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Use of spider silk fibres as an innovative material in a biocompatible artificial nerve conduit

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Abstract

Defects of peripheral nerves still represent a challenge for surgical nerve reconstruction. Recent studies concentrated on replacement by artificial nerve conduits from different synthetic or biological materials. In our study, we describe for the first time the use of spider silk fibres as a new material in nerve tissue engineering. Schwann cells (SC) were cultivated on spider silk fibres. Cells adhered quickly on the fibres compared to polydioxanone monofilaments (PDS). SC survival and proliferation was normal in Live/Dead assays. The silk fibres were ensheathed completely with cells. We developed composite nerve grafts of acellularized veins, spider silk fibres and SC diluted in matrigel. These artificial nerve grafts could be cultivated *in vitro* for one week. Histological analysis showed that the cells were vital and formed distinct columns along the silk fibres. In conclusion, our results show that artificial nerve grafts can be constructed successfully from spider silk, acellularized veins and SC mixed with matrigel.

Keywords: spider silk • Schwann cell • biocompatibility • nerve tissue engineering • nerve regeneration • nerve guide

Introduction

The axons of the peripheral nerve systems are able to form new sprouts and in many cases find their original target tissues rebuilding functional synapsis. This process of axonal regeneration is assisted by Schwann cells (SC) in numerous ways. One of the early events is the migration of SC to form a column in bands of Büngner. The cells are derived from a degenerative process at the distal stump called Wallerian degeneration, which induces proliferation of SC. They guide the regrowth of the proximal end of the axon by the production of many different bioactive substrates including growth factors and extracellular matrix proteins like collagen IV and laminin [18, 19]. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6) have been shown to be active factors during regeneration [27]. Axon-Schwann cell attachment is mediated by adhesion molecules like nerve cell adhesion molecule (N-CAM) and protein 0 [16, 21]. Finally, SC adjacent to axons provide essentially functional structures

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like myelination and signal to the appropriate expression of sodium channels at nodes of Ranvier in the reconstituted nerves [22].

Nevertheless, direct axonal repair is limited to small gaps and impaired by scarring and neuroma formation. Extensive nerve gaps must be reconstructed with structures creating a permissive environment for the axonal outgrowth. Current clinical practice uses autologous nerve grafts to compensate for lost nerval tissue. But the use of autologous nerve grafts is limited and inevitably associated with a loss of sensibility at the donor site.

Recent research concentrated on the development of alternatives based on either natural or synthetic material. Ideally, any used substrate does neither exert toxic effects on the neuronal cells nor evokes fibrotic or immune response. It allows migration of supporting cells like SC by providing endoneural-like structures and is permeable for influx of nutrients and revascularization. Natural materials are favourable concerning reduced cytotoxity and biocompatibility but must be immunologically compliant [11]. Autologous veins have been used in experimental and clinical evaluations as an interposition graft [7, 8, 12, 27]. The implementation of SC allowed nerval regeneration through a 6 cm gap in a rabbit model [27]. Unfortunately, a lack of endogenous structures in veins resulted in an impaired regeneration [13].

In our study, we developed a new type of artificial nerve grafts. We compared the feasibility of spider silk fibres as a growth material for SC to polydioxanone monofilaments (PDS). Good results concerning cellular adhesion, proliferation and cellular vitality prompted us to put spider silk fibres into acellularized veins as an intrinsic framework for artificial nerve grafts. SC mixed with matrigel were added and the constructs were cultivated *in vitro* and analysed histologically.

Materials and methods

Collection of spider silk

For collection of spider silk fibres we used adult females of the genus Nephila grown at our local animal facility. For experimental practice we used the major-ampullate-dragline, which serves the spider as security rope and building material. We stimulated the major ampullate gland of immobilized animals by pulling out the fibres mechanically.

Schwann cell cultivation

Adult human SC were isolated from peripheral nerves obtained with patients' consent in surgical procedures as transplantation of myocutaneous free-flaps. The epineurium and perineurium were stripped, and the nerves were cut into pieces of 1 mm length. The minced nerves were incubated in DMEM (Dulbecco`s modified Eagle`s medium) /F12 (PAA, Cölbe, Germany) supplemented with 20% FCS (fetal calf serum) (Biochrom, Berlin, Germany) and 100 u/ml penicillin and streptomycin (PAA) in humidified atmosphere at 37°C and 5% CO_2 for 3 weeks. Minced nerves were dissociated with Collagenase X (Sigma) and Dispase (Roche, Mannheim, Germany) and homogenised. The isolated SC were cultured in melanocyte growth medium (M2, Promocell, Heidelberg, Germany) on poly-L-lysine (Biochrom) coated dishes. SC were observed daily with an inverted phase contrast microscope.

Biocompatibility assays

About 10 spider silk fibres of approximate 10 µm diameter were added to a culture dish. 10^6 SC were dropped gently onto the culture dish. Bioresorbable polydioxanone monofilaments (PDS, Ethicon) were used as controls. Attachment and cell morphology were observed microscopically for two days. Photographs were taken regularly. Afterwards the viability of SC was assessed with a Live/Dead viability assay (Molecular Probes, Leiden, The Netherlands). Briefly, the cells were washed in PBS and incubated with 1:1 homodimer (1:1000) and calcein AM (1:8000). The cell viability was determined fluorescopically *in situ*. The living cells fluoresce green while dead cells have red fluorescent nuclei. All relevant steps were independently repeated three times at least.

Acellularizing of the venules

The venules were taken from veins of the lower extremities of pigs which were sacrificed for experimental purposes others than postulated in our study design. The venules had a diameter of approximately 2-3 mm and a

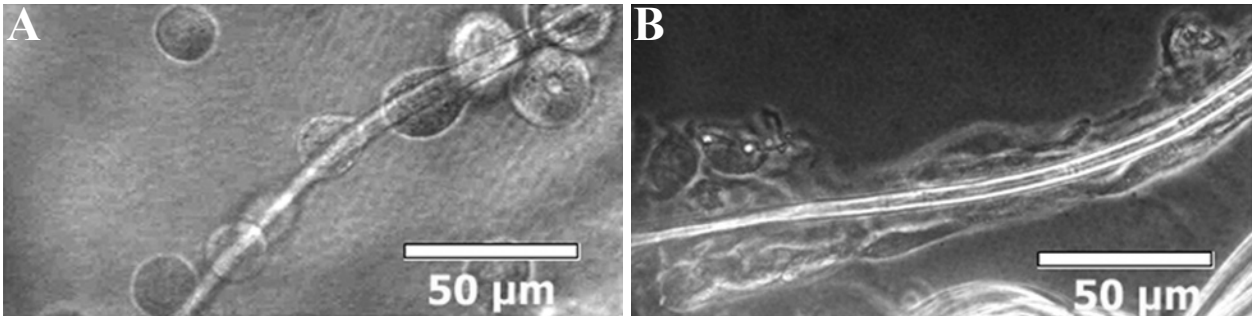

Fig. 1 SC adhesion on spider silk fibres. A cellular suspension was incubated on spider silk fibres. **A**: After 15 minutes cells appear still rounded but attached to the silk filaments. **B**: After 24 hrs SC are firmly attached to the silk fibres. Cells have a bipolar morphology oriented along the silk fibres ensheathing them completely. (Phase contrast micrograph, x 400)

length of 5 cm. For acellularization, freshly prepared veins were incubated for 24 hrs in trypsin/EDTA in PBS (phosphate-buffered saline) on a rocking platform at 37°C. The procedure was repeated after washing with PBS. After extensive washing the venules were frozen at -80°C until usage. Successful acellularization was histologically controlled.

Nerve constructs

The spider silk was cut to the appropriate length and pulled through the acellularized venules until filling out one quarter of the venule diameter. 300.000–500.000 SC were diluted in 200 µl matrigel (Basement Membrane Matrix, BD Biosiences, Heidelberg, Germany) and injected into the silk-prepared venule. The venule was filled out completely until having a cylindrical shape. In control samples, spider silk fibres were replaced by PDS filaments. The constructs were cultured in M2 for one week and analyzed. Results are representative for at least three independent experiments.

Immunofluorescence

Nerve constructs were frozen and cut into slices. Slices were fixed with 4% (w/v) paraformaldehyde for 20 minutes. Blocking was performed with 2% FCS in PBS. Incubation with the S-100 antibodies was done at a 1/400 dilution in PBS/1% FCS overnight at 4°C or, alternatively, for 1 hour at 37°C in a humidified chamber. The secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (approximate absorption 495 nm and fluorescent emission 519 nm, green fluorescent) (Molecular Probes), at a 1/800 dilution in PBS/1% FCS. After being washed, slices were examined with an inverse fluorescence microscope (Olympus) equipped with the appropriate barrier filters.

Histology

Cellular orientation and vitality were examined histologically. Nerve grafts were frozen and cut into 50 µm slices. Slices were air-dried and stained with hematoxilin-eosin.

Results

Determination of biocompatibiliy of spider silk *in vitro*

Spider silk has been discussed as a suitable biological scaffold in literature but until now was never integrated into experimental designs [1, 15, 30]. Some studies demonstrate that living animal tissue tolerates natural and untreated spider silk in ways comparable to synthetic protein polymer webs. Spider silk is integrated into the extracellular matrix and slowly degraded [29].

To preclude any toxic effects on the neuronal cells we tried to cultivate adult human SC on spider silk. Spider silk fibres were gained from specimen of the genus Nephila kept under standardized conditions. A cell suspension with cultivated cells was dropped gently onto the filaments. After 5 minutes resting the culture dishes were filled completely with appropriate medium. The proper attachment of SC onto the

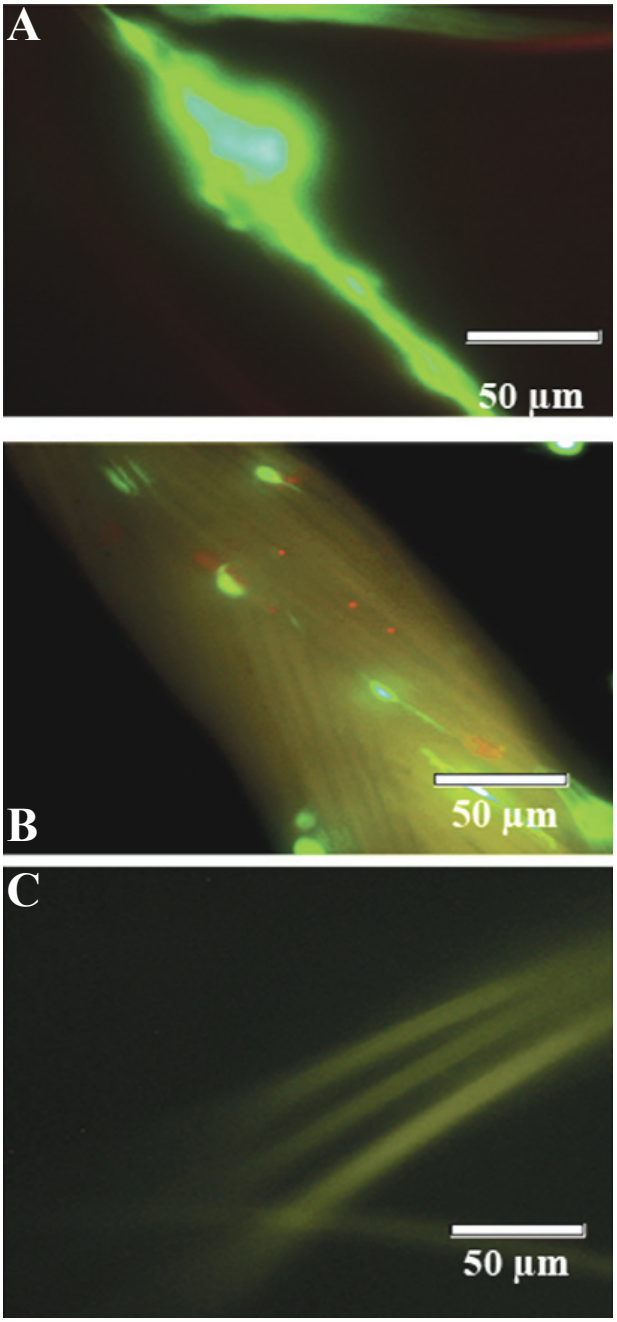

Fig. 2 Live/Dead assay of SC on filaments. **A**: SC were seeded onto spider silk for 48 hrs. All cell fluoresce in green indicating vital cells. Cells are forming a complete multilayer around the fibres. **B**: SC were seeded onto polydioxanone monofilaments (PDS) for 48 hrs. Most cells fluoresce in green. Cell debris is visible as fluorescent red granules. Cellular shapes vary between spherical and bipolar. The cell layer is not closed. **C**: Negative control without SC. Spider silk fibers show a slight autofluorescence. (Fluorescence micrograph, x 100)

silk could be observed after 15 minutes (Fig. 1A), whereas they needed 120 minutes to adhere to the PDS (polydioxanone monofilaments) control. After 24 hrs of incubation, sprouts of the cells ensheathed the thin silk filaments completely (Fig. 1B) and also an increased proliferation was observed.

After 48 hrs, the spider silk fibres were completely covered with multilayers of SC (Fig. 2A). We evaluated the viability of the cells in a fluorescent Live/Dead assay. All cells attached to the spider silk were viable, the cells were distributed along the fibres in a dense and longitudinal fashion forming a complete sheath (Fig. 2A). The thicker PDS fibres, however, were sparely covered with cells. While most of the cells were also viable, not all the cells examined had the typical long bipolar shape. Many cells appeared rounded up and traces of cell debris were visible as fluorescent red granules (Fig. 2B). Spider silk fibres without SC served as a negative control. A slight autofluorescence was visible (Fig. 2C)

Construction of artificial nerve constructs based on spider silk

As our results show that spider silk is a suited material for SC adhesion and proliferation, we constructed artificial nerve conduits based on spider silk fibres and acellularized veins as outer guiding tubes. Acellularization was achieved by repetitive enzymatic digestion. Histological analysis showed that the procedure resulted in a complete removal of living cells in the xenogenic veins (Fig. 3A). Recent literature stated that removal of cellular structures eliminates immunogenic response [2, 14]. To provide an intrinsic framework for cell adhesion, the veins were filled with spider silk. SC were mixed with diluted matrigel and injected into the conduits (Fig. 3B). The resulting construct showed the anatomic properties of nerves and proved to be strong and flexible (Fig. 3B).

Purity and adhesion to the silk fibres was examined immunofluorescopically on frozen sections after 48 hrs (Fig. 4A). Staining for the SC specific protein S-100 showed that the cell population stained on the whole while silk fibres showed no fluorescence. Cells were vital and oriented in longitudinal bundles along the fibres. Fibroblast contamination could not be observed. The untreated negative control shows a slight autofluorescence (Fig. 4B).

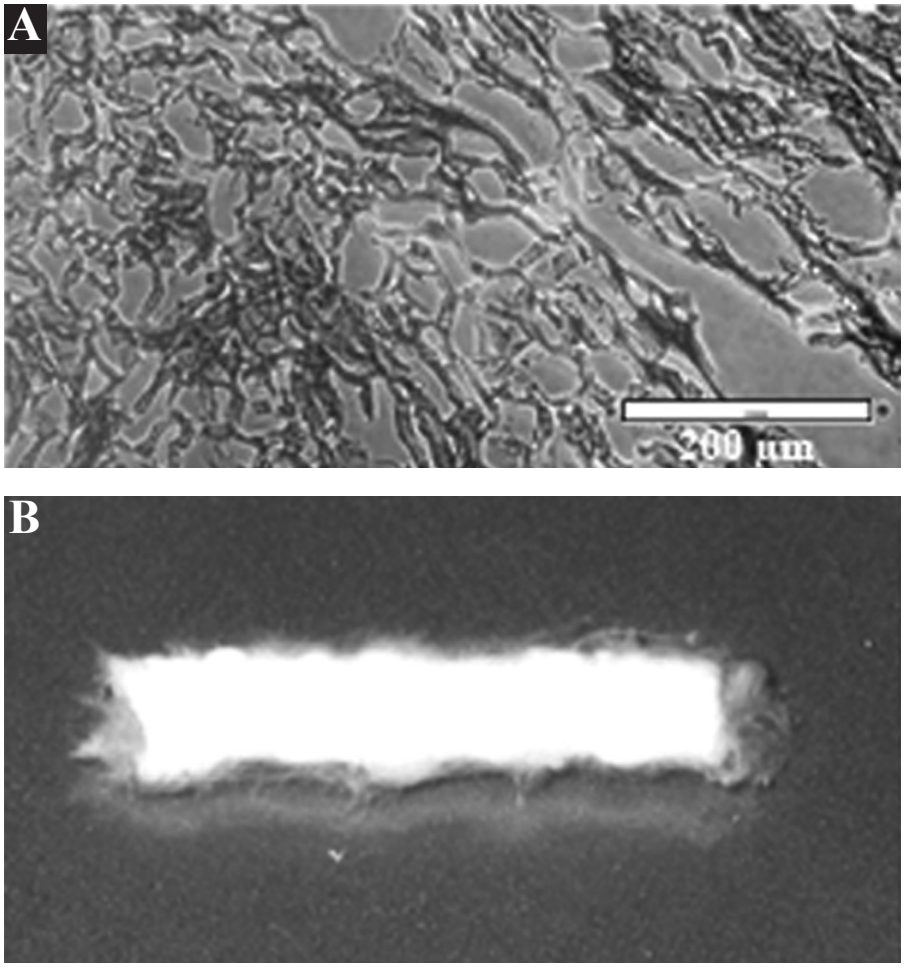

Fig. 3 Combined artificial nerve construct. **A**: Xenogenic veins were acellularized and cut in frozen sections. Sections were stained with hematoxilin /eosine. Cells are completely removed, the extracellular matrix is maintained. (Light micrograph, x 100) **B**: Artificial nerve construct of acellularized veins, spider silk fibres and SC diluted in matrigel

After one week of *in vitro* cultivation, the constructs were analyzed histologically. The cross-sections revealed cell orientation in bundles (Fig. 5A). The lumina of the venules were almost filled with cells indicating a high proliferation rate. In longitudinal sections, SC enclosed the silk fibres completely (Fig. 5B). Cells showed polar and elongated shapes. In all sections nuclei were clearly stained, being characteristic for vital cells (Fig. 5A and B).

Discussion

Nerve regeneration is a complex biological process and optimization is important for the clinical outcome. Artificial nerve grafts have to fulfil several requirements to meet these conditions concerning biocompatibiliy. Cellular and axonal migration is a prerequisite for the formation of Büngner bands and regrowth of the axon. Grafts should promote cellular and axonal migration. Additionally, all used materials should be bioresorbable without disturbing the healing process [3, 10].

In the published literature many synthetic materials have been tested as artificial nerve grafts, such as lactate polymers, polyglactin and polydioxanone scaffolds, silicone and polyethylene. Other groups worked on natural or modified material like chitosan, rat-tail glue, laminin, collagen and autologous or acellularized grafts consisting of arteria, veins or muscle [4–6, 9, 11, 12, 14, 23, 25, 28, 31]. Concordantly, the authors could show that constructs seeded with SC and implemented internal structures were more suitable as a nerve guiding conduit than singular tubes. Survival of supplemented SC was a determining factor for successful axonal outgrowth. Evans *et al.* used poly(L-lactic acid) (PLLA) conduits seeded with SC in a 12 mm sciatic nerve gap in rats. He could achieve a strong axonal outgrowth at the proximal end of the nerve but nerve fibres were unable to migrate along the con-

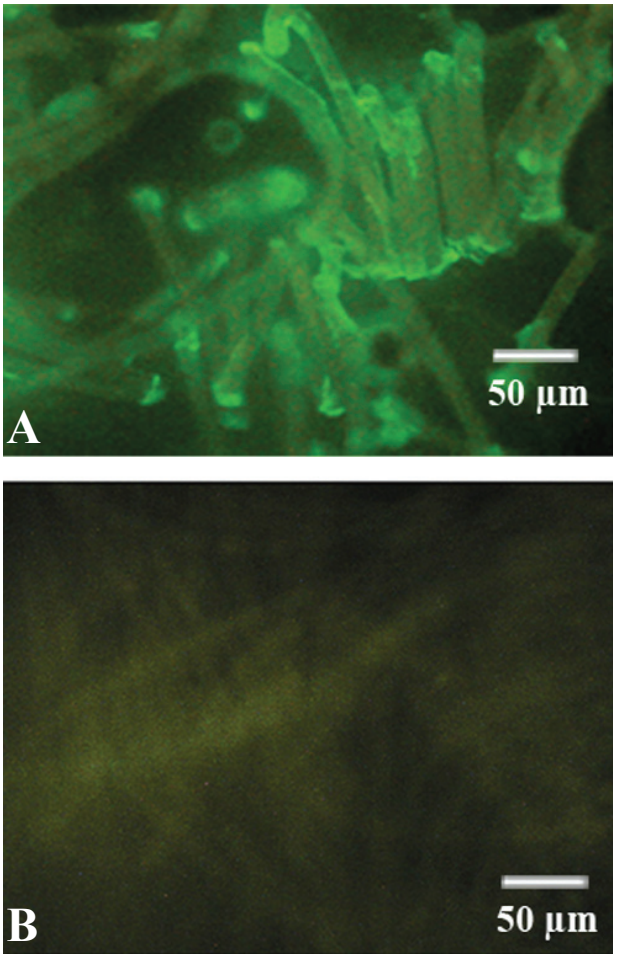

Fig. 4 Combined artificial nerve constructs stained for S-100 on frozen sections. **A**: Green fluorescence indicates S-100 expression. SC are aligned along the silk fibres. Section shows bundled silk fibres. **B**: Negative control without SC. Spider silk fibers show a slight autofluorescence. (Fluorescence micrograph, x 100)

duit. This was probably due to a decreased vitality of the grafted SC and the lack of endoneural structures like the formation of Büngner bands [11]. Biological material like veins, muscles or combined conduits provide environments favorable for SC attachment and proliferation. These materials provide molecular structures composed of or resembling to extracellular matrix favorable for cellular attachment, migration and differentiation. Neither of the materials used allowed nerve regeneration comparable to autologous nerve transplantation, however.

In own preparative studies polyglactin woven filaments and polydioxanone monofilaments were used to provide internal structures for a bioartificial nerve graft [25]. But while SC adhered better to PDS filaments and were more densely distributed acidic biodegradation impaired nerve regeneration data showed that optimization of cellular density on the internal fibres is important for the outcome of nerve regeneration. In our search for a material which efficiently promotes cellular adherence we successfully tested spider silk fibres as a growth material for SC.

Our results show that SC adhere quickly to spider silk. Harmful or toxic effects could not be observed. The cells attached firmly onto the fibres and had a typical bipolar shape. They showed normal survival and proliferation rates. Further studies are needed to differentiate between the possible effectes of spider silk composition of structure concerning the positive stimulation of cellular adherence and proliferation compared to PDS monofilaments. Yuan *et al.* could show that chitosan fibres serve as a biocompatible attachment matrix for SC. They report two differently shaped cell populations, spherical and bipolar, however, which could not be observed in our study. This could be due to differences in surface topography between the two materials.

In consequence, we used spider silk fibres as an intrinsic framework in a newly developed combined bioartificial nerve graft consisting of acellularized xenogenic veins and human SC. We tested SC survival, migration and proliferation over a period of seven days. The cells formed columns along the fibres ensheathening the fibres in short time in structures analogous to Büngner bands. These results are promising for the application of spider silk as endoneural structural analogon in nerve grafts. Migration and survival of SC producing neurotrophic factors and adhesion molecules are mandatory factors in peripheral nerve regeneration.

Spider silk-based tissue engineering has not come into clinical or experimental practice until now due to the predatory nature of spiders and the relatively low levels of silk production by individual animals. Nevertheless, spider silk has many attractive features as a biomaterial. The silk of spider species Nephila clavipes is less immunogenic than silkworm silk [29]. Its tensile strength is 4 x 10^9 N/m, being six times the breaking strength of steel. Degradation of bioresorbable polymers is usually accompanied by a switch to unphysiologically pH [17], which is avoided in proteolytically

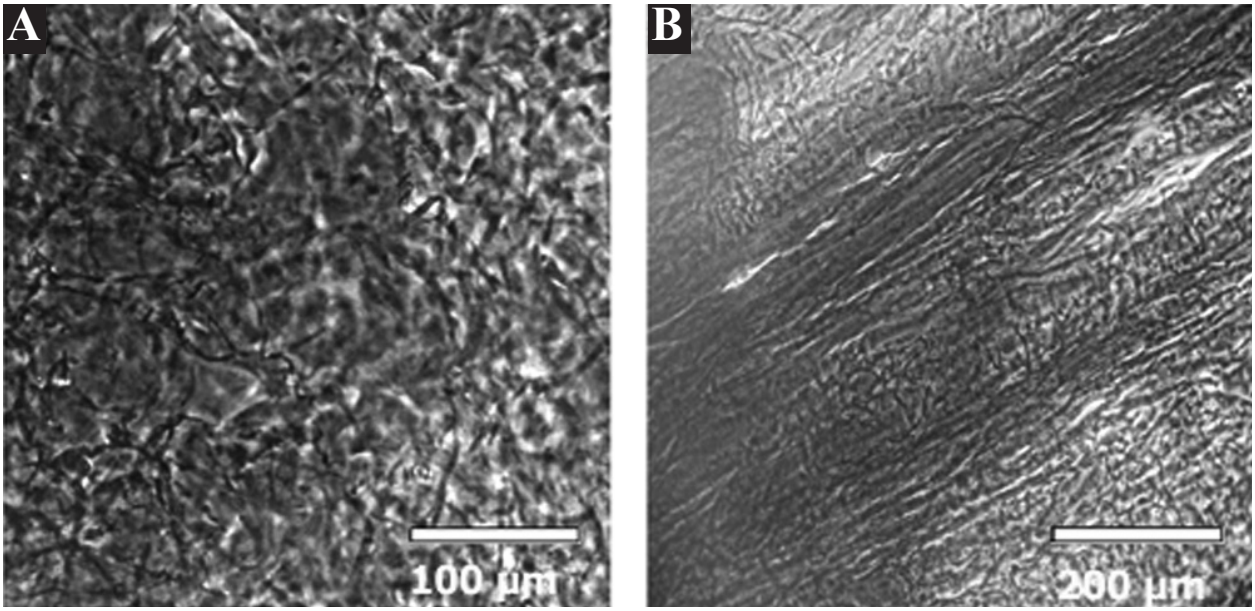

Fig. 5 Histological examination of combined artificial nerve constructs. **A** Cross section stained with hematoxilin/eosin. Cells are oriented in bundles. The lumina of the venules are almost filled. (Light micrograph, x 400) **B**: Longitudinal section stained with hematoxilin/eosin. SC enclose the silk fibres completely. Cells showed polar and elongated shapes. (Light micrograph, x 200)

degraded spider silk [26]. Furthermore, spider silk is resistant to fungial and bacterial decomposition for weeks and does not swell. The molecular structures promote cellular adhesion and migration. These factors provide a good microenvironment for nerves to regenerate.

A major drawback for spider silk as a biomaterial is the laborious harvest of the fibres. Furthermore, the precise amino acid composition varies between the species and even within the same individuals according to the spider's diet and environmental conditions. However, attempts have been made to artificially produce spider silk fibres. The use of native or synthetic spider silk genes have been reported in a variety of expression systems, including bacteria, yeast, insect cells, plants and mammalian cells [15, 20]. Spider silk proteins produced in transgenic plants have been shown to support chondrocyte attachment and proliferation [24].

The limited quantity required for the construction of artificial nerves allows application of native spider silk. So our presented technique of nerve graft construction was independent of the production of synthetic silk fibres. Further studies will be made to prove the feasibility of the constructs *in vivo*.

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