Biocompatibility studies of silk fibroin-based artificial nerve grafts *in vitro* and *in vivo* *

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Abstract Silk fibroin (SF) has been used extensively in the biomedical field including tissue engineering for the generation of artificial bones, skins or ligaments. We have previously reported on good in vitro biocompatibility of SF fibers with peripheral nerve tissues and cells. In the present study, we developed a novel design of the SF-based artificial nerve graft (SF graft) which was composed of a SF-nerve guidance conduit (NGC) inserted with SF fibers. MTT assay was performed to determine the cytotoxicity of the SF-NGC extract fluid on the cultured L929 cells derived from an immortalized mouse fibroblast cell line. In addition, this SF graft was implanted into adult rats for bridging a 10-mm long sciatic nerve defect. The following-up experiments at initial stage (1—4 week) of nerve regeneration including routine blood tests and histochemical investigation were conducted to evaluate the *in vivo* biocompatibility of the SF graft with peripheral nerves. The results demonstrated that the SF-NGC graft was biocompatible with the surrounding tissues and cells due to its low inflammatory potential with a grade 0 under the U.S. Pharmacopeia guidelines and it was generally suitable to a certain degree for bridging peripheral nerve defects in virtue of supporting Schwann cell adherence, expansion and migration. Therefore the SF graft is a promising alternative to classical autografts for peripheral nerve repair.

Keywords: silk fibroin, artificial nerve graft, nerve guidance conduit, biocompatibility, cytotoxicity.

Peripheral nerve injuries are commonly encountered in clinical practice due to trauma or deliberate surgical resection. For large nerve defects or gaps, implantation of a nerve graft is often required to bridge the proximal and distal nerve stumps for facilitating nerve regeneration and functional recovery. However, this useful treatment strategy is limited by some factors including graft availability, immunosuppression, etc. The research interest has thus been concentrated on the development of artificial nerve grafts using tissue-engineered biomaterials[1—3].

Silkworm silk has been used in the medical field as surgical sutures for centuries. Silk fibers consist of a core structural protein called silk fibroin (SF) which is coated with a glue-like protein called sericin. SF derived from silk after removing sericin which is identified as the source of undesired immunological responses can be dissolved in a mixed solvent followed by dialysis to obtain an aqueous solution that is further processed into nets, sponges, powders, or membranes[4—6]. Hence purified SF has found rapidly increasing applications in pharmaceutical and biomedical fields, such as controlled release of drugs, enzyme immobilization, biosensors, and tissue engineered artificial bones, skins or ligaments[7—9]. We have previously reported on good biocompatibility of SF fibers with peripheral tissues and cells under *in vitro* conditions[10]. In the present study, an artificial nerve graft composed of a SF-nerve guidance conduit (NGC) inserted with longitudinal SF filaments was prepared according to well-established procedures. In order to evaluate the *in vitro* and *in vivo* biocompatibility of the SF-based artificial nerve graft (SF graft), we determined the cytotoxicity of the SF-NGC extract fluid on the cultured L929 cells using MTT colorimetry; on the other hand, we implanted the SF graft to bridge rat sciatic nerve across a 10-mm long defect and examined the *in vivo* responses elicited by nerve grafting at different times post-implantation using a combination of routine blood test and histochemical investigation.

1 Materials and methods

1.1 Preparation of SF graft

Raw silk fibers (*Bombyx mori* cocoons) were

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bought from Xinyuan Sericulture Company, Hai'an, Jiangsu, China. Their sericin coating was removed via degumming process of boiling in aqueous Na_2CO_3 solution as previously described[10]. Degummed SF fibers were first dissolved in a tertiary solvent system of $CaCl_2/H_2O$/EtOH solution (mole ratio 1∶8∶2) at 80℃ for 1 hour and then dialyzed against distilled water in a cellulose tube (molecular cutoff=12000—14000) at room temperature for 3 days. The resulting SF aqueous solution was concentrated with a rotary evaporator under vacuum at 40℃ and ready for preparing the SF-NGC.

A stainless-steel casting mold was used, which consisting of an inner pillar and an outer tube, both of which were fixed on the mold bottom and which determined the diameter and thickness of SF-NGCs. After SF fibers had been wound evenly around its inner pillar, the SF solution was injected into the mold, followed by demolding under lyophilization in a stepwise manner. The resulting SF-NGC was further treated in 80% methanol solution for 15 min to achieve SF insolubility in water.

A novel artificial nerve graft, SF graft, was fabricated by filling the SF-NGC with about 20 longitudinal aligned SF fibers. It was sterilized by exposure to radiation of 20 kGy $_{60}$Co, and immersed in sterile saline for 30 minutes prior to use. For the preparation details of the SF graft, please refer to the patent (No. PCT/CN2005/002423).

1.2 Preparation of SF-NGC extract fluid

The SF-NGC, as the extract phase, was placed in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) that served as the extract medium, according to the ratio of 6 cm^2 of extract phase surface area in 1 mL of extract medium. And the extraction was allowed to proceed at 37℃ for 72±0.5 h[10,11].

1.3 MTT assay

The L929 cells at logarithmic growth phase, obtained following digestion and suspension, were plated at a cell density of 5×10^3/well in a 96-well culture plate containing DMEM supplemented with 10% FBS. After 24 h of culture the medium was removed from wells and replaced by 150 μL of either SF-NGC extract fluid or culture medium for an additional 48 or 72 hours. The viability of L929 cells was then assessed using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test, in which the yellow MTT was reduced to a purple formazan by mitochondrial dehydrogenase in cells. Briefly, the culture medium in the wells of plate was replaced with 100 μL plain medium and 25 μL MTT (5 mg/mL in PBS). After 4 h incubation at 37℃, the reaction solution was carefully removed from the wells and 100 μL of DMSO were added with gentle agitation, followed by measurement of OD values by spectrophotometry at 570 nm with an ElX-800 Microelisa reader (Bio-Tek Inc., USA). The assessments were performed at least in triplicate.

1.4 Animals and surgical procedures

In the present study, all experimental procedures involving animals were conducted following Institutional Animal Care guidelines and approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

Adult male Spragus-Dawely (SD) rats, weighing 200—230 g, were randomized into 4 groups (n=24): an SF graft group, an autograft group (served as positive control), a non-grafted group (served as negative control) and a normal group.

All animals except those in normal group were anaesthetized by an intraperitoneal injection of 3% sodium pentobarbital solution (30 mg/kg body weight) before the sciatic nerve was exposed by making a skin incision and splitting the underlying muscles in the left lateral thigh. A segment of sciatic nerve was resected, leaving a 10-mm long defect following retraction of the nerve ends. In the SF graft group the nerve defect was bridged with the SF graft; in the autograft group the resected nerve segment was reversed 180° and re-implanted as an autologous nerve graft across the nerve defect. After either implant was sutured to both the proximal and distal nerve stumps with 9/0 nylon suture, surgical incisions were closed in routine fashion. As to the non-grafted group, the nerve defect was left unbridged. And animals of the normal group underwent no surgery. After surgery all animals were housed and fed routinely.

1.5 Blood test

A small sample of blood was taken from the heart of the deeply anaesthetized animal in 4 groups at 1-, 2-, 3- or 4- week post-surgery. The blood sample

collected in an evacuated plastic tube was mixed with pre-added sodium heparin to prevent coagulation and subsequently tested under the automatic hematological analysis system XE-2100 to obtain total and differential leucocyte counts.

1.6 Histochemical investigation

Immediately after blood collecting for routine blood tests, the animal in the SF graft group was transcardially perfused sequentially with saline and 4% paraformaldehyde in 0.01 mol/L phosphate buffered saline (PBS, pH 7.4). The implant (SF graft or autograft) together with the segments of sciatic nerve 5 mm outside either end of graft was taken out. The procured specimens were postfixed in buffered 4% paraformaldehyde and transferred to buffered 30% sucrose, followed by frozen sectioning on a cryostat into 12-μm-thick serial longitudinal sections, which were subjected to haematoxylin and eosin (HE) staining or immunohistochemical staining with the antibodies against S-100 and neurofilament 200 (NF), respectively.

For immnohistochemistry, the nerve sections 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 either rabbit anti-rat S-100 antibody (1∶400 dilution, Sigma, USA) or rabbit anti-rat NF-200 antibody (1∶150 dilution, Sigma) overnight at 4℃ in a humidified chamber. After being washed three times with PBS, the samples were further reacted with the second antibody FITC-labeled goat anti-rabbit IgG (1∶200 dilution, Sigma) for 6 h at room temperature. 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). Negative control included leaving out the primary antibody and using non-labeled secondary antibodies to confirm inexistence of nonspecific binding, and positive control involved treatment of the nerve sample from the normal group.

1.7 Statistical analysis

The data were presented as means ± SEM. One-way analysis of variance (ANOVA) using Stata 6.0 software package (Stata Corp., USA), in combination with pairwise comparisons between groups, was performed. A p value less than 0.05 was considered statistically significant.

2 Results

2.1 Cytotoxicity evaluation

After the L929 cells were cultivated in the SF-NGC extract fluid and plain medium, respectively, for 48 and 72 hr, light microscopic visualization revealed that the L929 cells cultured in the two mediums were not obviously different in their morphological appearance throughout the culture process, namely, the L929 cells showed clear edges and shiny, transparent bodies in either medium.

MTT assay results indicated that the viability of the L929 cells cultured in the SF-NGC extract fluid was significantly higher than that in the plain medium at 48 h after culture initiation, while no significant difference in the cell viability was observed between the two mediums at 72 h after culture initiation (Fig. 1)

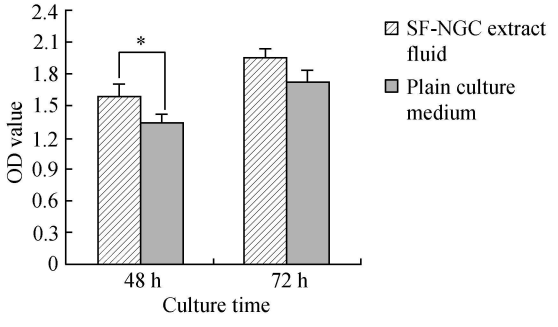

Fig. 1. The changes in the cell viability of the L929 cells after they were cultured in the plain DMEM medium or SF-NGC extraction fluid for 48 or 72 h, respectively. * $p < 0.05$.

2.2 General observation after surgery

The animals except those in the normal group were only able to walk with a limp and they exhibited no obvious sighs of systemic or regional infection during 4 weeks of post-surgery observation. At 1—2 week after implantation, the implant in the rats of the SF graft and autograft groups was covered with a thin layer of connective tissue, which did not grow thicker at 3—4 week after implantation, and no swelling, collapse, or broken implant was noticed.

2.3 Total and differential leucocyte counts

Fig. 2 compares the results of routine blood tests

for all four groups at 1-, 2-, 3- or 4- week post-surgery. The four groups exhibited no significant difference in the eosinophil percentage at different time points ($p > 0.05$). Either the total leukocyte number or the neutrophil percentage in three treatment groups (namely the SF graft, autograft and non-grafted groups) showed a slight increase as compared to the normal group. There was no statistically significant difference found in the total leukocyte number and the percentage of eosinophil or neutrophil between all three treated groups.

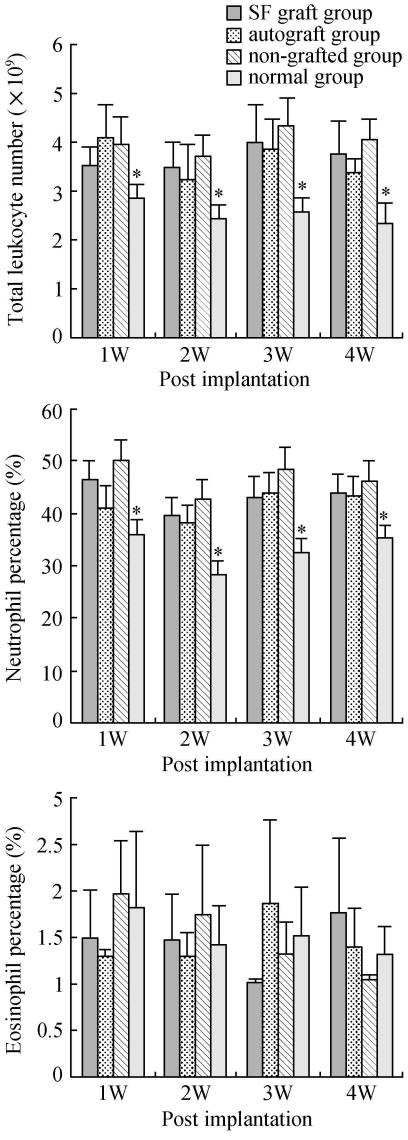

Fig. 2. The comparison of the total and differential leucocyte counts for the SF graft, autograft, non-grafted, and normal groups at 1-, 2-, 3- or 4- week post-surgery. * $p < 0.05$ versus three treatment groups.

2.4 Histochemical evaluation of implantation of SF graft

2.4.1 Microscopic examination of HE staining

One week after implantation with the SF graft, partial nerve degeneration existed in the proximal and distal stumps of cut sciatic nerve, while a small number of spindle cells crawled towards the cavity of the SF-NGC. Two to three weeks after implantation, more number of spindle cells occurred, migrating from both stumps along the scaffold made up of SF fibers filled in SF-NGC. Four weeks after implantation, a large number of spindle cells migrated into the lumen of the SF-NGC and aligned along the SF fibers, while the processes of the spindle cells interconnected to each other to display a bundle- or band-shaped appearance.

In addition, there was an infiltration of a small quantity of leucocytes with high ratio of neutrophils in the SF-NGC at one week after implantation, and a small quantity of macrophages and multinucleated giant cells was seen in the SF-NGC at four weeks after implantation.

2.4.2 Immunofluorescence microscopy

One week after implantation with the SF graft, a lower number of regenerating axons grew into the graft, accompanied by migration of Schwann cells. Two to three weeks after implantation, a dramatic enhancement in the number of regenerating axons and progressive directional migration of Schwann cells towards the distal portion were seen. Four weeks after implantation, regenerating axons were noted to approach the distal stump along the scaffold, as shown in Fig. 3(a) and (c); and Schwann cells continued an oriented migration along the scaffold with crosslinking to give a cell structure having a string-like shape, as shown in Fig. 3(b) and (d).

In immunofluoresecent staining procedures, the SF materials could show the similar green color as the immunofluorescence emitted by axons or Schwann cells upon laser illumination at 488 nm wavelength, but the SF conduits or fibers would change into the homogeneous blue color due to their autofluorescence induced by the ensuing ultraviolet illumination, thus being easily distinguishable from the nerve cells.

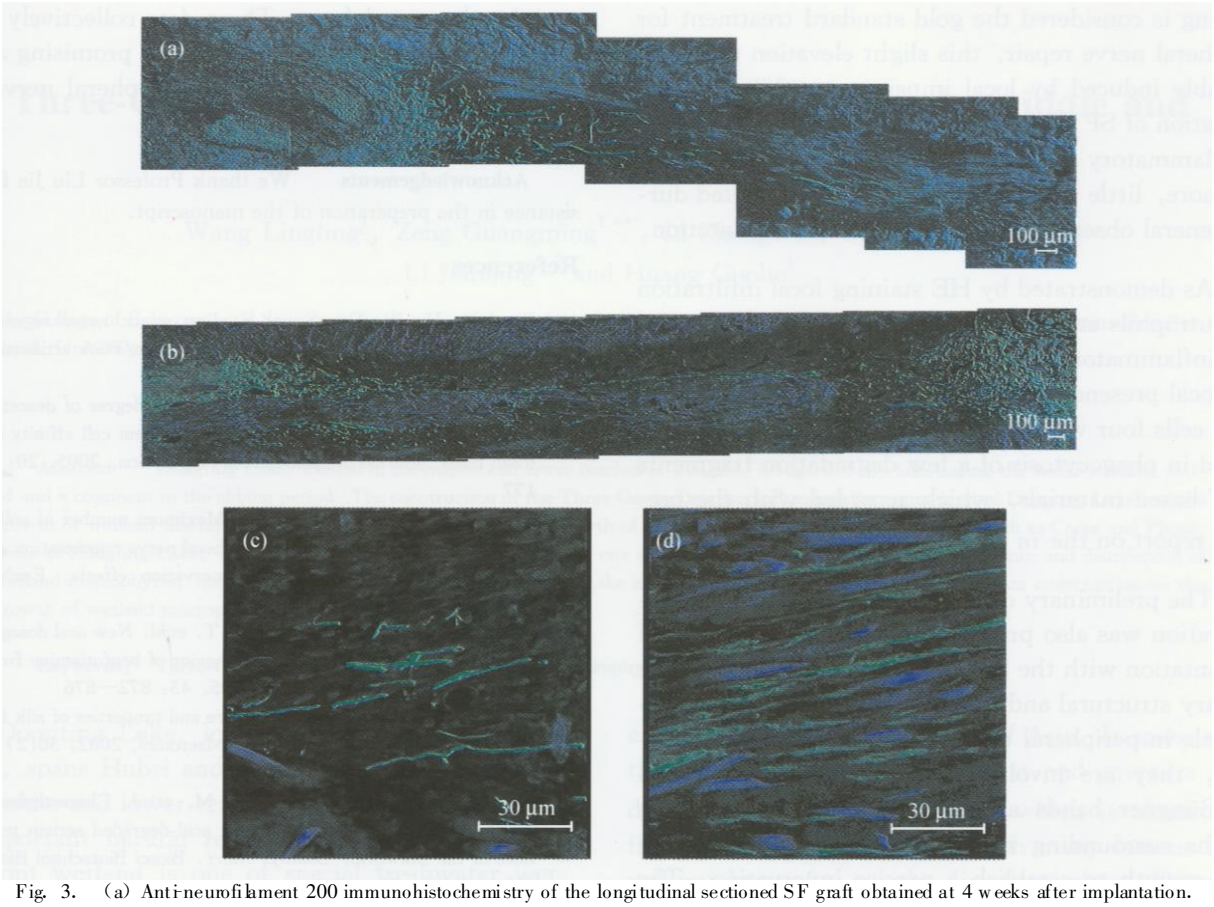

Fig. 3. (a) Anti-neurofilament 200 immunohistochemistry of the longitudinal sectioned SF graft obtained at 4 weeks after implantation. (b) Anti-S-100 immunohistochemistry of the longitudinal sectioned SF graft obtained at 4 weeks after implantation. (c) and (d) are local magnifications of (a) and (b), respectively.

3 Discussion

SF has proven to be an important biomaterial without toxicity and adverse reactions. Its emerging application in the tissue engineering field stems from its unique properties including air permeability and cell adherence. However, to our knowledge, there have hitherto been few reports on the utilization of SF-based materials for peripheral nerve repair. The present study was designed to develop an artificial nerve graft made up of SF materials in the hope of opening a new avenue for peripheral nerve repair by bridging extensive nerve defects.

Despite good biocompatibility of SF fibers or their extraction fluid with peripheral nerve tissues or cells (Schwann cells) that has been reported by our previous study[10], we did not know whether the SF graft fabricated through a series of physical and chemical steps could have some toxicity on peripheral nerve system, so biocompatibility of the graft was evaluated *in vitro* and *in vivo* in the present study. At first, the cytotoxicity of the main component of the SF graft, SF-NGC, was assessed *in vitro* using an internationally accepted method from International Standards ISO 10993-5 in couple with an internationally recognized and routinely used cell model, namely, the L929 cells derived from an immortalized mouse fibroblast cell line, because another graft component made up of SF fibers has been actually evaluated for its cytotoxicity[10]. The data of MTT assay indicated that after 48 and 72 hr of culture the relative viability of L929 cells in the SF-NGC extract fluid, as compared to the control, was larger than 100%, suggesting the non-cytotoxicity of the SF-NGC (grade 0) in accordance with the U.S. Pharmacopeia (USP) guidelines[12].

Secondly, the rat implantation experiment with the SF graft was performed to evaluate the *in vivo* biocompatibility of the graft. Blood tests revealed that there was no significant difference in the total and differential leucocyte counts between three treatment groups despite a slight elevation in the total leucocyte number and neutrophil percentage for three treatment groups as compared to the normal group. Since auto-

grafting is considered the gold standard treatment for peripheral nerve repair, this slight elevation might be probably induced by local impairment while the implantation of SF graft resulted in no obvious systematic inflammatory symptoms or allergic responses. Furthermore, little inflammatory reaction was noted during general observation of animals after implantation.

As demonstrated by HE staining local infiltration of neutrophils at one week after implantation implied low inflammatory potential of SF-based materials; and local presence of macrophages and multinucleated giant cells four weeks after implantation might be involved in phagocytosis of a few degradation fragments of SF-based materials, which accorded with the previous report on the *in vivo* foreign body response[15].

The preliminary data at initial stages of nerve regeneration was also presented on the repair effects of implantation with the SF graft. Schwann cells are the primary structural and functional cells that play a crucial role in peripheral nerve regeneration. More especially, they are involved in Wallerian degeneration and Büngner bands and capable of forming myelin sheaths surrounding axons, guiding and promoting axon growth to establish a precise innervation. The ideal biomaterials for preparing the scaffold of artificial nerve grafts must be beneficial to the attachment, expansion and migration of Schwann cells, creating an appropriate microenvironment for axon growth and regeneration. In the present study, immunochemistry of the marker protein S-100 was used to show Schwann cells distributed along the scaffold of SF fibers. The results showed that SF fibers were biocompatible with Schwann cells, and could become a scaffold material for supporting their adherence and migration to form an ordered cell structure arranged in a string shape.

In summary, we developed a new artificial nerve graft (SF graft) composed of a SF-NGC inserted with SF fibers. The *in vitro* cytotoxicity assessment indicated the non-toxicity of the SF-NGC. The rat implantation experiment with the SF graft and the following-up evaluations showed that the graft was quite biocompatible with the surrounding tissues or cells and generally suitable to a certain degree for bridging peripheral nerve defects. These data collectively raise a potential possibility of SF graft as a promising alternative to classical autografts for peripheral nerve repair.

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