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Biocompatibility evaluation of silk fibroin with peripheral nerve tissues and cells in vitro

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Abstract

Silk-based materials have been used in the field of bone or ligament tissue engineering. In order to explore the feasibility of using purified silk fibroin to construct artificial nerve grafts, it is necessary to evaluate the biocompatibility of silk fibroin material with peripheral nerve tissues and cells. We cultured rat dorsal root ganglia (DRG) on the substrate made up of silk fibroin fibers and observed the cell outgrowth from DRG during culture by using light and electron microscopy coupled with immunocytochemistry. On the other hand, we cultured Schwann cells from rat sciatic nerves in the silk fibroin extract fluid and examined the changes of Schwann cells after different times of culture. The results of light microscopy, MTT test and cell cycle analysis showed that Schwann cells cultured in the silk fibroin extract fluid showed no significant difference in their morphology, cell viability and proliferation as compared to that in plain L15 medium. Furthermore, no significant difference was found in expression of the factors secreted by Schwann cells, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and S-100, between Schwann cells cultured in the silk fibroin extraction fluid and in plain L15 medium by the aid of immunocytochemistry, RT-PCR and Western analysis. Collectively, these data indicate that silk fibroin has good biocompatibility with DRG and is also beneficial to the survival of Schwann cells without exerting any significant cytotoxic effects on their phenotype or functions, thus providing an experimental foundation for the development of silk fibroin as a candidate material for nerve tissue engineering applications.

Keywords: Silk fibroin; Dorsal root ganglia; Schwann cell; Biocompatibility; Cytotoxicity

1. Introduction

Peripheral nerve repair remains a common but challenging clinical problem. Direct end-to-end suturing is suggested for a short nerve injury. For larger nerve defects or gaps, implantation of a nerve graft is often necessary to bridge the proximal and distal nerve stumps for facilitating nerve regeneration and functional recovery. The typical choice is a nerve autograft that is harvested from another site in the body. However, this recognized ''gold standard'' technique for peripheral nerve repair is limited by tissue availability, donor-site morbidity, secondary deformities, as well as potential differences in tissue structure and size [1,2]. Xenografts and allografts have been tried, but they are subject to immunosuppression and have achieved very poor success [3–7].

Much effort has been devoted to seeking promising alternatives to conventional nerve autografts, and the emergence of tissue engineering has greatly stimulated the development of artificial nerve grafts, or nerve guidance channels [8–10]. In recent years, many synthetic or natural biopolymers such as polyglycolic acid, polylactic acid, chitosan, alginate and their composites or derivatives have been utilized to construct nerve guidance channels, showing considerable potential of these materials to find their applications in nerve tissue engineering [11–15].

Native silkworm silk protein from *Bombyx mori* consists of a core structural fibroin protein surrounded by sericin, a family of glue-like proteins. A highly repeated hydrophobic and crystallizable sequence has been described for the primary structure of fibroin heavy chain: Gly-Ala-Gly-Ala-Gly-*X* (*X* stands for Ser or Tyr). Sericin is a more

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hydrophilic protein, whose primary structure is richer in polar residues, but some of its fractions are not completely water-soluble due to β-sheet portions [16–19].

Silks have a long history of use as suture materials in the medical field because of their impressive mechanical properties and flexibility [20–23]. However, undesirable immunological reactions associated with silks diminished their clinical applications during the last years [16,24,25]. The identification of contaminating sericin as the source of immunological problems sparks new interest in silk fibroin as a native biomaterial. Hence purified silk fibroin has found broad applications in pharmaceutical and biomedical fields [26–31], especially in tissue engineering for the generation of artificial bones or ligaments [16,32–38]. However, to our knowledge, there have hitherto been few reports on the utility of silk-based or silk-coated materials for peripheral nerve repair. Based upon this, we cultured rat dorsal root ganglia (DRG) on the substrate made up of silk fibroin fibers to examine the in vitro biocompatibility of silk-based materials with peripheral nerve tissues, and also cultured Schwann cells from rat sciatic nerves in the silk fibroin extract fluid to evaluate (as per International Standards ISO 10993-5) the in vitro cytotoxicity of silk-based materials for peripheral nerve cells.

2. Materials and methods

2.1. Preparation of silk fibroin and its extract fluid

Bombyx mori silk, obtained from Xinyuan sericulture company, Nantong, Jiangsu, China, was degummed by boiling twice, 0.5 h each, in aqueous Na_2CO_3 solution (0.5%), in order to remove the sericins. Degummed silk was thoroughly washed in hot double distillated water for several times, and then dried at room temperature. The resulting silk fibroin needed to be sterilized by autoclave prior to use [39,40].

After sterilization, silk fibroin (1 g) was placed in an extraction container with an addition of 10 ml of L15 medium (Sigma) and allowed to incubate at $37\pm0.5\,^{\circ}C$ for 72 ± 0.5 h [39]. The resulting silk fibroin extract fluid had to be used within 24-h period. For control, the extraction fluids of hydroxyapatite, obtained from Sigma, or organotin, from Hongding Chemicals Company, Nantong, Jiangsu, China, were prepared in the same manner, respectively.

2.2. Tissue and cell culture

In this study, all experimental procedures involving animals were conducted as per Institutional Animal Care guidelines and approved ethically by the Administration committee of experimental animals, Jiangsu Province, China.

For tissue culture, silk fibroin fibers were sheared into 0.7–0.8 cm long fragments which were then soaked in L15 medium for about 30 min. The rat DRG were obtained from neonatal Sprague-Dawley rats, 1–2-d old, and stripped with connective tissue carefully at their surface. The treated DRG together with silk fibroin fragments were placed onto a 24-well culture plate. Afterwards, L15 medium supplemented with 15% fetal bovine serum (FBS) was added to the culture plate (800 μl per well), followed by incubation at 37 °C in a humidified, 5% CO_2 atmosphere. Half of the medium was replaced every other day. At different culture times, the cell growth of DRG was observed under an inverted light microscope (Olympus IX-70, Japan).

For cell culture, rat Schwann cells were harvested as follows. The bilateral sciatic nerves of neonatal Sprague-Dawley rats (1–2-d old) were excised out under sterile condition. After the epineurium and perineurium were removed from sciatic nerves carefully, the nerves were sheared into 0.01 mm^3 tissue fragments which were then planted onto a polylysine-coated coverslip in a 6-well tissue culture plate. L15 medium supplemented with 15% FBS was added to the plate (2 ml per well), followed by incubation at 37 °C, with humidity and 5% CO_2. Half of the medium was replaced every other day. After 7 d incubation, the subcultured Schwann cells from sciatic nerves of neonatal Sprague-Dawley rats were seeded in a 96-well plate at a cell density of 1×10^4/well and the plain L15 medium or different extract fluids were added to the plate, respectively. At different times of culture, the morphological changes of Schwann cells in these different culture mediums were observed under an inverted light microscope.

2.3. Immunocytochemistry

After 4 or 7 ds of culture of DRG on silk fibroin, the samples were fixed with a fresh-prepared 4% paraformaldehyde solution in PBS for 30 min at room temperature. The fixed samples were incubated for 1 h in a solution containing 10% goat serum, 3% bovine serum albumin and 0.1% Triton-X 100 at room temperature to block nonspecific binding. Then, they were allowed to incubate with mouse monoclonal anti-S-100 antibody (1:1200 dilution, Sigma) overnight at 4 °C in a humidified chamber. After being washed three times with PBS, the samples were further reacted with second antibody FITC-labeled goat anti-mouse IgG (1:250 dilution, Sigma) for 2 h at 37 °C. The samples were washed three times with PBS, mounted in fluorescent mounting medium and observed under a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany). Controls included leaving out the primary antibody and using non-labeled secondary antibodies to confirm inexistence of nonspecific binding.

After 24-, 48- or 72-h culture either in the plain L15 medium or the silk fibroin extract fluid, Schwann cells were incubated with 5 μg/ml Hoechst 33342 (Sigma) at 37 °C for 1 h and then fixed and blocked as the above mentioned. Afterwards, they were incubated with mouse anti-S-100 antibody (1:1200 dilution, Sigma) and rabbit polyclonal anti-rat nerve growth factor (NGF) antibody (1:1000, Chemicon, Temecula, CA, USA) or with mouse anti-S-100 antibody (1:1200 dilution, Sigma) and rabbit anti-rat brain-derived neurotrophic factor (BDNF) polyantibody (1:1000, Chemicon) at 4 °C overnight in a humidified chamber, respectively. After being washed with PBS three times, the samples were further reacted with FITC-labeled goat anti-mouse IgG (1:250 dilution, Sigma) and Cy3-labeled goat anti-rabbit IgG (1:100) at 37 °C for 2 h. Finally, the samples were viewed under a confocal laser scanning microscope. Controls were treated similarly as the above mentioned.

2.4. Electron microscopy

After 4- or 7-d incubation of DRG on the silk fibroin fibers, the samples were washed twice with PBS and fixed in 4% glutaraldehyde. They were then post-fixed with 1% OsO_4, dehydrated stepwise in increasing concentrations of ethanol, and dried in a critical point drier (Hitachi, Tokyo, Japan). Afterwards, the samples were coated with gold in a JFC-1100 unit (Jeol Inc., Japan) and observed under a scanning electron microscope (JEM-T300, Jeol Inc., Japan).

After 21-d incubation of DRG onto the silk fibroin fibers, the samples were washed twice with PBS and fixed in 4% glutaraldehyde. They were then post-fixed with 1% OsO_4, dehydrated stepwise in increasing concentrations of ethanol, and embedded in Epon 812 epoxy resin. Semi-thin sections were stained with toluidine blue, and ultrathin sections were then localized and viewed with a transmission electron microscope (JEM-1230, JEOL Ltd., Japan).

2.5. MTT assay

A modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, in which the yellow MTT is reduced to a purple formazan

by mitochondrial dehydrogenase in cells, was used to assess the cell viability.

After 12-, 24-, 48- or 72-h or 7-d incubation in different mediums, the viability of Schwann cells was assessed. Briefly, Schwann cells were washed three times with L15 medium. The culture medium in each well of the plate was replaced with 100 μl L15 medium and 25 μl MTT (5 mg/ml in PBS). After 4 h incubation at 37 °C, the reaction solution was carefully removed from each well and 100 μl dimethyl sulfoxide was added. The plates were gently agitated until the formazan precipitate was dissolved, followed by measurement of OD values by spectrophotometry at 490 nm with an ElX-800 Microelisa reader (Bio-Tek Inc., USA).

The serum concentration in the silk fibroin extract fluid was decreased in a graded manner to 10%, 5% even 2.5%, and the serum concentration in the plain L15 culture medium was also decreased in the same manner.

2.6. Cell cycle test

After 24-, 48- or 72-h incubation either in the plain L15 medium or the silk fibroin extract fluid, the Schwann cells were collected by trypsinization (10^6 per tube) and washed twice (5 min per time) with PBS via centrifugation at 1000 rpm. The cells were suspended in PBS, fixed with 70% ethanol (v/v) at −20 °C. Samples were washed with PBS and stained with PI/RNase Staining Buffer (BD PharMingen) for 30 min in dark at 4 °C. The number of cells at different phases of the cell cycle was analyzed using a flow cytometer (BD FACScalibur, BD Bioscience, USA).

2.7. RT-PCR analysis

After 12-, 24- or 48-h culture either in the plain L15 medium or the silk fibroin extract fluid, the Schwann cells were homogenized in Trizol Reagent (Gibco, Gaithersburg, MD, USA). Total RNA was extracted and reverse transcriptized according to the manufacturer's instructions. The sequences of primers for S-100, NGF, BDNF and GAPDH (used as an internal control) are shown in Table 1. The PCR reaction was conducted in a total volume of 25 μl containing 1 μl cDNA of samples, 2.5 μl 10 × PCR buffer, 2.0 μl $MgSO_4$, 2.5 μl dNTPmix (2 mmol/l), 0.5 μl sense primer (10 μmol/l), 0.5 μl antisense primer (10 μmol/l), 0.2 μl Taq DNA Polymerase (2 U/μl) and 15.8 μl H_2O. The reaction mixture was heated to 94 °C for 2.5 min and then amplified for 31 cycles (S-100), 31 cycles (NGF), 33 cycles (BDNF), or 26 cycles (GAPDH), respectively, as specified: 94 °C for 40 s (denaturation), 53 °C for 40 s (annealing) and 72 °C for 35 s (extension). Amplification products were separated by a 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.8. Western blot analysis

After 24-, 48- or 72-h culture either in the plain L15 medium or the silk fibroin extract fluid, the Schwann cells were washed with PBS and lysed with lysis buffer containing protease inhibitors (Promega, Madison, WI, USA). Protein concentration was detected by BCA method (calibrated on bovine serum albumin) to maintain the same loads. Protein extracts were heat denatured at 100 °C for 5 min, electrophoretically separated on a 12% SDS-PAGE, and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBST buffer (50 mM Tris–HCl, 100 mM NaCl, and 0.1% Tween-20, pH 7.4) and incubated with a 1:600 dilution of rabbit anti-rat S-100 polyclonal antibody in 5% non-fat dry milk in TBST buffer at 4 °C overnight. The membranes were washed with TBST buffer (5 min × 3), and further incubated with a 1:200 dilution of HRP-conjugated goat anti-rabbit IgG at room temperature for 1.5 h. After the membrane was washed, the HRP activity was detected using an ECL kit. The image was scanned with GS800 Densitometer Scanner (Bio-Rad, Hercules, CA, USA), and the data of optical density were analyzed using PDQuest 7.2.0 software (Bio-Rad). β-actin (1:4000) was used as an internal control.

2.9. Statistical analysis

At least three repetitive tests were performed, and all data were expressed with means ± SEM. A one-way ANOVA with the Stata 6.0 software package (Stata Corp., College Station, TX, USA) was used to conduct statistical analysis, and statistical significance was accepted at the 0.05 confidence level.

3. Results

3.1. Silk fibroin fibers as substrate for cell outgrowth of DRG

Transmission electron microscopy showed that the transverse section of the degummed silk threads was a homogeneous entity in an olivary shape with a maximum diameter of about 15 μm and had smooth and complete edges without surface attachments. But undegummed silk threads were wrapped with a layer of materials of a varied thickness, which were assumed as sericin (Fig. 1).

Light microscopy was used for visualizing the cell growth of DRG on silk fibroin fibers. After 24-h culture of DRG on the silk fibroin bundles (3–8 fibers per bundle), cells started to crawl out of DRG. Among them, some cells were spherical in shape and adhesive to the silk fibroin material; some cells were spindle in shape and suspended in the culture medium. After 3–4-d culture, the large number of cells migrated out of DRG. Some of them enwrapped the silk fibroin material in cell groups; some of them singly attached to the material and gradually turned into a spindle shape encircling the material; while the neurites were noted to elongate from the neurons of DRG and extend along the material. After 7 and more days of culture, more number of cells migrated out of DRG, and they spiraled tightly along the silk fibroin material and enwrapped it in a multilayer fashion, forming long cell chains. Immunocytochemistry further indicated that there existed S-100 positive cells, identified as Schwann cells, which attached to and encircled the silk fibroin material in virtue of their relatively strong green fluorescence emission (Fig. 2).

Table 1
Oligonucleotide primers for detecting mRNA levels of GAPDH, NGF, BDNF and S100

	Sense (5′–3′)	Anti-sense (5′–3′)
GAPDH	tggtgaaggtcggtgtgaac	ttcccattctcagccttgac
S-100	tgcttgtcctctgtgcaaac	ccatcattcctcagcagctc
NGF	gatcggcgtacaggcagaac	ggctcggcacttggtctcaa
BDNF	gtcaagtgccttggagcct	cttatgaaccgccagccaat

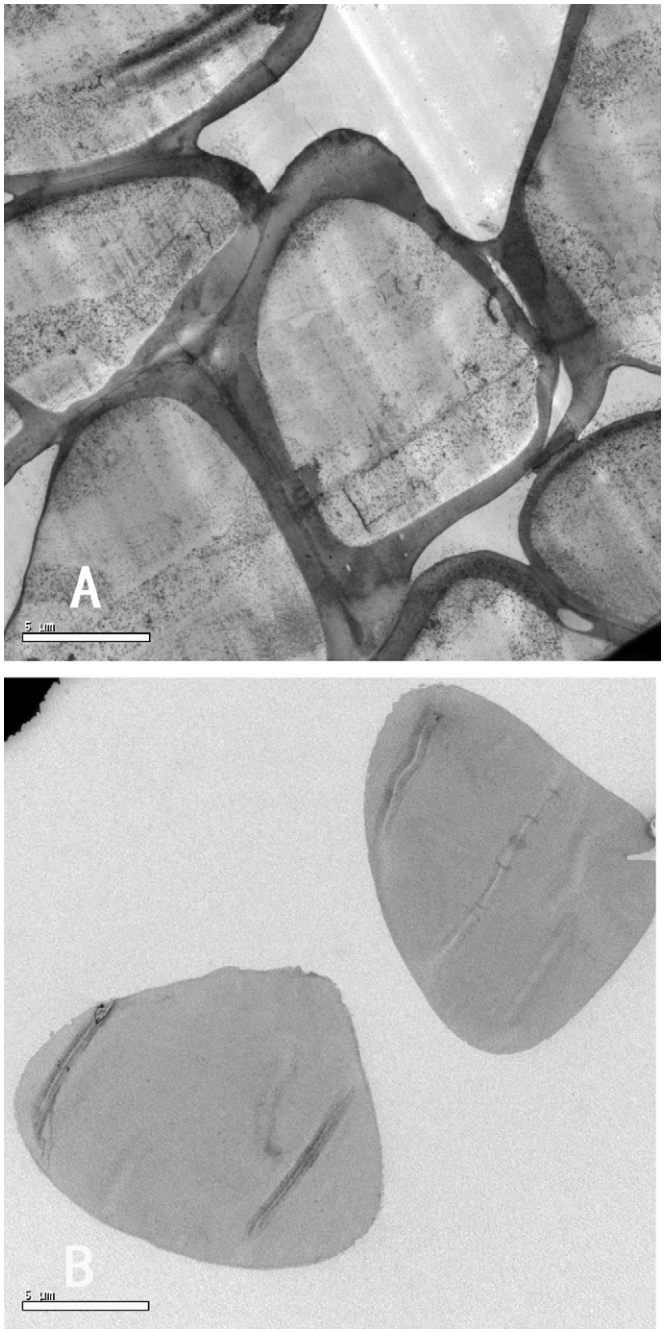

Fig. 1. Transmission electron micrographs of transverse sections of the undegummed (A) and degummed (B) silk threads.

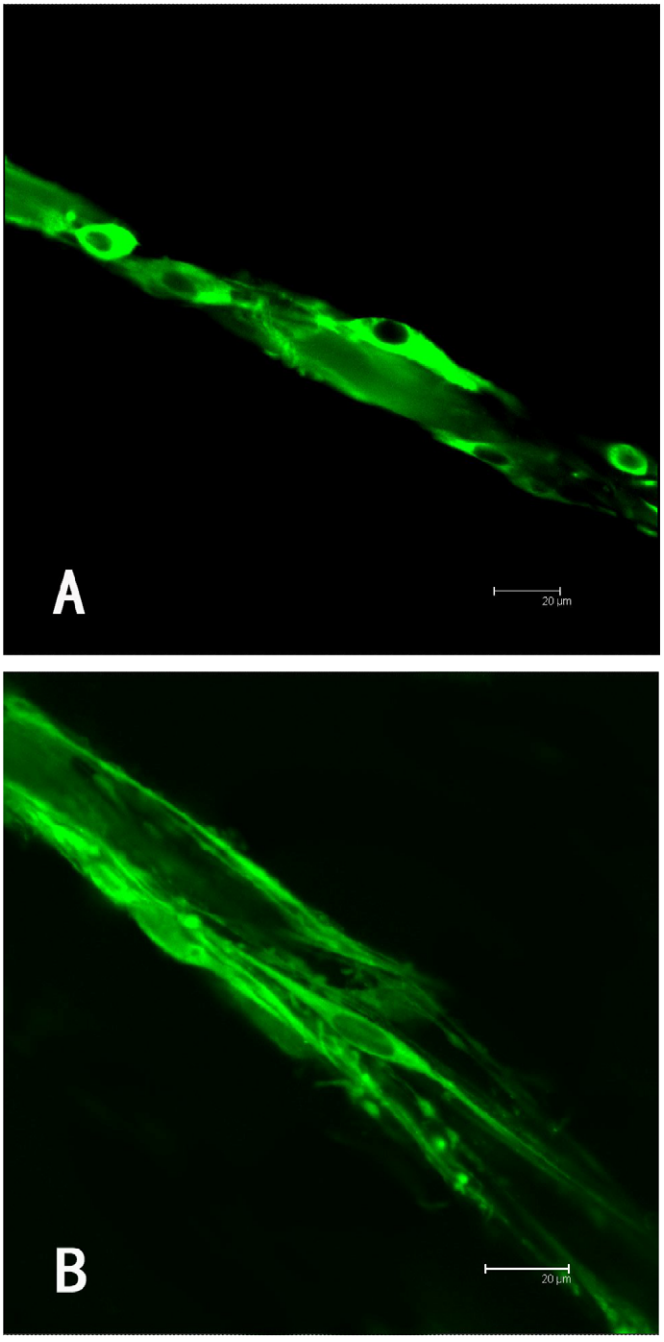

Fig. 2. Immunocytochemistry obtained after 4-d (A) or 7-d (B) culture of DRG on the silk fibroin fibers. Green color represents S-100 immunopositive cells that might be considered Schwann cells.

Visual inspection under scanning electron microscopy further showed that after 4-d culture, cells tightly attached to and partly coiled about the silk fibroin fibers and they exhibited either a spherical or spindle shape; after 7-d culture, a large quantity of cells took compact arrangements of either side-by-side or end-to-end configuration, forming a single- or multi-layer structure to enwrap the silk fibroin fibers (Fig. 3).

Transmission electron micrograph of the transverse sections of silk fibroin fibers demonstrated that Schwann cells enwrapped the silk fibroin fibers completely or partly, and they had a clear and complete inner structure and a normal appearance; on the other hand, axons grew in parallel along the silk fibroin fibers, while Schwann cells separated the axons into bundles and enwrapped some of the axons completely or partly (Fig. 4). The typical arrangement of non-myelinated nerve fibers that were enveloped by the plasmalemma of Schwann cells was also observed in Fig. 4B.

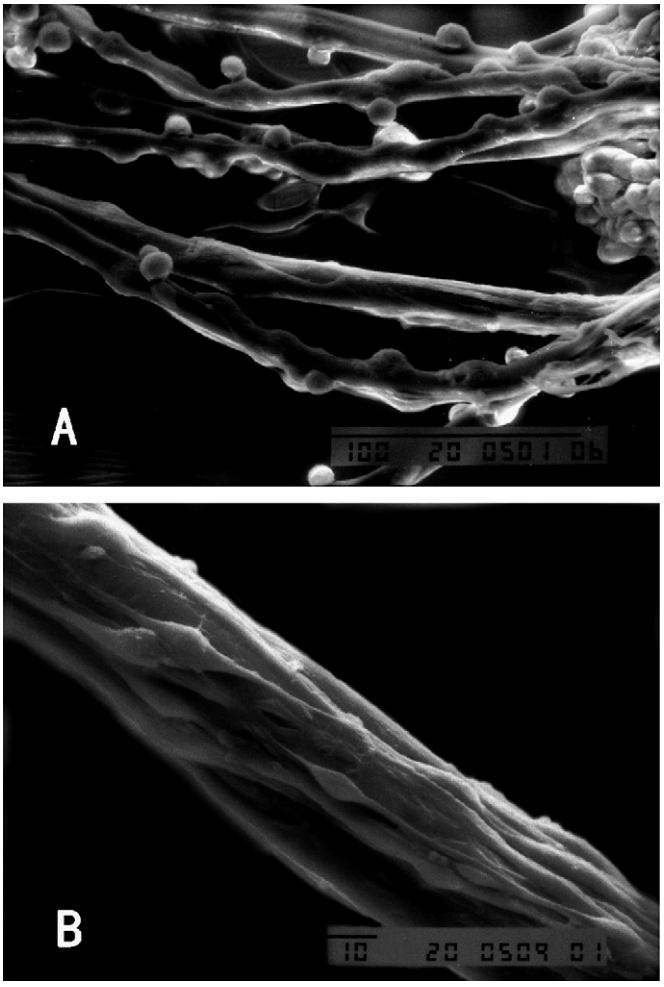

Fig. 3. Scanning electron micrographs of DRG cultured on the silk fibroin substrate, obtained after 4-d (A) or 7-d (B) culture.

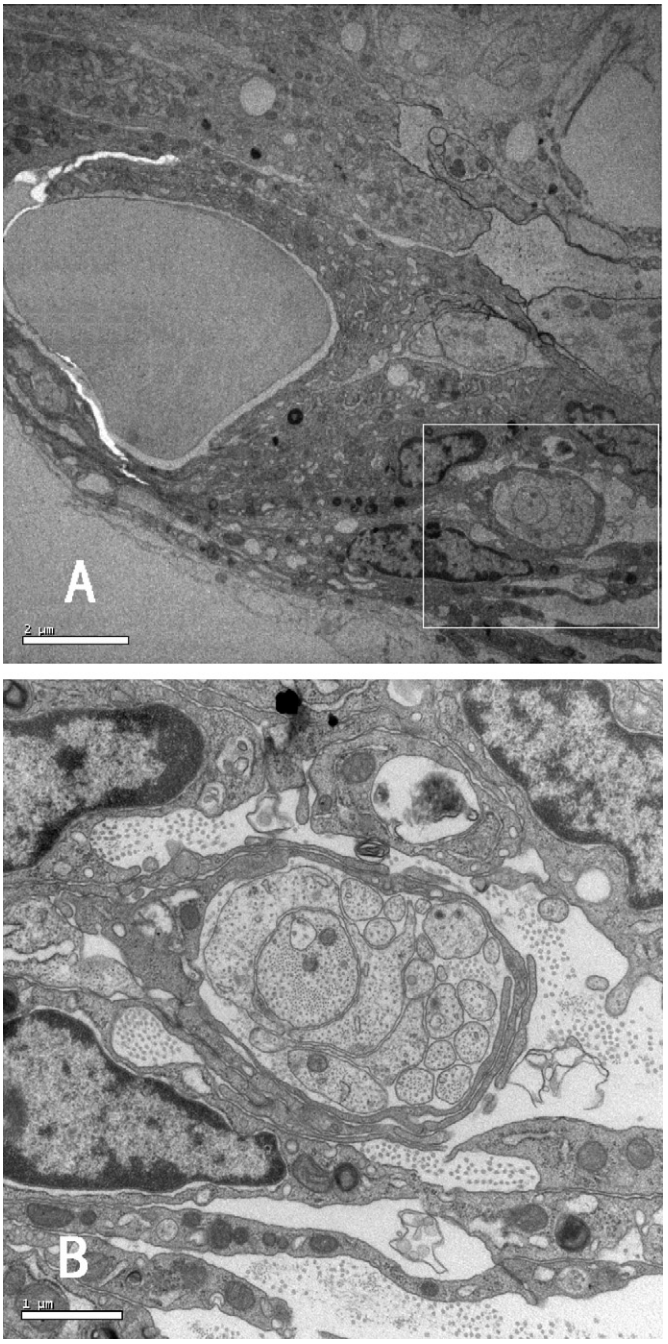

Fig. 4. (A) Transmission electron micrographs after 21-d culture of DRG on the silk fibroin fibers. (B) The higher magnification of the boxed area in (A) which showed the typical arrangement of non-myelinated nerve fibers that were enveloped by the plasmalemma of Schwann cells.

3.2. Morphology and survival analysis of Schwann cells in silk fibroin extraction fluid

Schwann cells were cultivated in the plain L15 culture medium and the silk fibroin extract fluid, respectively, for 12, 24, 48, 72 h and 7 d. No significant difference was found for the cell morphological status between these two different mediums throughout the cultivation process, namely, Schwann cells took a spindle shape with clear edges and shiny, transparent bodies in each medium. Schwann cells were also unchanged when cultivated in the hydroxyapatite extract fluid. In the organotin extract fluid, however, after 0.5-h culture a portion of Schwann cells retracted their processes; after more 0.5-h culture, the cell body of Schwann cells turned into round in shape, even some of Schwann cells detached from the culture plate; and after more 1-h culture, a majority of Schwann cells died and floated off, suspending in the organotin fluid.

Fig. 5 presents MTT assay results. The viability of Schwann cells cultured in the silk fibroin extract fluid was not significantly different from that in the plain L15 medium at all time points after plating. The viability of Schwann cells cultured in the hydroxyapatite extract fluid was lower after 12 h culture but is not significantly different after 24, 48, or 72 h or 7 d of culture, when compared to that either in the plain L15 medium or silk fibroin extract fluid. Contrarily, the viability of Schwann cells cultured in the organotin extract fluid was significantly lower than that in each of other culture mediums at all time points after plating.

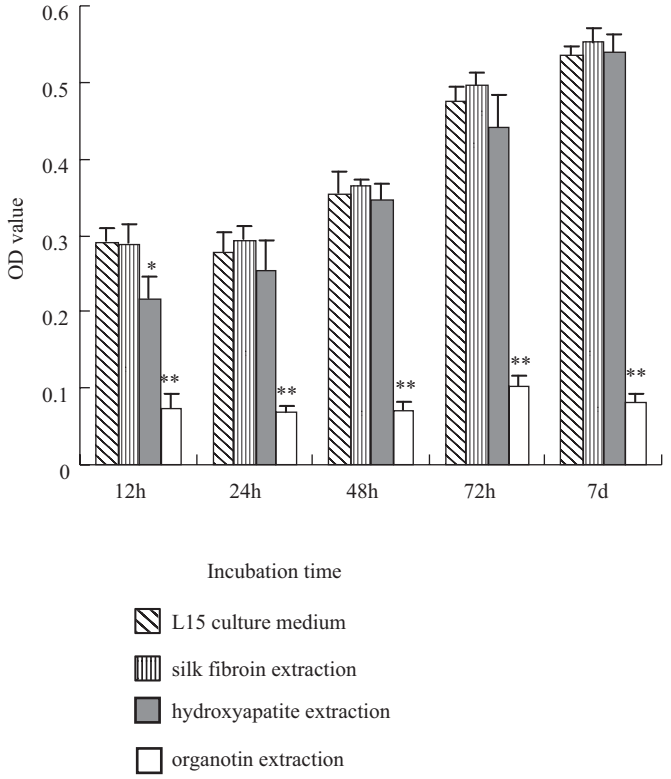

Fig. 5. The changes in the cell viability of Schwann cells after they were cultured in the plain L15 medium or different extraction fluids for 12, 24, 48, 72 h or 7 ds, respectively. Both $^{*}p<0.05$ and $^{**}p<0.01$ versus the corresponding values in the plain L15 medium.

3.3. Proliferation of Schwann cells in silk fibroin extraction fluid

According to the results of cell cycle analysis, no significant difference in cell proliferation, as indicated by proliferation index ((G2M + S)/(G0G1 + G2M + S), %), was found between Schwann cells cultured in the silk fibroin extract fluid and in the plain L15 medium after 24, 48, or 72 h of culture (Fig. 6).

3.4. Expression of the factors secreted by Schwann cells in silk fibroin extraction fluid

The mRNA and protein levels of NGF or BDNF secreted by Schwann cells that were cultured either in the plain L15 medium or the silk fibroin extract fluid were determined, respectively, by using immunocytochemistry, RT-PCR and Western blot analyses. No significant difference either in mRNA or protein levels was shown between in two culture mediums (Figs. 7 and 8). Similarly, Schwann cells cultured in the silk fibroin extract fluid and in the plain L15 medium exhibited no significant difference in the levels of S-100 mRNA or protein that is an important cell marker for Schwann cells (Figs. 8 and 9).

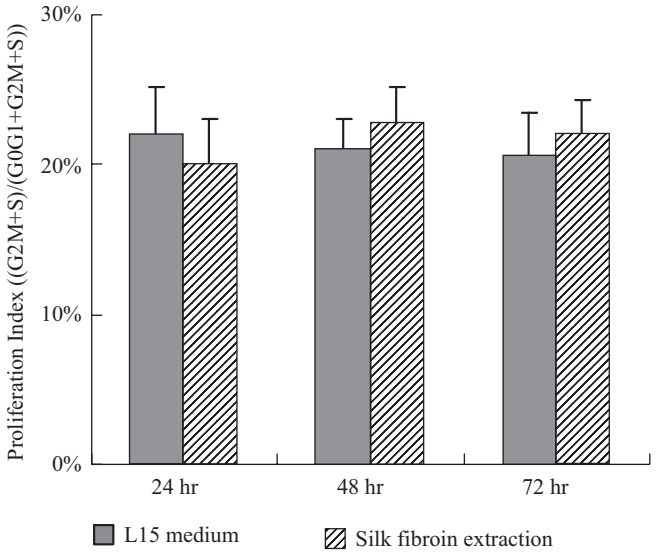

Fig. 6. Proliferation index ((G2M + S)/(G0G1 + G2M + S), %) of Schwann cells after they were cultured in the plain L15 medium or silk fibroin extraction fluid for 24, 48, 72 h, respectively.

4. Discussion

Since the contaminating sericin that surrounds the core silk fibroin protein is identified as the source of undesirable immunological reactions associated with silks, it is important to develop procedures for silk purification by removing sericin and retaining silk fibroin. This purification is commonly referred to as a degumming process of silk. In this study, boiling in aqueous Na_2CO_3 solution was used as a degumming method, which has been proved successful to achieve the complete removal of sericin and preserve the material homogeneity without effects on the structure of silk fibroin.

In order to examine the adhesion, spread and growth of neural tissue-derived cells on the support of silk fibroin, culture of rat DRG on silk fibroin fibers was conducted. After 4 d of culture, light microscopy revealed that in addition to oriented migration of Schwann cells along the silk fibroin fibers, also observed was the outgrowth of neurites within DRG and their attachments to the silk fibroin fibers. After 21 d of culture, transmission electronic microscopy revealed that axons in bundles were paralleled to the silk fibroin fibers and were wrapped with layers of Schwann cell processes. These results suggest a good biocompatibility existing between silk fibroin and neurites or Schwann cells of peripheral nerve tissues.

Schwann cells are the primary structural and functional cells that play a crucial role in peripheral nerve regeneration. Once a peripheral nerve is damaged, Schwann cells will alter their morphology, behavior and proliferation, being involved in Wallerian degeneration and Büngner bands. Schwann cells form myelin sheaths surrounding axons, guide and promote axon

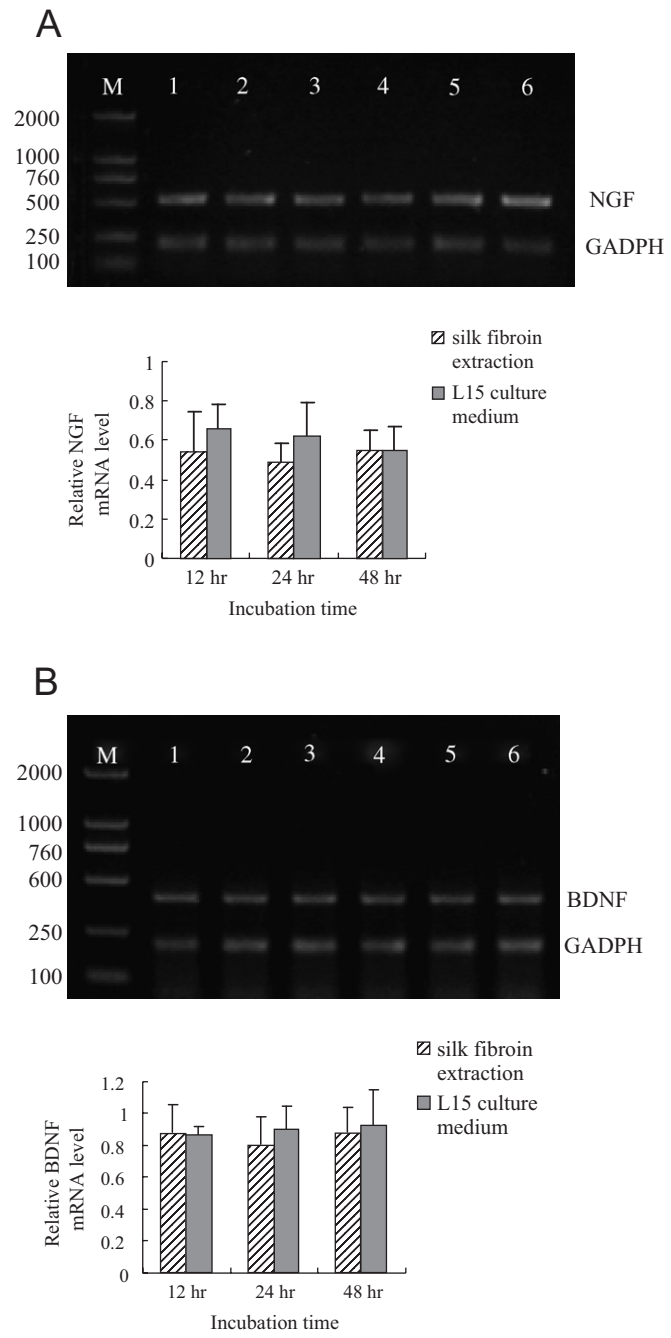

Fig. 7. The changes in mRNA levels of NGF (A) and BDNF (B) secreted by Schwann cells after they were cultured in the silk fibroin extraction fluid for 12, 24, 48 h (marked with 1, 2, 3) or in the plain L15 medium for 12, 24, 48 h (marked with 4, 5, 6). Lane M: DNA molecular weight standard marker (DL 2000).

growth to establish a precise innervation [41–43]. Therefore, it is important to gain a thorough insight into the in vitro biocompatibility between silk fibroin and Schwann cells, especially to carry out the cytotoxicity test as per International Standards compiled in ISO 10993-5. For this reason, we focused on testing whether or not the silk fibroin extract fluid had cytotoxic effects on Schwann cells that were harvested from rat sciatic nerves.

In this study, MTT assay results suggest that silk fibroin extract fluid has no effects on the survival of Schwann cells cultured in it. It was suspected that in MTT tests, the cytotoxic effects might be completely or partially masked by high concentration of serum in the culture medium. To check this suspicion, the serum concentration in the silk fibroin extract fluid or the plain L15 culture medium was changed in a graded manner. The comparison of Schwann cell viability between silk fibroin extract fluid and L15 medium were shown to be independent on the serum concentration despite a decrease in Schwann cell viability for both mediums with the serum concentration decreasing.

Since the proliferation index, referred to as the ratio of (G2M + S) to (G2M + G0G1 + S), is used to evaluate the proliferative status of cells, flow cytometric analysis on cell cycle was performed in this study to compare the proliferation index of Schwann cells cultured in the silk fibroin extract fluid with those in L15 medium at different times of culture. The results showed no significant difference, verifying that silk fibroin extract fluid had no cytotoxic effects on the in vitro proliferation of Schwann cells.

Considering that Schwann cells secrete a variety of neurotrophic factors, cell adhesion molecules, and other associated factors, among which NGF, BDNF are important for the development, maintenance and response to injury of peripheral nervous system [44–48], we investigated the expression of NGF and BDNF secreted by Schwann cells that were cultured in the silk fibroin extract fluid by the aid of immunocytochemistry and RT-PCR. Moreover, since S-100 protein is an important, albeit nonspecific, marker for Schwann cells, we also determined the expression of S-100 associated with Schwann cells that were cultured in the silk fibroin extract fluid by the aid of immunocytochemistry, RT-PCR and Western blot analysis. All the experimental results showed that the silk fibroin extract fluid did not influence expressions of neurotrophic factors or cell marker associated with Schwann cells, providing further evidence for little cytotoxicity of silk fibroin on Schwann cells.

5. Conclusion

As has been documented over decades, silk protein exhibits high mechanical strength and flexibility, permeability to water and oxygen and can be made into nets, sponges or membranes, being easily handled, manipulated and sterilized [23]. Purified silk fibroin proteins exhibit a high biocompatibility with some biological tissues and do not show the unwanted immunological responses that have been attributed to the silk sericin protein [17]. In addition, recent research has shown that a slow degradation of silk takes place in vivo [49,50].

In this study, we have shown that silk fibroin supports the cell growth of DRG and facilitates the survival of Schwann cells without exerting any

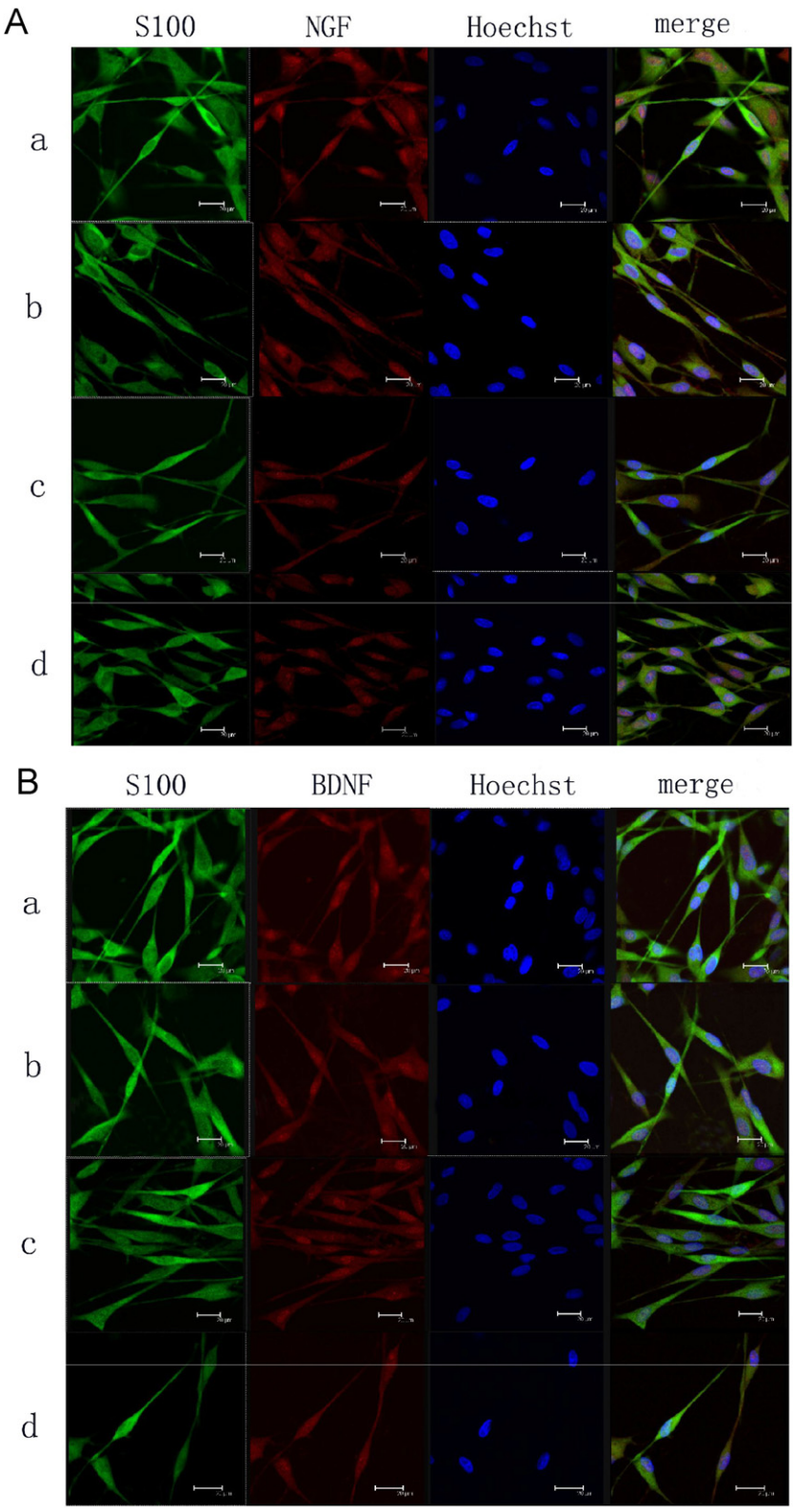

Fig. 8. Immunocytochemistry obtained after Schwann cells were cultured in the plain L15 medium (a) and in the silk fibroin extraction fluid for 24 h (b), 48 h (c) or 72 h (d): (A) immunostained for S-100, NGF proteins and their overlay. (B) Immunostained for S-100, BDNF proteins and their overlay. Also shown were the nuclei labeled with Hoechst 33342.

significant cytotoxic effects on the cell phenotype or cell functions. These attractive results, together with all the above mentioned positive characteristics of silk fibroin indicate that silk fibroin might be used as scaffold material to prepare the tissue engineered nerve grafts for promoting peripheral nerve regeneration. Obviously, however, further

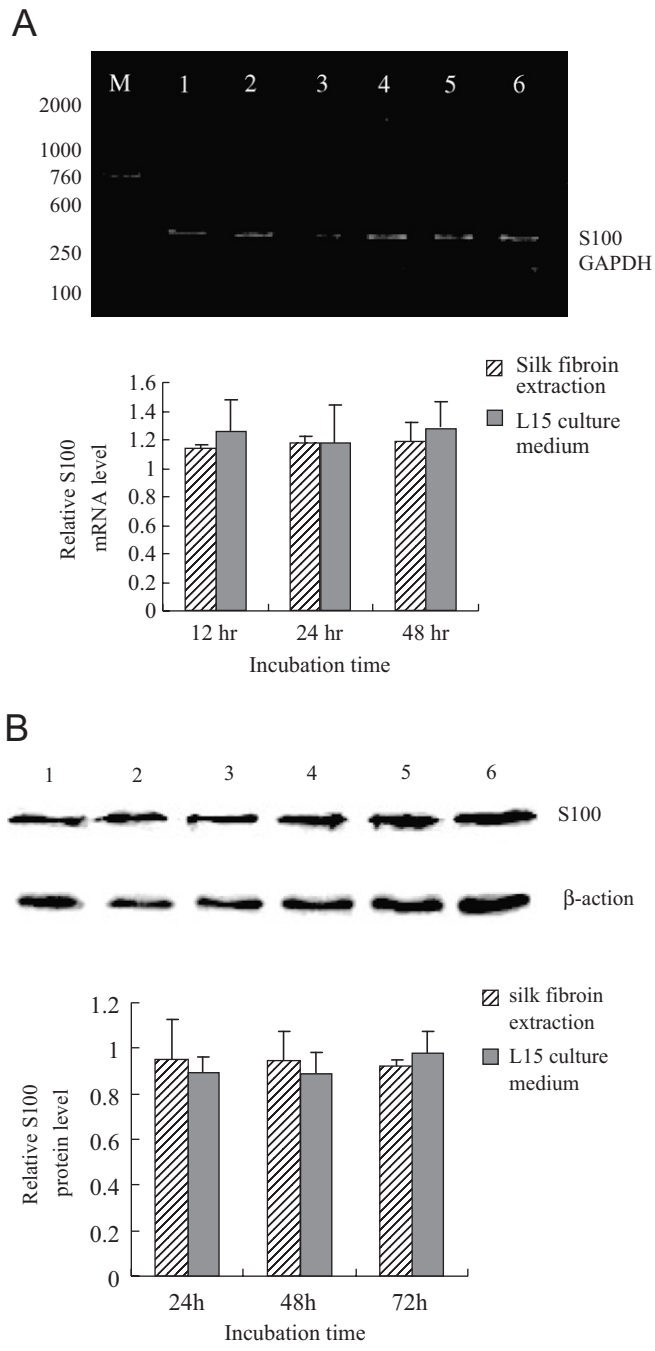

Fig. 9. (A) S-100 mRNA expression of Schwann cells, as determined by RT-PCR, after they were cultured in the silk fibroin extraction fluid for 12, 24, 48 h (marked with 1, 2, 3) or in the plain L15 medium for 12, 24, 48 h (marked with 4, 5, 6). Lane M: DNA molecular weight standard marker (DL 2000). (B) S-100 protein expression of Schwann cells, as determined by Western blot analysis, after they were cultured in the silk fibroin extraction fluid for 24, 48, 72 h (marked with 1, 2, 3) or in the plain L15 medium for 24, 48, 72 h (marked with 4, 5, 6).

works are necessary to expand the overall biocompatibility evaluation of silk fibroin with peripheral nerve from in vitro to in vivo studies and to seek effective approaches for improving the biodegradability of this biomaterial.

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