Novel use of biodegradable casein conduits for guided peripheral nerve regeneration

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ABSTRACT

Recent advances in nerve repair technology have focused on finding more biocompatible, non-toxic materials to imitate natural peripheral nerve components. In this study, casein protein crosslinked by naturally occurring genipin (GCC) was used for the first time to make a biodegradable conduit for peripheral nerve repair. The GCC conduit was dark blue in appearance with a concentric and round lumen. Water uptake, contact angle and mechanical tests indicated that the conduit had a high stability in water and did not collapse and cramped with a sufficiently high level of

mechanical properties. Cytotoxic testing and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showed the GCC was non-toxic and non-apoptotic which could maintain the survival and outgrowth of Schwann cells. Noninvasive real-time nuclear factor-κB (NF-κB) bioluminescence imaging accompanied with histochemical assessment showed the GCC was highly biocompatible after subcutaneous implantation in transgenic mice. Effectiveness of the GCC conduit as a guidance channel was examined as it was used to repair a 10 mm gap in the rat sciatic nerve. Electrophysiology, labeling of calcitonin gene-related peptide (CGRP) in the lumbar spinal cord and histology analysis all showed a rapid morphological and functional recovery for the disrupted nerves. Therefore, we conclude that the GCC can offer great nerve regeneration characteristics and can be a promising material for the successful repair of peripheral nerve defects.

Keywords: Casein; Nerve conduit; Nerve regeneration; Nerve injury

1. INTRODUCTION

For improving peripheral nerve regeneration, the development of new biodegradable materials to make nerve conduits has attracted considerable attention in recent years. In particular, such materials as polypyrrole (Ateh *et al.* 2006), poly-lactic acid (Lu *et al.* 2009; Wang *et al.* 2009; Wang *et al.* 2010a) and poly-glycolic acid

(Waitayawinyu *et al.* 2007; Hu *et al.* 2008; Huang *et al.* 2009) are of special interest because of their soft tissue biocompatibility and the easy control of their physical and chemical properties of the polymer network. Recent advances in nerve conduit technology have focused on finding more biocompatible, non-toxic natural materials to imitate natural peripheral nerve components (Mano *et al.* 2007), such as collagen (Madduri *et al.* 2010; Pereira Lopes *et al.* 2010; Yao *et al.* 2010), gelatin (Ghasemi-Mobarakeh *et al.* 2008; Alvarez-Perez *et al.* 2010; Wang *et al.* 2010b), and chitosan (Li *et al.* 2010; Shen *et al.* 2010; Yu *et al.* 2010; Wang *et al.* 2010c). In this work, our group developed a novel protein-based biodegradable conduit for nerve repair. For this purpose, casein, a predominant phosphoprotein accounting for nearly 80% of proteins in cow milk (Eigel *et al.* 1979; Aimutis *et al.* 1982) was crosslinked by genipin, which is a naturally occurring and low-cytotoxic crosslinking agent that can be obtained from its parent compound geniposide isolated from the fruits of Gardenia jasminoides ELLIS (Chen *et al.* 2005; Bispo *et al.* 2010; Harris *et al.* 2010).

In order to understand physical characteristics of the genipin-crosslinked casein (GCC) conduits, we evaluated their mechanical function, water uptake ratio, and hydrophilicity. Cytotoxic testing and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of the conduits were determined by using the Schwann cell line, which has been extensively adopted to study cell differentiation and neurite

outgrowth (Chi *et al.* 2010; Liu *et al.* 2010; Wang *et al.* 2010d), to study its neuronal characteristics upon exposure to the substances released from soaked GCC conduits. Nuclear factor-κB (NF-κB)-dependent luminescent signal in transgenic mice carrying the luciferase genes accompanied with histochemical assessment were used as the guide to assess the host-GCC interaction. Finally, the effectiveness of GCC conduits as a guidance channel was evaluated by examining calcitonin gene-related peptide (CGRP) in the lumbar spinal cord by immunohistochemistry, and correlating morphometric and electrophysiological data after sciatic nerve transaction combined with subsequent neurorrhaphy in adult rats.

2. METHODS

2.1. Fabrication of GCC conduits

A 23% (w/w) solution of casein (Sigma #C5890, Saint Louis, MO) in 0.2 M Na_2HPO_4 buffer was mixed with 1.5% (w/w) solution of genipin (Challenge Bioproducts Co., Taichung, Taiwan) at 60°C by magnetic stirring. A silicone rubber tube (1.96 mm OD; Helix Medical, Inc., Carpinteria, CA) was used as a mandrel vertically dipped into the GCC solution at a constant speed where it remained for 30 sec. The mandrel was then withdrawn slowly and allowed to stand for 30 sec. The mandrel was rotated horizontally consistently to reduce variations in the wall thickness along the axis of the tube. Nine coating steps were used to obtain a GCC conduit with wall thickness of about 280 μm. The coated mandrel was air-drying for 1 week and the GCC conduits were slipped off the silicone rubber mandrel and cut to

15 mm length. To allow fixation of the nerve tissue to the conduit, two small holes were drilled at both ends of the GCC conduits. Finally, the GCC conduits were sterilized with 25 kGy of γ-ray for subsequent implantation.

2.2. Cross-linking degree of GCC conduits

Ninhydrin assay was used to evaluate the cross-linking degree of GCC conduits. Ninhydrin (2,2-dihydroxy-1,3-indanedione) was used to determine the amount of amino groups of each test sample. The test GCC conduits were heated with a ninhydrin solution for 20 min. After heating with ninhydrin, the optical absorbance of the solution was recorded using a spectrophotometer (Model Genesys™ 10, Spectronic Unicam, New York, NY) at 570 nm (wavelength of the blue-purple color) using casein at various known concentrations as standard. The amount of free amino groups in the residual casein, after heating with ninhydrin, is proportional to the optical absorbance of the solution. The cross-linking degree of GCC conduits was then determined.

2.3. Macroscopic observation of GCC conduits

To examine the morphology of the GCC explants with scanning electron microscopy (SEM), the samples were gold-coated using a Hitachi E-1010 Ion Sputter and micrographs were obtained using a Hitachi S3000N scanning electron microscope at an accelerating voltage of 5 kV.

2.4. Mechanical function of GCC samples

The mechanical properties of GCC were determined in a dry condition. All test samples were preconditioned at 50% humidity and 23°C for 48 h. The maximum tensile force was determined by the universal testing machines (AG-IS, Shimadzu Co., Japan). All test samples, cut into dumbbell shape (Fig. 1), were pulled at an extension rate of 0.6 mm/min. Measurements were made five times for each sample and averages were reported.

2.5. Water Contact Angle Analysis of GCC samples

Drops of distilled water were placed on the GCC films and contact angles were measured using a static contact angle meter (CA-D, Kyowa, Japan). An auto pipette was employed with the meter to ensure that the volume of the distilled water droplet was the same (20 μL) for each specimen.

2.6. Water uptake ratio *of GCC conduits*

The weight equilibrium water uptake ratio was experimentally determined using the following equation:

water uptake ratio = $(Wt-W_0)/W_0$

where Wt is the weight of the swollen test sample and W_0 is the weight of the dried

test sample. The measuring of water uptake ratio in each step is carefully conducted six times at 0.5, 1, 3, 6, 12, 24, 48, 60, 72 and 84 h after GCC conduits were soaked in 10 ml of de-ionized water of pH 7.4 at room temperature.

2.7. Cytotoxicity and apoptosis of soaking solution of GCC conduits

The indirect cytotoxicity was conducted in adaptation from the ISO10993-12 standard test method. GCC conduits of 6 cm^2 were washed twice with sterilized 1× PBS and dried in a laminar flow. GCC extraction solution was prepared by incubating the conduit in 1 ml of DMEM-serum free medium at 37°C for 24 h in an incubator with 75% humidity containing 5% CO_2. RSC96 Schwann cells were seeded at 1×10^4 cells/well in a 96-well tissue-culture polystyrene plate (TCPP; Corning, USA) at 37°C for 24 h in an incubator with 75% humidity containing 5% CO_2. After that, the culture medium was removed and replaced with the GCC extraction solution (200 μL/well). After 24 h and 48 h of cell incubation with the GCC extraction solution, the solution was removed, replaced with 110 μL/well of 5 mg/ml of MTT solution in 1× PBS and further incubated in an incubator at 37°C for 4 h. Then, the MTT solution was removed and replaced with 50 μL of DMSO to dissolve the formazan. The color intensity was measured using a microplate reader (ELx800TM, Bio-Tek Instrument, Inc., Winoski, VT, USA) at the absorbance of 550 nm. Data were then expressed as a

percent of control level of the optical density within an individual experiment.

Apoptotic cell death was also confirmed in the present study. After treating with the GCC extraction solution for 48 h, the Schwann cells were washed with PBS twice, fixed in 2% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100/PBS for 30 min at room temperature. After washing with PBS, TUNEL assay was performed according to the manufacturer's instructions (Boehringer Mannheim). Cells were incubated in TUNEL reaction buffer in a 37°C humidified chamber for 1 h in the dark, then rinsed twice with PBS and incubated with DAPI (1 mg/ml) at 37°C for 10 min, stained cells were visualized using a fluorescence microscope (Olympus DP70/U-RFLT50, Olympus Optical Co., Ltd., Japan). TUNEL-positive cells were counted as apoptotic cells.

2.8. Biocompatibility of GCC conduits

Prior to the beginning of the *in vivo* testing, the protocol was approved by the ethical committee for animal experiments of the China Medical University, Taichung, Taiwan. Transgenic mice, carrying the luciferase gene driven by NF-κB-responsive elements, were constructed as described previously (Ho *et al.* 2007; Hsiang *et al.* 2009). All transgenic mice were crossed with wild-type F1 mice to yield NF-κB-luc heterozygous mice with the FVB genetic background. For insertion of the GCC

implant, transgenic mice were anesthetized with 0.12 g ketamine/kg body weight and one incision (3 mm in length) on the back was made. The GCC conduit was then implanted subcutaneously into the incision and the skin was closed with silk sutures. Six transgenic mice were randomly divided into two groups of three mice: (1) sham, the incision was made and nothing was implanted and (2) GCC, the incision was made and the GCC conduit was implanted. The mice were imaged for the luciferase activity at various time points: 1 d, 3 d, 7 d, and 28 d and subsequently sacrificed for histochemical staining. For *in vivo* imaging, mice were anesthetized with isoflurane and injected intraperitoneally with 150 mg luciferin/kg body weight. Five minutes later, mice were placed facing down in the chamber and imaged for 5 min with the camera set at the highest sensitivity by IVIS Imaging System® 200 Series (Xenogen, Hopkinton, MA). Photons emitted from tissues were quantified using Living Image® software (Xenogen, Hopkinton, MA). Signal intensity was quantified as the sum of all detected photon counts per second within the region of interest after subtracting the background luminescence and presented as photons/sec/cm^2/steradian (photons/s/cm^2/sr). For histochemical staining, the GCC implants were retrieved and fixed in 10% formalin for 2 d. Tissue was rinsed in saline and dehydrated in a series of graded alcohols (50%, 70%, and 95%) for 30 min each. Samples were then embedded in paraffin and cut into thin 12-μm sections. For histomorphometric

evaluation, sections were stained with hematoxylin and eosin. The tissue reactions to the implants in the subcutaneous tissue were evaluated for uniformity and thickness of the foreign body capsule as well as the inflammation responses under optical microscopy (Olympus IX70, Olympus Optical Co., Ltd., Japan).

2.9. GCC conduits implantation

Thirty adult Sprague-Dawley rats underwent placement of GCC conduits, which were removed upon sacrifice at various time points: 2 weeks, 5 weeks, and 8 weeks. At each implantation time, 10 rats were operated on. The animals were anesthetized with an inhalational anesthetic technique (AErrane®, Baxter, USA). Following the skin incision, fascia and muscle groups were separated using blunt dissection, and the right sciatic nerve was severed into proximal and distal segments. The proximal stump was then secured with a single 9-0 nylon suture through the epineurium and the outer wall of the GCC conduits. The distal stump was secured similarly into the other end of the chamber. Both the proximal and distal stumps were secured to a depth of 2.5 mm into the chamber, leaving a 10-mm gap between the stumps. The muscle layer was re-approximated with 4-0 chromic gut sutures, and the skin was closed with 2-0 silk sutures. All animals were housed in temperature (22°C) and humidity (45%) controlled rooms with 12-hour light cycles, and they had access to food and water *ad libitum*.

2.10. Electrophysiological techniques

All the animals with apparent nerve regeneration were re-anaesthetized and the

sciatic nerve exposed. The stimulating cathode was a stainless-steel monopolar needle, which was placed directly on the sciatic nerve trunk, 5-mm proximal to the transection site. The anode was another stainless-steel monopolar needle placed 3-mm proximally to the cathode. Amplitude, latency, and nerve conductive velocity (NCV) of the evoked muscle action potentials (MAP) were recorded from gastrocnemius muscles with micro-needle electrodes linked to a computer system (Biopac Systems, Inc., USA). The latency was measured from stimulus to the takeoff of the first negative deflection and the amplitude from the baseline to the maximal negative peak. The NCV was carried out by placing the recording electrodes in the gastrocnemius muscles and stimulating the sciatic nerve proximally and distally to the nerve conduit and calculated by dividing the distance between the stimulating sites by the difference in latency time.

2.11. Histological processing

Immediately after the recording of muscle action potential, all of the rats were perfused transacrdially with 150 ml normal saline followed by 300 ml 4% paraformaldehtde in 0.1 M phosphate buffer, pH 7.4. After perfusion, the L4 spinal cord was quickly removed and post-fixed in the same fixative for 3-4 h. Tissue samples were placed overnight in 30% sucrose for cryoprotection at 4°C, followed by

embedding in optimal cutting temperature solution. Samples were the kept at -20°C until preparation of 18 μm sections was performed using a cryostat, with samples placed upon poly-L-lysine-coated slide. Immunohistochemistry of frozen sections was carried out using a two-step protocol according to the manufacturer's instructions (Novolink Polymer Detection System, Novocastra). Briefly, frozen sections were required endogenous peroxidase activity was blocked with incubation of the slides in 0.3% H_2O_2, and nonspecific binding sites were blocked with Protein Block (RE7102; Novocastra). After serial incubation with rabbit- anti-CGRP polyclonal antibody 1:1000 (calbiochem, Germany), Post Primary Block (RE7111; Novocastra), and secondary antibody (Novolink Polymer RE7112), the sections were developed in diaminobenzidine solution under a microscope and counterstained with hematoxylin. Sciatic nerve sections were taken from the middle regions of the regenerated nerve in the chamber. After the fixation, the nerve tissue was post-fixed in 0.5% osmium tetroxide, dehydrated, and embedded in spurs. The tissue was then cut to 5-µm thickness by using a microtome with a dry glass knife, stained with toluidine blue.

2.12. Image analysis

All tissue samples were observed under optical microscopy. CGRP-immunoreactivity (IR) in dorsal horn in the lumbar spinal cord was detected

by immunohistochemistry as described previously (Zheng *et al.* 2008). The immuno-products were confirmed positive-labeled if their density level was over five times background levels. Under a 100x magnification, the ratio of area occupied by positive CGRP-IR in dorsal horn ipsilateral to the injury following neurorrhaphy relative to the lumbar spinal cord was measured using an image analyzer system (Image-Pro Lite, Media Cybernetics, USA) coupled to the microscope.

As counting the myelinated axons, at least 30 to 50 percent of the sciatic nerve section area randomly selected from each nerve specimen at a magnification of 400x was observed. The axon counts were extrapolated by using the area algorithm to estimate the total number of axons for each nerve. Axon density was then obtained by dividing the axon counts by the total nerve areas. All data are expressed as mean ± standard deviation. Statistical comparisons between groups were made by the one-way analysis of variance.

3. RESULTS

3.1. Macroscopic observation of GCC conduits

GCC conduits were dark blue in appearance caused by the reaction between genipin and amino acids or proteins. Figure 2 shows the GCC conduit was concentric and round with a smooth inner lumen and outer wall surface.

3.2. Physical characteristics of GCC conduits

The cross-linking index of GCC conduits, expressed as a percentage of free amino groups lost during cross-linking, was 13.6±5.2%. It means that 1.0 wt.% genipin was sufficient to cross-link about 13.6% of the amino groups. The maximum tensile force and the water contact angle of GCC conduits were 165.7±24.9 N and 59.0±4.5 degree. These results showed that the GCC conduits could provide enough mechanical strength to resist muscular contraction and their surface was hydrophilic which was conducive to cell adhesion and growth. Throughout the experimental period, the water uptake ratios of the soaked GCC conduits increased markedly (Fig. 3A). Though the walls of the GCC conduits were swelled caused by absorption of soaking solution, they still kept their tubular structure without occlusion even after 72 hr of soaking (Fig. 3B), indicating that the genipin cross-linked casein matrix provided a framework with suitable mechanical strength.

3.3. Cytotoxicity and apoptosis of GCC conduits

Spindle-shaped cellular morphology of Schwann cells cultured on the culture plate was viable and there was no sign of infection. Treatment with the soaking solution of GCC conduits did not induce apoptotic cell death since only very few TUNEL positive cells were seen, suggesting that the DNA fragmentation did not occur in these Schwann cells (Fig. 4A). This result was supported by the cytotoxic test that all GCC scaffolds were considered non-toxic to Schwann cells as cell viability was in a range of 102.3–104.1%, indicated that these GCC scaffolds were suitable for cell culture (Fig. 4B).

3.4. Biocompatibility of GCC conduits

No clinical problems were seen for any of the rats in the postoperative period. The GCC implant was implanted subcutaneously on the back of the mice and the NF-κB-driven bioluminescent signals were monitored by luminescent imaging on the indicated periods (Fig. 5A). As a result, luminescent signal in the implanted region was initially increased and dramatically decreased (Fig. 5B). NF-κB activity reached a maximal activation at 3 d where a strong and specific in vivo bioluminescence around the implantation site was observed. In consistent with the bioluminescent signals, an acute inflammatory response was observed at the site between GCC conduits and their surrounding tissues even just 1 d post-implantation under optical microscopy, characterized by a rapid accumulation of inflammatory cells (Fig. 6A). Phagocytising reaction was still obvious at the interfaces between the disintegrated GCC materials and tissues after 3-7 d of implantation (Fig. 6B, 6C). At 28 d, the GCC conduits had been degraded completely (Fig. 6D).

3.5. Electrophysiological measurements

MAPs were recorded at postoperative intervals of 2, 5, and 8 weeks. All of the electrophysiological indexes, including amplitude, latency, and NCV of the

regenerated nerves were improved as a function of the experimental period (Fig. 7A-7C). Specifically, the difference of the NCV between the nerves at postoperative intervals of 2 and 8 weeks reached the significant level at $P<0.05$. In addition, the regenerated nerves at 8 weeks postoperatively had a significantly shorter latency as compared to those at 2 and 5 weeks of recovery ($P<0.05$). However, the lack of statistically significant amplitude difference could imply that the atrophy of the muscle was still serious after 8 weeks of recovery, even if muscle fibers had been reinnervated.

3.6. CGRP-IR in the dorsal horn following injury

Immunohistochemical staining showed that lamina I-II regions in the dorsal horn ipsilateral to the injury were strongly CGRP-immunolabeled on week 2, and then notably decreased on weeks 5 to 8 (Fig. 8A, 8B). CGRP-labeled fibers were also noted in the area of lamina III-V (Fig. 8C). These results indicated CGRP expression dynamics in the lumbar spinal cord differed depending upon the recovery stage of regenerating sciatic nerve in the GCC conduit.

3.7. Sciatic nerve regeneration

Throughout the 8 weeks of experimental period, no nerve dislocation out of the

GCC conduits was seen for all of the rats. Brownish fibrous tissue encapsulation was noted, covering all over the GCC conduits (Fig. 9). After trimming the fibrous tissue, cutting the wall of the tube, the regenerated nerve was exposed and then retrieved. Observing the muscle tissue surrounding the conduit, no obvious inflammation or adhesion was found. Overall gross examination of the GCC conduits at the three observation time points all revealed 100% of nerve formation in the tubes.

3.7.1. After 2 weeks

At 2 weeks post-implantation, the GCC conduits had been well integrated into the regenerating nerve tissue. Regenerated nerves in the GCC conduits were still immature composed of fibrin matrices, which were populated by mast cells and red blood cells (Fig. 10A). This fibrin bridge could provide a framework for subsequent migration of fibroblasts, Schwann cells, and axons.

3.7.2. After 5 weeks

At 5 weeks, the process of degradation of the GCC conduits was obvious. Only a small amount of wall residues was seen surrounding the regenerating nerve. Up to this time, the regenerated nerves became more mature, displaying a structure with a symmetric and thin epineurium, surrounding a cellular and vascularized endoneurium

in which numerous myelinated axons had been seen (Fig. 10B). This area was surrounded by a collagen-rich encapsulating structure in which remnants of the GCC conduit wall and numerous large round cells were observed. It appeared the GCC conduit was being broken down by these large round cells.

3.7.3. After 8 weeks

The GCC conduits had almost totally been degraded, exposing slender regenerated nerves inside. As seen at 5 weeks of regeneration, the nerves at this stage had a mature structure with a large number of myelinated axons interposed in the endoneurium with rich neovascularization (Fig. 10C). Although maturation of the regenerated nerve tissue progressed with time, it was still noted an outer encapsulating structure which contained fragments of GCC and large round cells (Fig. 10D).

3.8. Morphometric measurements

As aforementioned results, nerve features in the GCC conduits at 2 weeks of implantation were too immature to be included in comparisons of their morphometric measurements. By comparison, morphometric studies revealed available data in regenerated nerves in both the tube groups after 5 and 8 weeks of implantation for

their mean values of myelinated axon number, axon area, axon density, and total nerve area (Fig. 11A-11D). Especially, it was noted that a significant increase in the axon density and the axon area at the significant level of 0.05 for both the postoperative intervals. In addition, large variations of total nerve area and axon number occurred in the regenerated nerves at 5 weeks postoperatively, indicating that a relatively immature structure happened at this time point.

4. DISCUSSION

For a short nerve injury, the end-to-end and the fascicular suture repair techniques are suggested. However, if the nerve injury is extensive, forming an irreducible gap between the injured proximal and distal stumps, a nerve graft or a nerve bridge is preferred. It is difficult to acquire donor nerves for grafting; therefore, considerable research has been conducted on peripheral nerve repair using the nerve bridge technique. Most of the successful studies with the nerve bridging model have used a short nerve gap. The inherent regenerative capacity of the nerve in animals could be so efficient over shorter gaps that the benefits of different modifications of the nerve bridging conduit may not be fully revealed. To demonstrate the efficiency of nerve conduits in bridging damaged nerves, a larger gap is therefore suggested.

For improving peripheral nerve regeneration with a large gap, degradable

polymer conduits have attracted considerable interest. It is conceivable that different stratagems should be designed for the degradable conduits to assist the growth of regenerating nerves. Ideally, a nerve guide should be composed of a biodegradable material that degrades at a rate in accordance with the rate of axonal elongation during early phases of regeneration. At this stage, the nerve guides should persist their structure for enough time to allow formation of a fibrin matrix to connect the proximal and distal nerve stumps. Once the initial fibrin matrix is formed, the nerve guides should degrade within reasonable time. Otherwise, delayed nerve regeneration could happen, resulting from the compression by the guide lumen, causing epineural fibrosis thus hampering nerve regeneration and maturation (Chang *et al.* 2009).

In this work, a novel protein-based degradable nerve conduit has been prepared and characterized. Naturally occurring genipin was used to crosslink casein, a phosphoprotein that precipitates from raw skim milk by acidification (Bansal *et al.* 2007). The affinity of the GCC to the Schwann cells was assessed by cytotoxic testing, TUNEL assay, the contact angle, and the water uptake ratio of the materials. As a result, the GCC could maintain the survival and outgrowth of Schwann cells, which had a good hydrophilicity and could keep its integrity even after 72 h of soaking in the de-ionized water. We also constructed transgenic mice carrying the luciferase gene under the control of NF-κB-responsive element to monitor the inflammatory

response following implantation of the GCC. The noninvasive real-time NF-κB bioluminescence imaging accompanied with histochemical assessment both showed the GCC was highly biocompatible, only evoking a mild tissue response. These results are not surprising since the casein has been shown a promising material for use in pharmaceutical applications (Song *et al.* 2010; Trejo & Harte 2010), and the genipin shown prominent neuritogenic activity in paraneurons such as PC12h cells (Yamazaki *et al.* 2004; Yamazaki *et al.* 2006).

From *in vivo* observations, we found the cellular activity within the GCC conduits appeared a promising medicinal product for repair of peripheral nerve defects. We can see that the GCC conduits degraded as a function of implantation period. However, they still kept their functional capability as a structural cuff even after 8 weeks of implantation. Since the luminal adequacy is paramount in determining the extent of nerve regeneration, we believe that the stable dimensions of the GCC conduits played a critical role in contribution to the high success of nerve regeneration in the present study. Therefore, the GCC can be considered as an ideal tubulization material since it can provide a suitable and continuous support to protect the regenerating axons from invasion by the surrounding connective tissue. The stable dimensions of the GCC conduits could result from the chemical crosslinking of genipin with the amino groups on the casein macromolecular chains (Song *et al.*

2009). After completion of their guiding function, the GCC conduits degraded and were well integrated with the regenerating nerve tissues. At 2 weeks of regeneration, regenerated nerve cables were composed of fibrin matrices which were populated by mast cells and red blood cells. After 5 weeks of recovery, the regenerated nerves were well vascularized and myelinated axons were numerous in their endoneurial areas which were surrounded by a collagen-rich encapsulating structure. The encapsulation tissue is commonly seen as using a biodegradable conduit for nerve regeneration which is coming from the cellular activity during the process of degradation of the nerve guide that evokes neural fibrosis (Den Dunnen *et al.* 1993). These histological results were supported by the protein levels of CGRP in associated spinal cord segments, which were gradually decreased during the test period. Since the CGRP has been recognized as a nerve regeneration-promoting peptide in vivo (Belyantseva & Lewin 1999; Blesch & Tuszynski 2001; Chen *et al.* 2010), it is conceivable that the declining CGRP expression in the spines may be attributable to the fact that, nerves at their late stage of regeneration in the GCC conduits were more mature; thus, injury-related signals derived from these nerves which could be retrogradely transported to neurons in the dorsal horn and subsequently trigger these cells to synthesize and release CGRP became less. Morphometric studies also indicated that the recovery of the regenerated nerves was progressing with the function of the

experimental period, which suggested that the transected nerve had undergone adequate regeneration in the GCC conduits. The experimental test details, used in several recent studies on biodegradable bridging conduits to repair injured rat sciatic nerves, were gleaned from the literature and summarized (Table 1). It is noted that the regenerated nerves in the GCC conduits are more mature with larger mean values of myelinated axon count (approximately 6,000) and axonal density (approximately 25,000/mm^2) than those in the conduits made of various biodegradable materials reported in the literature, such as the chitosan (Wang *et al.* 2010c), the polylactic acid (Lu *et al.* 2009), the polyglycolic acid (Waitayawinyu *et al.* 2007), the proanthocyanidin cross-linked gelatin (Liu 2008), and the genipin cross-linked gelatin (Chang *et al.* 2009; Chen *et al.* 2005). In addition, the temporal and spatial progresses of cellular activity within the GCC conduit are better than those seen for experiments using silicone rubber nerve guides (Yang *et al.* 2010; Xie *et al.* 2008), which have largely been used in clinical practice. These results again show the advantages of the GCC conduits which could promote the regeneration and maturity of injured nerves.

5. CONCLUSION

The current study is the first work dedicated to genipin-crosslinked casein conduits, a newly devised natural nerve bridge. Such nerve guides seem to be

promising candidates to be applied as an alternative material for the clinical repair of large peripheral nerve defects since they are well-integrated into the host tissue with a mild foreign body reaction and supports myelinated axonal regeneration and functional recovery.

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CAPTIONS

Table 1: Experimental details of recent studies on biodegradable bridging conduits to repair injured rat sciatic nerves

Figure 1: Schematic drawing of the dumbbell-shaped sample used in the mechanical testing (not for real scale).

Figure 2: SEM micrograph of the GCC conduit.

Figure 3: (A) Time effect on the water uptake ratio (%) of GCC conduits. (B)

Macrographs of the GCC conduits soaked in de-ionized water at different periods.

Figure 4: Induction of apoptosis and cytoxicity by soaking solution of GCC conduits. (A) Nuclei of Schwann cells were characterized by DAPI and TUNEL assay and investigated under a fluorescent microscopy. (B) Quantification of cytotoxic test of soaking solutions of GCC conduits relative to the controls on Schwann cells. Values are mean±standard error.

Figure 5: NF-κB-dependent bioluminescence in living mice implanted with GCC conduits. (A) Diagrams show the bioluminescent signal within a radius of 2.5 mm of implanted region (boxed area). The color overlay on the image represents the photons/s emitted from the animal, as indicated by the color scales. (B) Quantification of photon emission within the implanted region. Values are mean ± standard error of three mice. *P<0.05, significant difference from other examined time points.

Figure 6: Micrographs of interface area between the host and GCC conduits implanted for (A) 1 d, (B) 3 d, (C) 7d, and (D) 28 d. Note a rapid accumulation of inflammatory cells phagocytising the disintegrated GCC materials (black arrows). Scale bars = 100 μm.

Figure 7: Analysis of the evoked muscle action potentials, including (A) peak

amplitude, (B) latency, and (C) NCV. *P<0.05, significant difference from other examined time points.

Figure 8: CGRP-IR in dorsal horn in the lumbar spinal cord after injury. (A) CGRP-IR was detected by immunohistochemistry, and (B) the positive CGRP-IR area ratio was measured. (C) Photo shows the area of lamina III-V examined for CGRP-labeled fibers (black arrows). Shown in (C, b) is the higher magnification of the boxed area in (C, a). Scale bars = 200 μm for panel A, 100 μm for (C, a), and 25 μm for (C, b). *P<0.05, significant difference from other examined time points.

Figure 9: Macrographs of the GCC conduits at different implantation periods.

Figure 10: Light micrographs of regenerated nerve cross-sections at different implantation periods, (A) 2 weeks, (B) 5 weeks, and (C) 8 weeks. Note a great number of myelinated axons (black arrows) and blood vessels (white arrows) in the nerves after 5 weeks of regeneration. (D) An encapsulating structure in which fragments of GCC (long black arrows) and large round cells tissue (short black arrows) was noted surrounding the regenerated nerve (white arrows indicate the border area). Scale bars = 100 μm for panels A, D and 25 μm for B, C.

Figure 11: Morphometric analysis from the regenerated nerves in the GCC conduits,

including (A) axon number, (B) axon area, (C) axon density, and (D) total nerve area. *P<0.05, significant difference from other examined time points.

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