Dog sciatic nerve regeneration across a 30-mm defect bridged by a chitosan/PXA artificial nerve graft

Xiaodong Wang, Wen Hu, Yong Cao, Jian Yao, Jian Wu and Xiaosong Gu

Key Laboratory of Neuroregeneration, Nantong University, Nantong City, Jiangsu, People’s Republic of China

Correspondence to: Xiaosong Gu, Key Laboratory of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong, JS 226001, P.R. China
E-mail: neurongu@public.nt.js.cn

We have developed a dual-component artificial nerve graft comprising an outer microporous conduit of chitosan and internal oriented filaments of polyglycolic acid (PGA). The novel graft was used for bridging sciatic nerve across a 30-mm defect in six Beagle dogs, which were used as a chitosan/PXA graft group. The other Beagle dogs were divided into an autograft group (n = 6) as the positive control and a non-grafted group (n = 5) as the negative control. All animals of three groups were monitored for changes in their appearance and locomotion activities after surgery. Their posture and gait were recorded regularly with the aid of photographs and videotapes for each dog. Six months post-operatively, a combination of electrophysiological examination, FluoroGold retrograde tracing, histological assessment including light microscopy and transmission electron microscopy, immunohistochemistry as well as morphometric analyses to both regenerated nerves and target muscles was utilized to investigate the nerve repair effects of our artificial nerve graft. The results demonstrated that, in the chitosan/PXA graft group, the dog sciatic nerve trunk had been reconstructed with restoration of nerve continuity and functional recovery, and its target skeletal muscle had been re-innervated, improving locomotion activities of the operated limb. This study proves the feasibility of the chitosan/PXA artificial nerve graft for peripheral nerve regeneration by bridging a longer defect in a large animal model.

Keywords: artificial nerve graft; chitosan; polyglycolic acid; long sciatic nerve defects; dogs

Abbreviations: CMAP = compound muscle action potential; DG = distal graft; DN = distal sciatic nerve; DRG = dorsal root ganglia; FG = FluoroGold; NF = neurofilament; PBS = phosphate-buffered saline; PG = proximal graft; PGA = polyglycolic acid; PN = proximal sciatic nerve


Introduction

Peripheral nerve injuries are common in clinical practice due to trauma or deliberate surgical resection. In certain situations, especially when a nerve defect or gap is so long that end-to-end suturing becomes difficult, implantation of a nerve graft is often necessary to bridge the proximal and distal nerve stumps for promoting nerve regeneration. The typical graft of choice is a nerve autograft harvested from another site in the body. However, this recognized ‘gold standard’ technique for peripheral nerve repair, to which other treatments are compared, is limited by tissue availability, donor site morbidity and secondary deformities, as well as by potential differences in tissue structure and size (Ducker and Hayes, 1970; Evans, 2000). Allografts have been tried, but they are subject to immunosuppression and have achieved very poor success (Heath and Rutkowski, 1998). Autologous and allogeneic organs or tissues such as blood vessels and skeletal muscles have also been used for nerve grafting with varying levels of success, but they are still accompanied by some of the same problems as autografts and allografts (Brunelli et al., 1993; Tong et al., 1994; Fansa et al., 2002; Barcelos et al., 2003). This affects the development of various decellularization techniques applied to them.

A promising alternative to conventional grafting is the use of artificial nerve grafts, of which the Integra Neurosciences...
Type I collagen sheath (NeuraGen™) (Archibald et al., 1995) is commercially available for treating short nerve defects clinically. Artificial nerve grafts are usually in the form of nerve tubes and conduits (Fields et al., 1989; Keeley et al., 1993). Clinical trials demonstrated that tubular repair of 3–5 mm nerve gaps with silicone tubes yielded functional recovery at least as good as routine microsurgical repair (Lundborg et al., 1997b, 2004). To bridge larger nerve defects and support nerve regeneration, research interest in artificial nerve grafts has been focused on seeking bioresorbable materials to construct nerve conduits (their uniqueness is that they gradually degrade after functioning as temporary scaffolds for nerve regeneration).

Recent advances in nerve tissue engineering have greatly promoted the generation of nerve conduits, which may be implanted empty, or may be filled with growth factors, cells or fibres (Heath and Rutkowski, 1998). It is worth mentioning that longitudinal biomaterial filaments have also been introduced into a nerve conduit to facilitate nerve regeneration across extended nerve defects (Lundborg et al., 1997a; Terada et al., 1997; Arai et al., 2000). Accordingly, multi-component complex nerve guides are often referred to as ‘tissue engineered nerve grafts’.

Chitosan (the fully or partially deacetylated form of chitin) is the second most abundant polysaccharides in nature next to cellulose. In vitro studies have shown that chitosan is biocompatible to nerve cells and can be used as nerve conduit material to facilitate nerve cell attachment, differentiation and growth (Cheng et al., 2003; Yuan et al., 2004). A chitosan tube made from crab tendons (with additives) was used as an artificial nerve graft for bridging a 15-mm sciatic nerve gap in rats; the in vivo examination demonstrated that inflammatory cell infiltration occurred on the chitosan tube surface during the initial period after implantation and lasted for 6 weeks, despite a rapid decrease in tissue reactions with time (Itoh et al., 2003a, b). On the other hand, polyglycolic acid (PGA) is a synthetic polymer that has exhibited great promise for use in nerve tissue engineering and in nerve regeneration, research interest in artificial nerve grafts has been focused on seeking bioresorbable materials to construct nerve conduits (their uniqueness is that they gradually degrade after functioning as temporary scaffolds for nerve regeneration).

Chitosan (the fully or partially deacetylated form of chitin) is the second most abundant polysaccharides in nature next to cellulose. In vitro studies have shown that chitosan is biocompatible to nerve cells and can be used as nerve conduit material to facilitate nerve cell attachment, differentiation and growth (Cheng et al., 2003; Yuan et al., 2004). A chitosan tube made from crab tendons (with additives) was used as an artificial nerve graft for bridging a 15-mm sciatic nerve gap in rats; the in vivo examination demonstrated that inflammatory cell infiltration occurred on the chitosan tube surface during the initial period after implantation and lasted for 6 weeks, despite a rapid decrease in tissue reactions with time (Itoh et al., 2003a, b). On the other hand, polyglycolic acid (PGA) is a synthetic polymer that has exhibited great promise for use in nerve tissue engineering and in nerve regeneration, research interest in artificial nerve grafts has been focused on seeking bioresorbable materials to construct nerve conduits (their uniqueness is that they gradually degrade after functioning as temporary scaffolds for nerve regeneration).

Material and methods

Preparation of the artificial nerve graft

The chitosan/PGA artificial nerve graft was prepared according to our patent (Chinese patent ZL 0110820.9). Briefly, 3 g of chitosan (Nantong Xincheng Biochemical Company, Nantong, Jiangsu, P.R. China), with a deacetylation degree of 92.3% and an average molecular weight (Mw) of 2.2 × 10⁶ kDa, was dissolved in 100 ml of 0.15 M acetic acid and stirred to form a white viscous liquid. Following slow addition of 10 ml of 2.0% (w/v) formaldehyde solution into the liquid, a porous viscous gel formed.

The gel was immediately injected into a stainless-steel casting mould. The mould consisted of an inner pillar and an outer tube, which were fixed on the mould bottom and which determined the diameter and thickness of a chitosan conduit. After being demoulded through lyophilization, the resulting chitosan conduit was rinsed repeatedly with double-distilled water for 2 days to remove the formaldehyde residues. The microporous conduit had an inner diameter of 4.5 mm, a wall thickness of 0.6 mm and was 30 mm long.

An artificial nerve graft was obtained by filling the conduit with ~2000 longitudinally aligned PGA fibres, which were 14 μm in diameter and 30 mm long (Holycon Biochemical Instrument Co. Ltd., Nantong, Jiangsu, P.R. China). The graft was sterilized by exposure to radiation of 20 kGy 60Co for 12 h, and immersed in sterile saline for 30 min prior to implantation.

Animals and surgical procedure

Seventeen adult male Beagle dogs weighing 6–8 kg were obtained from the Comparative Medical Centre of Yangzhou University (Yangzhou, Jiangsu Province, China). All the animal tests were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Academy of Sciences (http://oacu.od.nih.gov/regs/index.htm) and approved by the Administration Committee of Experimental Animals, Jiangsu Province. The dogs were randomly divided into three groups: (i) six in a chitosan/PGA graft group; (ii) six in an autograft group as the positive control; and (iii) five in a non-grafted group as the negative control.

Each animal was anaesthetized by an intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight) before surgery on its sciatic nerve. This was exposed after making a skin incision and splitting the underlying muscles in the left lateral thigh. A 26-mm long segment of sciatic nerve was then resected, leaving a 30-mm long defect following retraction of the nerve ends.

For dogs in a chitosan/PGA graft group, the nerve defect was bridged by our artificial nerve graft with both the proximal and distal nerve stumps anastomosed to the graft at each junction (Fig. 1A). For an autograft group, the nerve defect was re-implanted with the resected nerve segment instead of the artificial nerve graft. For a non-grafted group, the nerve defect was left unbridged.

General observation

After surgery, all dogs were housed and fed routinely, and monitored for changes in their appearance, appetite, response and locomotion activities. The posture and gait of each dog were recorded by the aid of photos and videotapes at 1 week, 1 month, 3 months and 6 months after surgery.

Electrophysiological assessment

Six months after surgery, electrophysiological tests were performed on all animals. Under anaesthesia with sodium pentobarbital, the previous surgical site at the mid-thigh level was re-opened and the left sciatic nerve was re-exposed. Electrical stimuli (10 mA in strength) were applied to the sciatic nerve trunk at the proximal and distal ends of the graft sequentially, and compound muscle
Germany) with ultraviolet illumination so as to obtain micrographs.

under a DMR fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Sections were mounted on a cryostat (30 µm thick transverse sections for spinal cords and 20 µm thick longitudinal sections for DRG). Sections were examined with FluoroGold (FG) retrograde tracing. Fifty microlitres of a 5% FG solution (Fluorochrome Inc., Denver, CO, USA) was injected into the sciatic nerve trunk 10 mm from the distal end of the chitosan/PGA graft followed by suture of the incisions.

action potentials (CMAPs) were recorded on the gastrocnemius belly. The latency and the amplitude of CMAPs were obtained, and the distance between the distal and proximal stimulated sites was measured to calculate the conduction velocity of the motor nerves (Suzuki et al., 1999). Normal CMAPs were also recorded at the unoperated contralateral side.

The following procedures were carried out at the end of the electrophysiological tests.

**FluoroGold retrograde tracing**

Three dogs randomly selected from the chitosan/PGA graft group were examined with FluoroGold (FG) retrograde tracing. Fifty microlitres of a 5% FG solution (Fluorochrome Inc., Denver, CO, USA) was injected into the sciatic nerve trunk 10 mm from the distal end of the chitosan/PGA graft followed by suture of the incisions. After being kept routinely for 2 weeks, the dogs were transcardially perfused sequentially with saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The vertebral canal was opened before the lumbar spinal cord was exposed. Afterwards, the animal was transcardially perfused in the same way as described above. The regenerated nerve in the place of the graft, together with two segments of sciatic nerve 20 mm outside the proximal and distal ends of the graft, was dissected out. The contralateral sciatic nerve was used as a control. The specimens were fixed in buffered 4% paraformaldehyde, washed in water, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 6 µm thick sections.

For the nerve specimens, transverse sections were obtained at four sites of a specimen, i.e. the proximal sciatic nerve (PN), the proximal graft (PG), the distal graft (DG) and the distal sciatic nerve (DN) (Fig. 1B). If they could be obtained at the corresponding sites, the specimens from the non-grafted animal were treated in the same way. All nerve sections were made in triplicate: the first one was stained with haematoxylin and eosin (H&E); the second using a special trichrome technique developed by Meyer et al. (1995), for which three main dyes (haematoxylin, Fast Green FCF and Chromotrope 2R) were used; and the third by immunohistochemical methods. As for the gastrocnemius muscle specimens, both transverse and longitudinal sections were prepared, and Masson trichrome staining was applied to every sixth slice.

**Immunohistochemistry**

After being routinely deparaffinized and rinsed in 0.01 M phosphate-buffered saline (PBS) for 10 min, the nerve sections were blocked in a 10% normal goat serum for 30 min at room temperature, and allowed to incubate with mouse monoclonal anti-NF-200 antibody (1:400, Sigma, St Louis, MO, USA) at 4°C for 24 h before being washed three times in PBS.

The nerve sections were further reacted with alkaline phosphatase (AKP) labelled secondary antibody goat anti-mouse IgG (1:1000, Gibco, Grand Island, NY, USA) at 4°C for 24 h before being washed three times sequentially in PBS, 2 × 10 min in 0.1 M Tris buffer I (containing 0.1 M NaCl and 0.1 M MgCl₂, pH 8.0) and 2 × 10 min in 0.1 M Tris buffer II (containing 0.1 M NaCl and 0.1 M MgCl₂, pH 9.5). After the AKP chromogen, BCIP/NBT, had been applied to them at 37°C for 10 min, the sections were dehydrated and cleared before cover slips were applied.

DRG sections labelled in retrograde with FG were also immunostained for NF (specific to myelinated axons and their cell bodies) to determine the FG fluorescence localization. After being rinsed three times in PBS, the cryo-sections were blocked in a 10% normal goat serum for 30 min. Primary antibody monoclonal mouse anti-NF-200 (1:400, Sigma), was applied and the sections allowed to incubate at 4°C for 24 h. TRITC (tetramethylrhodamine isothiocyanate) labelled secondary antibody goat anti-mouse IgG (1:200, Sigma) was then added and the sections allowed to incubate at 4°C for 24 h. After each exposure to immunoreagents, the sections were routinely washed in PBS. The sections were mounted on slides and coverslipped followed by visualization under a TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

Some of DRG sections were suitable for immunofluorescent staining for neurofilament (NF) in the manner as described below.

**Histological assessment**

The gastrocnemius muscles of both sides were harvested from a deeply anaesthetized animal and their wet weights measured. Several pieces of the excised gastrocnemius muscle were cut from its mid-belly and fixed in buffered 4% paraformaldehyde. Afterwards, the animal was transcardially perfused in the same way as described above. The regenerated nerve in the place of the graft, together with two segments of sciatic nerve 20 mm outside the proximal and distal ends of the graft, was dissected out. The contralateral sciatic nerve was used as a control. The specimens were fixed in buffered 4% paraformaldehyde, washed in water, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 6 µm thick sections.

For the nerve specimens, transverse sections were obtained at four sites of a specimen, i.e. the proximal sciatic nerve (PN), the proximal graft (PG), the distal graft (DG) and the distal sciatic nerve (DN) (Fig. 1B). If they could be obtained at the corresponding sites, the specimens from the non-grafted animal were treated in the same way. All nerve sections were made in triplicate: the first one was stained with haematoxylin and eosin (H&E); the second using a special trichrome technique developed by Meyer et al. (1995), for which three main dyes (haematoxylin, Fast Green FCF and Chromotrope 2R) were used; and the third by immunohistochemical methods. As for the gastrocnemius muscle specimens, both transverse and longitudinal sections were prepared, and Masson trichrome staining was applied to every sixth slice.

---

Fig. 1 (A) Gross view obtained immediately after bridge implantation of the chitosan/PGA artificial nerve graft (asterisk) into a 30-mm dog sciatic nerve defect. The arrow and arrowhead indicate the proximal and the distal coaptations, respectively. Minimal scale = 1 mm. (B) Schematic diagram showing four sites (PN, PG, DG and DN) of a nerve specimen of the operated limb, at which transverse sectioning was performed 6 months post-operatively for histological assessment.
Electron microscopy

Electron microscopy was performed for nerve specimens of three randomly selected dogs from each group. The samples were fixed with pre-cooled 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide solution, dehydrated stepwise in increasing concentrations of ethanol, and embedded in Epon 812 epoxy resin. Ultra-thin sections were stained with lead citrate and uranyl acetate, followed by examination under a transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Morphometric analysis

The specimens from the distal sciatic nerve portions, which had been stained with Meyer’s modified trichrome technique, were photographed using a DC 300F colour digital camera and the images digitalized into a Q550 IW image analysis system (Leica Imaging Systems Ltd., Cambridge, England). Three visual fields of the oil-immersed lens were selected randomly for each specimen and the cross-section areas of >1000 myelinated nerve fibres were measured for each group. The diameter distribution of myelinated nerve fibres was then generated (Fine et al., 2002).

After being immunostained for NF, the transverse section of nerve tissues was analysed to determine the percentage of the total area that was NF immuno-positive. The density of myelinated axons was calculated by dividing the number of axons in a high magnification field by the field area.

Following examination under a transmission electron microscope, photographs from 10 random fields (3000× magnification) of each of the ultra-thin nerve sections were taken. These were digitalized into the image analysis system and analysed using the Leica QWin software package to obtain the diameters of the myelinated axons and myelinated nerve fibres. The thickness of myelin sheaths and the G ratio (myelinated axon diameter/total myelinated fibre diameter) were calculated (Fansa et al., 2001).

The image analysis system was also applied to the transverse sections of gastrocnemius muscle stained by Masson trichrome reagents in order to measure the cross-section areas of the muscle fibres. The percentage of the collagen fibre area that was expressed was calculated by dividing the collagen fibre area by the sum of muscle fibre area and collagen fibre area.

Statistical analysis

Morphometric data were analysed using one-way ANOVA (analysis of variance) with the Stata 6.0 software package (Stata Corp., College Station, TX, USA). If there was no significant overall difference between groups, pairwise comparisons were conducted using Schefé’s post hoc test. Values of \( P < 0.05 \) were considered statistically significant.

Results

Macroscopic appearance

Following implantation, all dogs in both the chitosan/PGA graft and autograft groups showed no obvious signs of systemic or regional inflammation. The locomotor function of their operated limbs was recovered gradually; animals began to stand with their palms at the operated side touching the ground at 4 weeks post-operatively and showed it was possible to walk at ease at 3 months post-operatively. Meanwhile, the non-grafted dog could stand with the operated limb suspended and walk only with a limp.

Six months after surgery, the chitosan/PGA graft dog could stand upright on two hind limbs (Fig. 2A), which coordinated well with each other in movements (Fig. 2D); the extent of the strength of the operated limb seemed to approximate to that of the unoperated limb. The calf muscle groups of the operated limb showed no remarkable atrophy in the chitosan/PGA graft group. The recovery in locomotor function of the operated limb for the autograft group was similar to that for the chitosan/PGA graft group (Fig. 2B and E). In the non-grafted group, however, the operated hind limb exhibited limping, calf muscle atrophy, incapability of load-bearing, abnormality of metatarsophalangeal joint plantar flexion (Fig. 2C) and uncoordinated motion with the unoperated hind limb (Fig. 2F) even at 6 months after surgery. Moreover, the non-grafted dog could not stand upright on two hind limbs.

Electrophysiological evaluation

Six months after surgery, CMAPs were detected at both the operated and unoperated sides for each dog in both the chitosan/PGA graft and autograft groups (Fig. 3A–D). The recovery index of CMAP amplitude in the chitosan/PGA graft dog was \( \sim 0.55 \) as calculated by the formula:

\[
\text{Recovery index} = \frac{\text{Peak amplitude of the operated side}}{\text{Peak amplitude of the unoperated side}}
\]

The difference in CMAP amplitude was not statistically significant between the chitosan/PGA graft and autograft groups (Fig. 3E). The motor nerve conduction velocities were 33.31 ± 11.67 and 32.27 ± 10.56 m/s (mean ± SD) for the chitosan/PGA graft and autograft groups, respectively. However, there was no statistically significant difference between them. The average conduction velocity in the chitosan/PGA graft dog was 33.5% of that of the normal nerve control (90.49 ± 17.26 m/s). In addition, no CMAP was recorded on the operated side of the non-grafted dog.

Retrograde tracing

Two weeks after an injection of FG tracer into a tested animal, golden-coloured FG fluorescence occurred in DRG sections ipsilateral to the operated sciatic nerve on illumination with ultra-violet light. More than a third of the DRG neurons were retrogradely labelled, and the FG fluorescence was found to concentrate within the NF immuno-positive neuron cell bodies (Fig. 4A–C). The FG-labelled particles were found to aggregate at one side within the neuron, yielding an uneven distribution of fluorescence intensity. Some of the FG positive cells were found within the cell groups under DRG capsula, either in singles or clusters. Other FG positive cells were dispersed among the nerve fibre bundles. In addition, FG labelled motoneuron cell bodies were found in the anterior horn of grey matter ipsilateral to the operated side (Fig. 4D) and, on average, there were eight cell bodies labelled in each transverse section of the spinal cord. In addition, the fluorescent particles aggregated at one side within these neurons.
Qualitative and quantitative morphological analyses of regenerated nerve tissues

Gross views

Six months after surgery it was observed that, in the chitosan/PGA graft group, the artificial nerve graft had been completely degraded and absorbed, and a piece of nerve-like regenerated tissue continuously bridged the sciatic nerve across the 30-mm defect without obvious enlargements at each junction (Fig. 5). In the autograft group, on the other hand, the graft had been integrated well to both stumps of sciatic nerve without remarkable demarcations. For the non-grafted dog, however, there was some loose tissue between the nerve stumps with remarkable enlargements at the proximal stump. Serious adhesion of both stumps to the epimysium of the underlying musculature was also observed.

Light microscopy and morphometric examination

In the chitosan/PGA graft animal, neither residual fragments of chitosan and PGA nor infiltration of inflammatory cells were found in either the proximal or distal portions of the graft, where massive bundles of myelinated nerve fibres and numerous blood vessels were regenerated. The regenerated myelinated nerve fibres were smaller in diameter and their myelin sheaths were relatively thin. In the autograft group, most of the nerve fibres regenerated into the original fascicles of the autograft with an even distribution; quite a few fibres regenerated outside the fascicles, showing an extrafascicular regeneration (McLean et al., 2002). In the non-grafted group, however, far fewer nerve fibres but a large quantity of connective tissues were visible between the nerve stumps, and axons were observed by chance at 10 mm proximal to the distal nerve stump only for two dogs among the five.

At the transversely sectioned distal portion of sciatic nerve of the chitosan/PGA graft dog, nerve fibres regenerated densely into the original fascicles with an even distribution. Very few fibres regenerated outside the fascicles (Fig. 6A and B; Fig. 7A and B), where quite a large number of regenerated fibres were observed for the autograft dog (Fig. 6C and Fig. 7C). With the non-grafted dog, there were obvious hyperplasia of connective tissues and dramatic enhancements of fusiform cells in degenerated fascicles in disoriented arrangement, and no regenerated nerve fibre was seen (Fig. 6E–F and Fig. 7E–F).

Fig. 2 Photographs (A–C) demonstrating locomotion functions of the operated hind limb of dogs at 6 months post-operatively and sequential video frames (D–F) showing coordination in the motion of the two hind legs when the dogs were walking at 6 months after surgery. In the chitosan/PGA graft group, dogs could stand upright on two hind limbs (A) and exhibited a good coordination of two hind limbs (D). In the autograft group, dogs behaved similarly to dogs in the chitosan/PGA graft group (B and E). The non-grafted dog (C) could not stand upright on its two hind limbs, and had a poor function recovery with incapability of load-bearing and abnormality of metatarsophalangeal joint plantar flexion (arrow). (F) It also exhibited a limping, a lack of coordination of the operated limb with the unoperated limb, and abnormality of metatarsophalangeal joint plantar flexion (arrows).
Following anti-NF immunohistochemical staining, the percentage of NF immuno-positive areas and myelinated axon density were measured in order to compare the three groups (data not shown). At the PG or DG, both the percentage of NF immunopositive areas and myelinated axon density showed no significant difference between the chitosan/PGA graft and autograft groups ($P > 0.05$), but they were significantly lower in the non-grafted group compared with the chitosan/PGA graft group ($P < 0.05$). At the DN of the chitosan/PGA graft dog, the percentage of NF immunopositive areas and myelinated axon density were 14.26% and 9895/mm², respectively. These data were slightly

---

**Fig. 3** CMAP examinations carried out 6 months post-operatively. Representative data detected at the operated side of the dog in the chitosan/PGA graft group (A) and the autograft group (C), and at their respective unoperated contralateral side (B and D). The traces recorded after stimulating the distal and proximal positions of the nerve trunk are marked with arrowheads and arrows, respectively. No reproducible CMAPs were recorded in the non-grafted group. (E) Comparison of CMAP amplitudes. *$P < 0.05$ for both the chitosan/PGA graft and the autograft group versus normal control. There was no significant difference between the chitosan/PGA graft group and the autograft group ($P > 0.05$).

Following anti-NF immunohistochemical staining, the percentage of NF immuno-positive areas and myelinated axon density were measured in order to compare the three groups (data not shown).
higher than those for either the autograft dog or the normal contralateral side, and differences were statistically significant ($P < 0.05$).

The diameter of myelinated nerve fibres at the DN was measured and the diameter distribution plotted (Fig. 8). In the chitosan/PGA graft group, myelinated fibres 3–4 $\mu$m in diameter were as much as 43% of the whole population, and those 2–5 $\mu$m in diameter accounted for the majority (~80% of the population). The distribution pattern in the autograft group was similar to that in the chitosan/PGA graft group, while the myelinated nerve fibre diameter in these two groups were smaller than that of the normal contralateral nerve, the diameter distribution of which had a peak at 6–7 $\mu$m. The mean diameter of myelinated fibres at the DN of dogs in the chitosan/PGA graft and autograft groups was 3.05 ± 0.26 and 2.93 ± 0.37 $\mu$m, respectively, suggesting no significant difference. However, the mean diameter in the chitosan/PGA graft group was as small as 50% of that of the normal nerves (6.12 ± 0.5 $\mu$m).

Fig. 4 FluoroGold retrograde tracing carried out 6 months after implantation of the chitosan/PGA graft. (A) DRG neurons labelled by FG. (B) Immunostained for NF-200. (C) Overlay of (A) and (B). FG fluorescent particles aggregated at one side within cell bodies (arrow); an asterisk indicates the DRG capsule. (D) FG-labelled motoneurons in the anterior horn of grey matter in the spinal cord. FG fluorescent particles also aggregated at one side within cell bodies (arrow). Scale bar: 40 $\mu$m for A, B and C; 80 $\mu$m for D.

Fig. 5 Gross view of the regenerated nerve 6 months after bridge implantation of the chitosan/PGA artificial nerve graft. The arrow and arrowhead mark the proximal and distal coaptation, respectively. Minimal scale = 1 mm.
Fig. 6 Light micrographs (Meyer’s modified trichrome staining) obtained 6 months post-operatively of the transversely sectioned distal sciatic nerve portion of the dog operated side in the chitosan/PGA graft group (A), the autograft group (C), the non-grafted group (E) and of the dog unoperated side (G), respectively. B, D, F and H are higher magnifications of the boxed areas in A, C, E and G, respectively. Arrows indicate well-developed myelinated nerve fibres (B, D), a large number of nerve fibres regenerating outside fascicles (C) and normal myelinated nerve fibres (H). Arrowheads indicate fusiform cells hyperplasia in (F). Scale bar = 100 μm for A, C, E and G; 10 μm for B, D, F and H. The insert is a schematic diagram of sectioning sites.
Electron microscopy and morphometric examination

In the chitosan/PGA graft group, transmission electron microscopy of the mid-portion of the graft revealed that regenerated myelinated fibres were dispersed densely in clusters and there were also numerous unmyelinated fibres. Despite their thinner thickness compared with the normal nerve, myelinated fibres had a compact and uniform structure, including a clear, electron-dense myelin sheath and

Fig. 7 Anti-NF immunohistochemistry obtained 6 months post-operatively of the transversely sectioned distal sciatic nerve portion of the dog-operated side in the chitosan/PGA graft group (A), the autograft group (C), the non-grafted group (E) and the dog-unoperated side (G). B, D, F and H are higher magnifications of the boxed areas in A, C, E and G, respectively. Arrows indicate regenerated (B, C and D) or normal axons (H). Scale bar: 100 μm for A, C, E and G; 10 μm for B, D, F and H. The insert is a schematic diagram of sectioning sites.

Electron microscopy and morphometric examination

In the chitosan/PGA graft group, transmission electron microscopy of the mid-portion of the graft revealed that regenerated myelinated fibres were dispersed densely in
perfect basal membrane of Schwann cells (Fig. 9A). In the autograft group, the regenerated nerve fibres with thin myelin sheaths dispersed in clusters (Fig. 9B). The average thickness of regenerated myelin sheath was measured as 0.63 \( \mu \text{m} \) in the chitosan/PGA graft group. This value is much smaller than that of normal nerves (1.02 \( \mu \text{m} \)), but it is similar to that in the autograft group (0.75 \( \mu \text{m} \)). In addition, the G ratios are 0.673 ± 0.083 and 0.651 ± 0.083 in the chitosan/PGA graft and autograft groups, respectively, suggesting no significant difference. However, both G ratios are larger than that of the normal nerve (0.596 ± 0.102).

Morphological and morphometric examinations of the gastrocnemius muscle

In all three groups at gross view, the size of the gastrocnemius muscle on the operated side was smaller than that on the unoperated side to varying degrees, and especially in the non-grafted group. The muscles on the operated side were red and lustrous in colour, and quite soft in texture for both the chitosan/PGA graft group and the autograft group. However, they were pale in colour, hard and tenacious in texture with the severely atrophied muscle belly for the non-grafted group. The wet weight ratio of gastrocnemius muscle (operated side/ unoperated side) showed no significant difference between the chitosan/PGA graft and autograft groups, but the ratio for the non-grafted group was only 0.44. This was significantly lower than 0.83, an average value of wet weight ratio for the chitosan/PGA graft group \((P < 0.05)\).

Morphometric analyses were performed on specimens of gastrocnemius muscle following Masson trichrome staining (Fig. 10). For the chitosan/PGA graft group, muscle cells of gastrocnemius on the operated side were uniform in their size with an average cross-sectional area of 585.6 mm\(^2\). Their nuclei were under the sarcolemma, with no remarkable increase in number, and they showed clear cross striations on longitudinal sections. Moreover, there was a small amount of collagen fibre between the gastrocnemius cells on the operated side with no obvious hyperplasia; the average percentage of the collagen fibre area was 2.2%. No significant difference was detected between the chitosan/PGA graft and the autograft groups. For the non-grafted group, however, the gastrocnemius cells of the operated side exhibited typical atrophy and degeneration resulting from denervation; the cross-sectional area of muscle cells was much smaller, being a half that of the chitosan/PGA graft group. However, the average percentage of the collagen fibre area was much higher (>10%).
Discussion

For peripheral nerve repair, much effort has been devoted to developing artificial nerve grafts to replace traditional autograft techniques, which exhibit some drawbacks. Although artificial nerve grafts constructed from non-resorbable materials (e.g. conduits made from silicone or polyethylene) have yielded some degree of functional recovery, long-term complications often mean that a second surgical procedure is necessary to remove the conduits. These may actually become detrimental by virtue of toxicity or tendency to constrict the nerve (Fields et al., 1989). A nerve graft made of bioresorbable materials is thus a promising alternative for promoting successful nerve regeneration. A variety of resorbable materials have been examined for nerve tissue engineering applications, including two classes of polymer. One class consists of natural polymers such as chitosan and alginate (Hashimoto et al., 2002; Itoh et al., 2003a,b). Another class is formed from synthetic polymers such as polyglycolic acid (PGA), poly l-lactic acid (PLLA), poly-3-hydroxybutyrate (PHB) and their copolymers or derivatives (Matsumoto et al., 2000; Evans et al., 2002; Young et al., 2002).

Chitosan \([(1\rightarrow4)-2\text{-amino-2-deoxy-\(\beta\)-d-glucan}] is a derivative of chitin, a biopolymer of \((1\rightarrow4)-N\text{-acetyl-2-amino-2-deoxy-\(\beta\)-d-glucose units linked by 1–4 glucosidic bonds, obtained by N-deacetylation of the latter. Experimental evidence suggests that chitosan is mainly depolymerized by lysozyme into glucosamine units in the body. Chitosan possesses not only beneficial biological properties, but also many other inherent characteristics of permeability, adsorption performance and easiness of processing, which make it suitable for a range of applications in fields such as materials, medicine, pharmaceuticals and the food industry. In recent years, chitosan has come into use for the development of tissue-engineered grafts (Lee et al., 2000; Cheng et al., 2003; Itoh et al., 2003a,b).

PGA, a member of the linear aliphatic polyester family, has been used widely as a synthetic polymer for tissue engineering. PGA degrades into glycolic acid \(in vivo\) through hydrolysis of its ester linkage, while changing its molecular weight or chemical composition may regulate the rate of degradation. Surgical sutures made from PGA have been commercially available since the 1970s and are approved for clinical use.
by the US Food and Drug Administration. This polymer has been processed into a variety of three-dimensional scaffolds that are useful for tissue engineering purposes (Freed et al., 1994; Gao et al., 1998), including nerve repair applications (Matsumoto et al., 2000; Weber et al., 2000). Neither the polymer nor its degradation product (glycolic acid) are toxic in vivo.

Considering the favourable biological properties of chitosan and PGA as mentioned above, we have developed a dual-component artificial nerve graft comprising an outer microporous conduit of chitosan and internal oriented filaments of PGA. In this novel design, the chitosan conduit is found to support the in-growth of blood vessels and to allow diffusion of nutrients and other molecules while preventing cells from entering the conduit. The PGA filaments could serve as a directional guide to facilitate Schwann cells functioning properly. In vivo degradation products of chitosan and PGA are basic glucosamine and acidic glycolic acid, respectively; these are assumed to undergo neutralization without local remarkable change in pH, maintaining a relatively stable microenvironment for nerve regeneration. Moreover, both the degradation products of chitosan and PGA can enter metabolic pathways within the body.

In this study, we have examined an implantation into dog sciatic nerve across a 30-mm defect with our artificial nerve grafts. There have recently been reports on the application of artificial nerve grafts bridging a peripheral nerve gap >25 mm (Kiyotani et al., 1996; Suzuki et al., 1999; Matsumoto et al., 2000). However, the peripheral nerve defect treated in this study was as long as 30-mm in length and was from sciatic nerve, the largest single nerve trunk of the body. It also came from a large animal model, namely the Beagle dog, rather than the smaller mouse, rat, cat or rabbit. On the other hand, our artificial nerve graft was of an ‘empty’ conduit without pre-introduced biological active molecules of either growth substrates or neurite-promoting materials such as fibronectin, laminin, collagen, neurotropic factors or even viable Schwann cells.

This study demonstrated that, 6 months after implantation, the chitosan/PGA artificial nerve graft was degraded and absorbed completely, with replacement by a tissue with nerve-like appearance, which passed through the defect and linked up both stumps. Light microscopy findings revealed that, at the site of the original artificial nerve graft, the regenerated nerve tissues were encapsulated in a well-organized epineurium and nerve fibres were arranged in many fascicles of different size and compartmented by a perineurium.

In the chitosan/PGA graft group, the percentage of NF immuno-positive areas and myelinated fibre density were similar to those of normal nerve. However, the myelinated fibre density in the distal portion of sciatic nerve was slightly higher than that of the normal nerve, which might be associated with a relatively large amount of regenerating axon sprouts and their rami. Among numerous sprouts and their rami, only one sprout could reach a connection with the target cells—the synaptic terminal—so as to re-innervate the target cells. This evidence shows that guided axonal growth plays a pivotal role in peripheral nerve regeneration (McLean et al., 2002). The fact that extrafascicular regeneration at the distal sciatic nerve portion in the chitosan/PGA graft group was much less than that in autograft group indicates a guiding role of the artificial nerve graft during nerve regeneration. It is the guiding role that is responsible for preventing or reducing disoriented growth of regenerated nerves and for facilitating the re-innervation of target cells.

As one of the most important aspects for measuring the extent of nerve repair, the maturity of regenerated myelinated nerve fibres is frequently assessed by means of such parameters as the diameter distribution of myelinated nerve fibres, G ratio and thickness of myelin sheath (Chamberlain et al., 1998; Fansa et al., 2001). The diameter of myelinated nerve fibres has been shown to increase gradually with time, approaching the normal level over a long term of regeneration (Mackinnon et al., 1985). For instance, Hashimoto et al. (2002) found the diameter of myelinated nerve fibres in the rat sciatic nerve model to be close to the normal level 21 months after implantation. However, several groups have showed that the diameter of myelinated nerve fibres was usually smaller during a short period of time (Kiyotani et al., 1996; Ceballos et al., 1999; Fine et al., 2002). With regard to canine peripheral nerve regeneration, Matsumoto et al. (2000) found that the average diameter of myelinated nerve fibres at the distal nerve was merely 4.0 μm up to 12 months after implantation.

The results from our study showed that the diameter distribution of myelinated nerve fibres at the distal sciatic nerve portion of the chitosan/PGA graft dog was similar to that of the autograft dog with a peak at ~3–4 μm. The average diameter in the chitosan/PGA graft group was 3.05 μm—a value similar to that in the autograft group, but greatly less than that of the normal nerve. This can probably be attributed to a shorter period of nerve regeneration. On the other hand, it is known that the G ratio or the thickness of myelin sheath could affect the conduction velocity of myelinated nerve fibres. The results in the present study indicated that the G ratio in the chitosan/PGA graft group was similar to that in the autograft group, but higher than that of the normal nerve. Collectively, these data prove reconstruction of dog sciatic nerve at 6 months following implantation of our artificial nerve graft.

Since the amplitude of the CMAP is directly proportional to the number of nerve fibres innervating the muscle and allows the conduction velocity of the motor nerve to be calculated, CMAP examinations offer an important index for the conduction function of peripheral nerve. In this study, CMAP was obtained in each dog of the chitosan/PGA graft group. The recovery index of either the CMAP amplitude or the conduction motor nerve velocity showed no significant difference between the chitosan/PGA graft and autograft groups. These results thus suggest that recovery level of electrophysiological properties in the chitosan/PGA graft dog was close to that in the autograft dog. The fact that recovery index was only 0.55 might be correlated with multiple factors such
as the smaller diameter of the regenerating axons, thinner myelin sheaths with shorter internodes and immaturity of myelinated nerve fibres as a whole (Matsumoto et al., 2000).

Many fluorescent reagents like FluoroGold, Fast Blue, toxins such as cholera toxin-B and enzymes like horseradish peroxidase have been used extensively for retrograde tracing of axonal transport as well as for establishing regenerated nerve continuity (Kiyotani et al., 1996; Llewellyn-Smith et al., 2000; Fine et al., 2002). The FluoroGold retrograde tracing carried out in this study revealed FG-labelled neuron cell bodies in both the DRG and the anterior horn of grey matter in the spinal cord ipsilateral to the operated side of the chitosan/PGA graft dog. This demonstrated a completeness of the regenerated nerve pathway and axonal transport function of regenerated nerve fibres.

It is known that denervation of a target muscle occurs as a consequence of damage to motor nerves, followed by alterations in various aspects including gene expression, protein metabolism, enzyme activity, cell ultrastructure and neuromuscular junction. This induces a shift of protein metabolism from protein synthesis toward protein degradation, muscle fibres decrease in size and there is final muscle atrophy and unweighting, accompanied by hyperplasia of connective tissues. If the muscle is re-innervated, its function is restored and atrophy stopped (Bodine-Fowler et al., 1996; Bodine et al., 2001; Ijkema-Paassen et al., 2001). To measure the degree of skeletal muscle atrophy, it is necessary to determine the wet weight ratio; the smaller the wet weight ratio, the more serious the skeletal muscle atrophy. The gastrocnemius muscle was taken as representative of the target muscles of the sciatic nerve in this study. Six months after implantation, the wet weight ratio showed no significant difference between the chitosan/PGA graft group and autograft group, but the wet weight ratio for the non-grafted group was significantly smaller than that for the chitosan/PGA graft group. Morphological observations also demonstrated the difference between the chitosan/PGA graft group and the non-grafted group. These findings are consistent with the above electrophysiological assessment, collectively providing evidence for sciatic nerve regeneration and the resulting re-innervation of target muscle.

The sciatic nerve, as a mixed function nerve, is made up of both sensory and motor nerve fibres and is involved in skeletal muscle movement and proprioception, skin sensation and smooth muscle function in the region it controls. Although the classical walking track analysis, which allows quantitative analysis, can be employed for evaluation of motor nerve function of the sciatic nerve for mice and rats (Bain et al., 1989; McLean et al., 2002), few quantitative analysis methods have been recognized as suitable for evaluating a large animal model such as a dog (Matsumoto et al., 2000). In this study, therefore, we persisted in monitoring for changes in locomotion activities after implantation. The posture and gait of each dog were recorded regularly with the aid of photos and videotapes. These observations show the favourable effects of sciatic nerve repair.

In conclusion, the chitosan/PGA artificial nerve graft we developed was implanted into dog sciatic nerve injury across the 30-mm long defect. After 6 months, it was observed that the sciatic nerve tract had been reconstructed with restoration of nerve continuity, functional recovery for conducting electrical impulses and transporting materials, and re-innervation of target skeletal muscle resulted, which improved the locomotion activities of the operated limb. Accordingly, this study proves the feasibility of the chitosan/PGA artificial nerve graft for promoting nerve regeneration, raises new possibilities of seeking alternatives to autograft for nerve repair, and establishes an experimental basis for constructing tissue engineered nerve grafts favourable to an implantation into peripheral nerve with a larger defect. Furthermore, it is possible that this study will allow improvements to meet clinical trial requirements in the future.

Acknowledgements

We wish to thank Professor Jie Liu for assistance in the preparation of this manuscript. This work was supported by Hi-Tech Research and Development Programme of China (863 Program, Grant No. 2003AA205030) and a National Natural Science Foundation of China (Grant No. 30070255).

References


