated

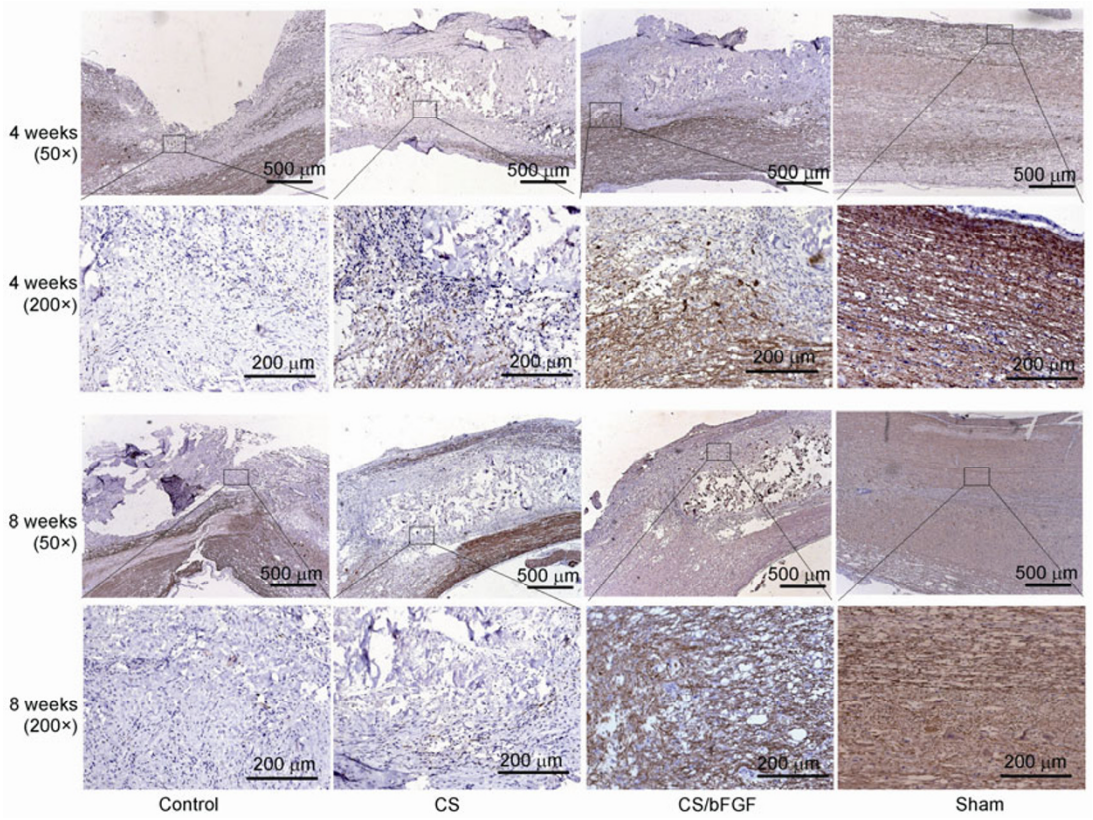

Figure 7 Immunohistochemical staining of neurofilament(NF) for spinal cords at four and eight weeks after operation.

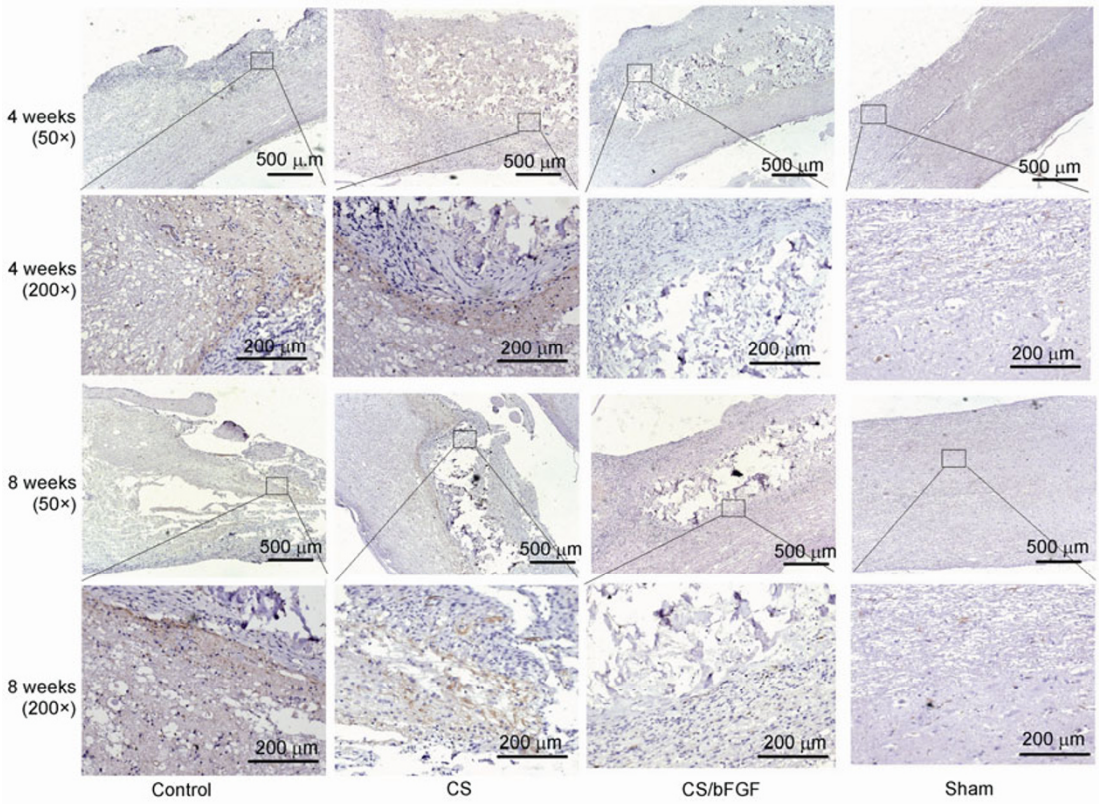

Figure 8 Immunohistochemical staining of glial fibrillary acidic protein (GFAP) for spinal cords at four and eight weeks after operation.

group. The degree of locomotor recovery of the CS/bFGF group was the highest among the SCI groups. The footprint analysis also showed the same trend in limb coordination and body weight support. The phenomenon might be related to the neuroprotective role of CS/bFGF implant and improvement of the systemic status function. BFGF can inhibit apoptosis and increase the survival of neurons, which may be the main mechanisms. BFGF activate some downstream signals, such as PI3K/Akt and ERK1/2 pathways, which are essential for the neuroprotective effect [44]. Particularly, the PI3K/Akt pathway is very important for mediating neuronal survival under conditions of glucolipotoxicity, inflammation, etc. [31,32].

Macroscopically, a large coloboma was formed in the injury spinal cord in the control group. As we expected, the coloboma was completely filled with the scaffold in the CS-treatd and CS/bFGF group. Nerve fiber regeneration was demonstrated by H&E and immunohistochemical staining. At the injury side of the spinal cord, there were more NF-positive neural fibers in the CS/bFGF implant than those in CS-only implant. This biopsy demonstrated that more fibers were guided to grow through the implants in CS/bFGF group than CS-only group. The combination of CS and bFGF through CBD might thus create a more suitable microenvironment for nerve fiber regeneration. In contrast, axonal growth was hardly seen in the control group, in which the higher density of GFAP staining was present than that in other groups. Similar to the results of previous study, the CS treatment decreases the accumulation of glial scar at the lesion site [7]. All the data on functional analysis and histological staining indicated that CS/bFGF treatment was benificial to the neural generation after the lateral hemisection SCI.

For the treatment of SCI, to lay the foundation for the environment permissive for axonal growth, bridging the hemicord gap has become one of the strategies to re-establish neurological structures and functions. Tissue engineering may play an important role in restoring integrity and function of the injured spinal cord. The restoration effect of the implantation system (CS/bFGF) may have many complicated mechanisms. In the early stage, CS not only could bridge the injury gap for axonal regeneration, but also may influence cell migration and act as a carrier for migrating stem cells (such as MSCs, NSPCs, et al.) [24]. Meanwhile, CS fused with bFGF through CBD could be avoided of the rapid diffusion of bFGF, which may be one of the important mechanisms to promote the growth of axons. In addition, as a common biodegradable material, CS fused with bFGF can release bFGF continuously during the recovery period after SCI [33]. In the future clinical applications, this implantation system probably will be considered to be used as a new delivery system of growth factors for SCI patients.

In conclusion, this study shows that implantation of CS/bFGF into a semi-transected SCI rat model can guide axon growth at the injury site and promote obvious improvement in functional recovery. As a result, it could be a promising alternative system for the clinical application of SCI repair.

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