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ORIGINAL ARTICLE

Preparation of the acellular scaffold of the spinal cord and the study of biocompatibility

S-Z Guo, X-J Ren, B Wu and T Jiang

Department of Orthopedics, Xinqiao Hospital, Third Military Medical University, Chongqing, China

Study design: Acellular spinal cord was prepared through chemical extraction, and its biocompatibility was studied.

Objective: Acellular scaffolds have been developed from various materials for tissue reconstruction, except for spinal cord. The objective of this study was to prepare acellular spinal cord and examine the biocompatibility of the scaffold.

Setting: This study was conducted at the Department of Orthopedics, Xinqiao Hospital, The Third Military Medical University, Chongqing, China.

Methods: The morphology of the acellular segments was revealed by scanning electron microscopy, immunohistochemistry, and hematoxylin and eosin stain. Biocompatibility was studied by immuno-histochemistry.

Results: Results show that in spinal cord scaffolds, cells, myelin sheath and axon of nerve fibers were eliminated, and three-dimensional supports of extracellular matrix were reserved. The component analytical results of the acellular spinal cord indicate that they contain laminin, fibronectin and collagen, which can facilitate and induce the regeneration of injured nerves, and enhance the adhesion and proliferation of cells. The acellular spinal cord has a three-dimensional structure and excellent biocompatibility.

Conclusion: Our data indicate that acellular spinal cord has certain biological properties and it may be a potential alternative scaffold for spinal cord tissue engineering.

Introduction

Spinal cord injury usually results in a devastating and permanent loss of function below the injured area. Because central nervous system axons lack the ability to spontaneously regenerate, further compounded by chemical (myelin inhibitors) and physical (for example, glial scar) barriers to regeneration after spinal cord injury, patients normally experience poor functional recovery.[1–2]

Several methods have been investigated to overcome this hostile environment for regeneration and to promote partial functional recovery. Strategies to promote axonal extension through a site of injury include both the provision of nervous system growth factors and the implantation of substrates to support axon extension, such as cellular grafts. In general, however, the growth of axons is highly random and does not extend beyond the lesion site and into host tissue.[3] Recently, researchers have realized that it is

difficult to achieve complete functional recovery by relying on a single method. This has presented tissue-engineering technology as an alternative strategy for the treatment of spinal cord injury. The scaffold can guide the linear growth of axons across a site of injury, in addition to providing neurotrophic and/or cellular support. This will help retain the native organization of regenerating axons across the lesion site and into distal host tissue, eventually increasing the probability of achieving function recovery.

Various natural and synthetic polymeric materials have been used to promote functional recovery after spinal cord injury. Nevertheless, further improvements are needed for all previously reported approaches, which mimic a native extracellular matrix (ECM). Acellular scaffolds are among the various materials that have been recently used in tissue reconstruction.[5] Acellular scaffolds are the noncellular part of a tissue and consist of such proteins as collagen and carbohydrate structures secreted by resident cells. They can be transplanted without rejection and can provide a conducive environment for normal cellular attachment, migration, proliferation, differentiation and angiogenesis, as well as provide a framework for tissue regeneration, as they

are completely replaced by the host tissue.[4,5] In the last few years, acellular matrices have been successfully used to substitute and repair skin,[6] bladder,[7,8] urethra,[9] small bowel,[10,11] cardiac valve,[12] blood vessel,[13] skeletal muscle[14] and peripheral nervous defects[15] among others. In an attempt to mimic the regenerative capacity of the spinal cord graft, we investigated the acellular spinal cord, which provides the physical pathway for axonal regeneration.

Materials and methods

Acellular spinal cord scaffold preparation

Spinal cord was harvested under aseptic conditions from 200–250 g Sprague–Dawley rats and immediately placed in phosphate-buffered saline (PBS) at 4 °C. All subsequent steps were conducted in a laminar flow hood for sterility. Fatty and connective tissue was removed from the nerve epineurium. Nerve tissue was cut into 20 mm segments and placed in deionization-distilled water. All washing steps were carried out at 25 °C with agitation. The spinal cord was rinsed twice (1 h each) in PBS. After 1 h, the water was replaced with 1% Triton X-100 solution. The spinal cord was agitated for 3 h. The tissue was then rinsed with washing solution 0.01% PBS thrice (1 h per rinse). Next, the washing solution was replaced by 1% sodium deoxycholate solution (Sigma, St Louis, MO, USA) for 3 h. After agitation for 3 h, the tissue was rinsed with the washing solution thrice (1 h per rinse). The nerve segments were again agitated in 1% Triton X-100 solution (3 h), washed once and agitated in 1% sodium deoxycholate solution (3 h). Finally, tissue segments were washed thrice (1 h each) in a 0.01% PBS solution and stored in the same solution at 4 °C until use.

All chemicals were purchased from Sigma unless otherwise noted. All solutions were autoclaved or filter sterilized before use.

HE stain and immunostaining

For histology and immunohistochemistry analysis, specimens were embedded in tissue-freezing medium, with temperature fixed at −20 °C. Sections of 8 mm thickness were obtained and routinely stained with haematoxylin and eosin (HE), and immunostained with myelin for collagen types IV, laminin (LM) and fibronectin (FN). The primary antibodies used were the following: (1) polyclonal rabbit anti-mouse collagen type IV, 1:100; (2) polyclonal rabbit anti-mouse Laminin, 1:100; (3) polyclonal rabbit anti-mouse Fibronectin, 1:100, at 4 °C overnight, in a wet chamber. Secondary antibody staining was performed with a SABC kit (Boster Ltd, Wuhan, China). Negative controls were similarly stained, with the primary antibodies replaced with PBS. All slides were counter-stained with haematoxylin and mounted before viewing.

Myelin staining

To visualize myelin, fresh rat spinal cord specimens were extracted and embedded in tissue-freezing medium, set at −20 °C. Sections of 10 mm thickness were obtained and stained using Weil's method. All slides were viewed under a microscope.

Scanning electron microscopy analysis

Scaffolds were sectioned in longitudinal or transverse planes and visualized by scanning electron microscopy (SEM). The sectioned scaffolds were attached to sample stubs, and sputter-coated with gold or palladium. The specimens were observed and photographed using a KYKY-3200 M scanning electron microscope.

Hemolysis test

Samples included the following: (1) acellular spinal cord scaffold 5 g; (2) diluted blood: fresh anticoagulated blood 8 ml was diluted by 10 ml normal sodium; (3) positive control: distilled water 10 ml, with diluted blood 0.2 ml added; (4) negative control: normal sodium 10 ml, with diluted blood 0.2 ml added.

The acellular scaffold was washed twice with distilled water in a test tube, with addition of 10 ml normal sodium. After 30 min in a 37 °C thermostat oven, diluted blood 0.2 ml was added before another 60 min of incubation, followed by 5 min (750 g) centrifugation. The supernatant was obtained and optical density was detected using a spectrophotometer in 545 nm wavelength. With the hemolysis rate $<5\%$, the scaffold is qualified to become a bio-tissue-engineering material; with the haemolysis rate $\geqslant 5\%$, the scaffold is considered to have hemoclasis.

Cytotoxicity test

In vitro biocompatibility was tested through co-incubation of scaffolds with NIH 3T3 cells. NIH 3T3 cells were plated at 50% confluency on 48-well culture plates. One scaffold was placed inside each well containing 2 ml of Dulbecco's modified eagle medium complex culture medium, supplemented with 10% FCS in a 5% CO_2 incubator at 37 °C. The scaffold was positioned in such a way that it was fully submerged in media, and yet physically detached from cells. Media were changed every other day, and the rate of cell proliferation was qualitatively compared with that of control wells. Evidence of cytotoxicity (rounding of cells and lifting from the well surface), if present, was recorded in eight separate wells.

Preliminary examination of in vivo biocompatibility

A bilateral surgical approach was made to implant the acellular spinal cord scaffolds into the subcutaneous back skins of SD rats. Anesthesia was induced with a celiac injection of pentobarbital (30 mg kg^{-1}). Before surgical incision, the back skin was shaved and disinfected with iodophors solution. Thereafter, a small skin incision was made (10 mm long), and a pocket was created through blunt dissection. The scaffolds were then implanted through these skin incisions subcutaneously into the mid-portion of the back areas. The incision was sewed using conventional cotton sutures. Tissue was obtained at 1, 2, 3 and 4 weeks after the operation, with inflammatory reaction evaluated by HE stain. The immunogenicity of the acellular scaffold was tested by immunohistochemical analysis examining the intensity of CD4^{+} and CD8^{+} cells that infiltrated the allografts.

Results

Macroscopic observation

A large amount of white-colored floss was secreted from the spinal cord during decellulation. After being treated, the spinal cord scaffold became ivory white in color and translucent, and yet still shaped as a circular cylinder. However, the diameter shrank to 2/3–4/5 of that of the original spinal cord and the strength decreased slightly, although the tenacity remained unchanged and viscosity increased slightly (Figure 1).

HE staining

Normal spinal cord has generous neurons, glial cells and myelin sheaths (Figure 2a). In a cross-section (Figure 2b), a network of the ECM was seen in the scaffold. The cells, myelin and axons disappeared after the spinal cord was treated with detergents TritonX-100 and deoxycholate. A typical network of empty tubes was viewed in longitudinal sections (Figure 2c).

Myelin staining

The normal myelin sheath of the spinal cord is black and has regulation shape (Figure 3a). In acellular spinal cord, either no myelin sheath is observed or only a small quantity of myelin sheath pieces is detected (Figure 3b).

SEM analysis

In the scaffold, cells have been removed completely, although the ECM and the pore have remained to form three-dimensional network structures. The pore and the channel of the scaffold diameter were 125 ± 25 and $119 \pm 26\,\mu m$, respectively (Figures 4a and b).

Immunohistochemistry

A positive reaction to laminin, fibronectin and IV collagen is seen in both normal spinal cord and acellular spinal cord. Staining is weaker in the acellular scaffold than in normal spinal cord, which indicates that the majority of the ECM is preserved after the decellulation treatment of spinal cords.

Biocompatibility in vitro

A haemolysis rate <5% qualifies scaffolds as bio-tissue-engineering materials. After scaffolds were co-incubated with

Figure 1 Normal spinal cord vs acellular spinal cord. The spinal cord scaffold became ivory white in color and translucent, and yet still shaped as a circular cylinder. The diameter shrank to 2/3–4/5 of that of the original spinal cord and its strength decreased slightly, its tenacity remained unchanged and viscosity increased slightly.
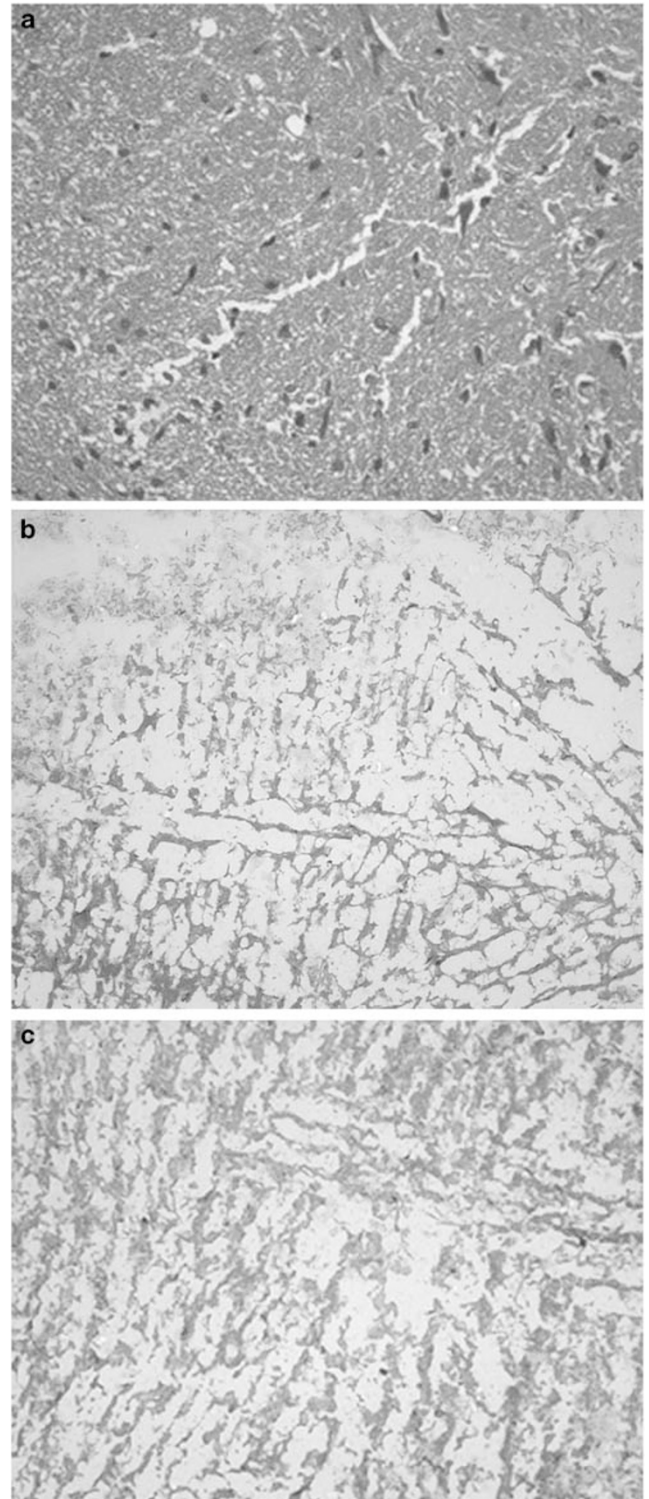
Figure 2 Spinal cord HE staining. Normal spinal cord has generous neurons, glial cells and myelin sheaths (**a**). In a cross-section, a network of the ECM was seen in the scaffold. The cells, myelin and axons disappeared (**b**). A typical network of empty tubes was viewed in longitudinal sections (**c**).
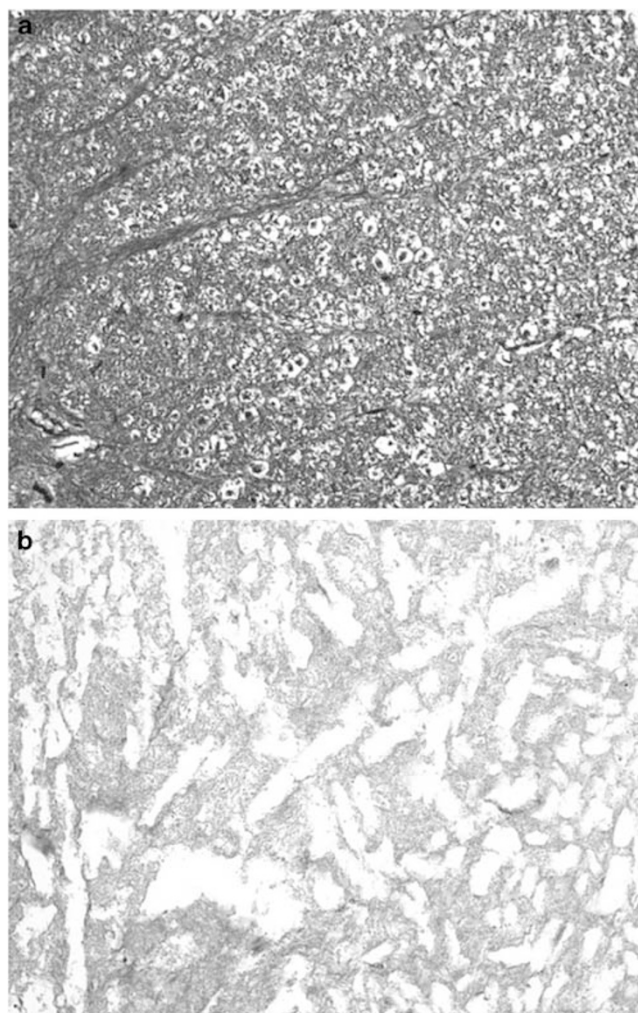
Figure 3 Spinal cord myelin staining. The normal myelin sheath of the spinal cord is black and has regulation shape (**a**). In acellular spinal cord, either no myelin sheath is observed or only a small quantity of myelin sheath pieces is detected (**b**).

Figure 4 Acellular spinal cord SEM observation. In a cross-section of the scaffold, cells have been removed completely (**a**); in longitudinal sections (**b**), the ECM and the pore have remained to form three-dimensional network structures.

NIH 3T3 cells for 72 h (Figure 5b), NIH 3T3 cells showed no signs of cytotoxicity (loss of adherence, nuclear condensation and cell soma contraction) and cells proliferated normally compared with those in control wells (Figure 5a), expanding from approximately 50–100% confluency within 72 h.

Histocompatibility in vivo
Lymphocytes, neutrophilic granulocytes and fibroblasts were seen in control groups (Figures 6b) and experimental group animals (Figure 6a). The degree of infiltration in experimental groups was significantly weaker than that in control groups after 1 week of implantation. There was no obvious increase in implanted infiltrated cells and neutrophilic granulocytes vanished after 4 weeks. However, there was multiplicity lymphocyte and neutrophilic granulocyte infiltration in control groups after 4 weeks of implantation.
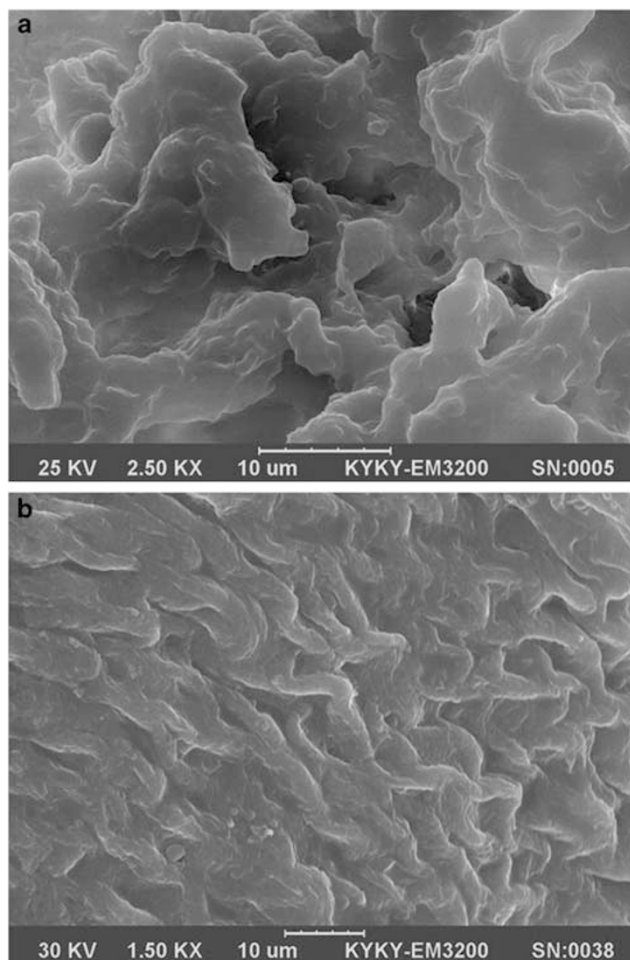
$CD4^+$ and $CD8^+$ leukomonocyte infiltration was spared 1 week and 2 weeks after implantation. Moreover, there was no obvious increase in experimental groups. There was massive $CD4^+$ and $CD8^+$ leukomonocyte infiltration after implantation, and the staining intensity of positive cells was obviously stronger compared with that of experimental groups.

Discussion

The disruption of spinal cord motor and sensory pathways after traumatic injury to the central nervous system has devastating consequences for injured patients. Tissue engineering is one of the most promising methods to restore central nerve systems in human health care. Tissue-engineering strategies that create nerve bridges through injured spinal cord involve the use of either cellular or cell-free bridges or a combination of both. Theoretically, scaffolds that have been created for guiding axonal regeneration should have pores small enough to physically align and
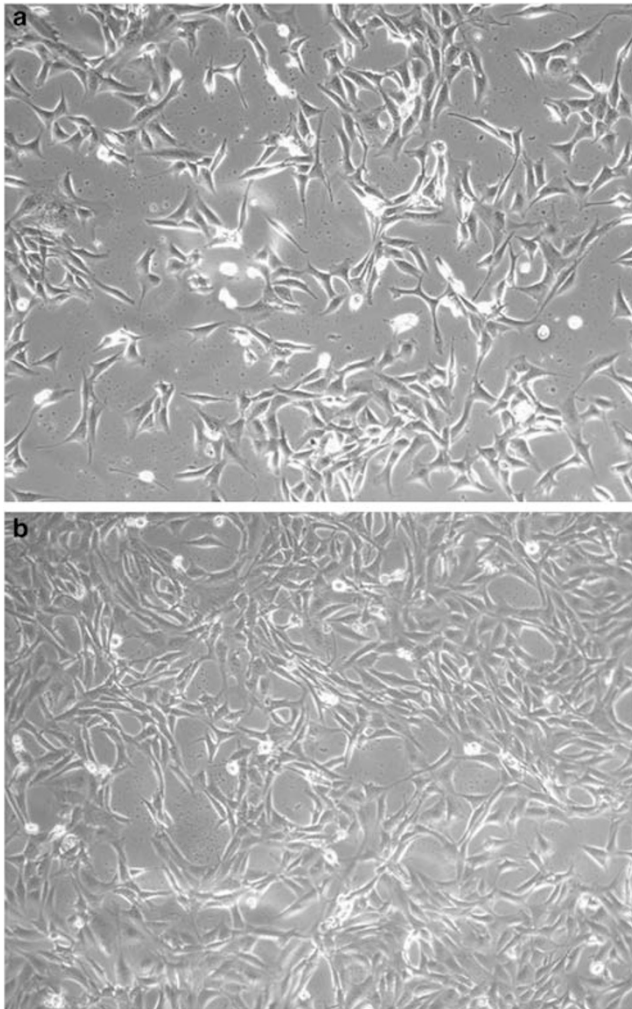
Figure 5 NIH3T3 cells co-incubated for 72 h. After scaffolds were co-incubated with NIH 3T3 cells for 72 h (**b**), NIH 3T3 cells showed no signs of cytotoxicity (loss of adherence, nuclear condensation and cell soma contraction) and cells proliferated normally compared with cells in control wells (**a**), expanding from approximately 50–100% confluency within 72 h.

Figure 6 Spinal cord allografted subcutaneouly embedded CD4$^+$ cells. Immunohistochemistry staining BC × 400. CD4$^+$ lymphocytes were seen in control groups (**b**) and experimental group animals (**a**). The degree of infiltration in experimental groups was significantly weaker than that in control groups after 2 weeks of implantation.

restrict the direction of growing axons, yet large enough to allow for vascularization and the infiltration of cells, which might support regeneration. Nevertheless, so far, no materials have been found that can meet the requirement desirably.

Presently, natural or artificial synthetic materials are used as the tissue-engineering scaffold of spinal cord.[16–19] As their pores and channels are artificial, it is difficult to completely simulate the conduction channels of normal spinal cords. Moreover, these scaffold materials have their respective disadvantages, such as degradation products that are unfit for neuronal survival and axonal regeneration; low adhesiveness of cells; and much poorer biological and physical characteristics. Because of the limitation of the manufacturing technique and the complexity of spinal cord tissue structure, it is hard to create a three-dimensional network structure that is highly similar to normal spinal cord.

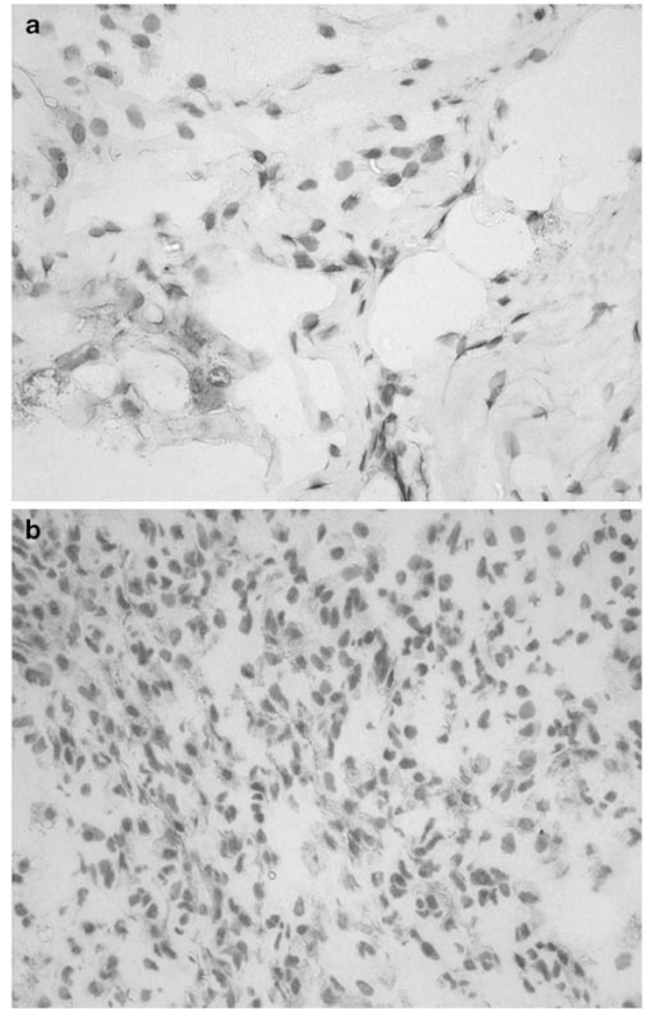A recent tendency involves the use of the natural decellularated matrix (bio-derived materials) as repair materials for nerve tissue. Many types of tissues, including skin, cornea, mucosal membranes, cartilage, peripheral nerve and skeletal tissues, have been engineered using acellular scaffold. Some reports have shown that nerves made myelin-free by extraction have very low immunogenicity, which supports regeneration and leads to functional recovery.[6,14] Ribatti D et al.[20] successfully prepared acellular brain scaffolds using chemical extraction and found that acellular brain scaffolds induce a strong angiogenic response.

Our study first showed that segments from the spinal cord of Sprague–Dawley rats can be successfully extracted to become acellular. The outcome of the extraction was monitored by morphological methods and immunohistochemistry. The extraction procedure involved the removal of myelin following Weil's myelin staining, and cells, leaving a largely intact ECM. Immunohistochemistry analysis revealed the presence of laminin, fibronectin and IV collagen in the ECM.

The scaffold of spinal cord is an emulated three-dimensional natural spinal cord, which has fundamentally distinct and innate superiority over biological degradation materials. It is easy to obtain and is not confined by length or caliber. It can undergo isoloci transplantation and anatomy transplantation.

The extracted spinal cord was invaded by $CD4^+$ and $CD8^+$ cells to a much lesser extent than an allologous spinal cord graft. We speculate that this difference is mainly because myelin and cells were removed during the extraction procedure.

The acellular spinal cord scaffolds created in this study have a number of positive properties that can potentially support axonal regeneration after nervous system injury. The scaffolds are soft and flexible, containing linear guidance pores extending through their full length. Because the scaffolds are stable under physiological conditions, there is no risk of introducing toxic molecules to the site of injury. On the basis of current findings, we believe that extracted spinal cord could be used as allografts, with the possibility of becoming useful for spinal cord repair in the future. Ongoing work *in vivo* will test their ability to support axonal regeneration after spinal cord injury. The experiments of functional nerve recovery and microscopical (and morphometric) analysis will be evaluated in more detail.

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