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reprogramming is surely to
understand neural
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*John Gurdon
23 Sept 2015.*

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Does glioblastoma cyst fluid promote sciatic nerve regeneration?

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Abstract

Glioblastoma cyst fluid contains growth factors and extracellular matrix proteins which are known as neurotrophic and neurite-promoting agents. Therefore, we hypothesized that glioblastoma cyst fluid can promote the regeneration of injured peripheral nerves. To validate this hypothesis, we transected rat sciatic nerve, performed epineural anastomosis, and wrapped the injured sciatic nerve with glioblastoma cyst fluid- or saline-soaked gelatin sponges. Neurological function and histomorphological examinations showed that compared with the rats receiving local saline treatment, those receiving local glioblastoma cyst fluid treatment had better sciatic nerve function, fewer scars, greater axon area, counts and diameter as well as fiber diameter. These findings suggest that glioblastoma cyst fluid can promote the regeneration of injured sciatic nerve and has the potential for future clinical application in patients with peripheral nerve injury.

Key Words: nerve regeneration; peripheral nerve injury; sciatic nerve injury; cyst fluid; glioblastoma; growth factors; neural regeneration

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Introduction

Physicians have studied peripheral nerve injury (PNI) since the Hippocratic era (Terzis et al., 1997). An injury to any peripheral nerve may lead to complete or partial loss of sensory, motor or autonomic function, which can lead to a substantially reduced quality of life and loss of function (Devesa et al., 2012). Due to these catastrophic consequences, many studies have investigated the regeneration of peripheral nerves (Navarro et al., 2007). Several techniques have been confirmed useful in improving PNI, including the administration of neurotrophic factors (Raivich et al., 1991; Yin et al., 2001; Oya et al., 2002; Pierson et al., 2002), the blockade of axonal regeneration inhibitory molecules (Deumens et al., 2006; Radtke et al., 2009), stem cells (Cuevas et al., 2002; Uemura et al., 2012), and the use of nerve conduits (Weber et al., 2000; Taras et al., 2011). Fibrosis is also an important issue in patients with PNI, and many methods, such as low-dose external radiation (Petersen et al., 1996; Görgülü et al., 2003) and agents that are immunosuppressive (Wang et al., 1997; Lee et al., 2000; Ozgenel, 2003; Ozay et al., 2007) have been applied in this context. Despite these extensive studies, striking axon regeneration after PNI has not yet been achieved.

Glioblastoma multiforme is the most common primary central nervous system cancer. This condition may present a cystic lesion (Wen and Kesari, 2008), and glioblastoma cyst

fluid (GCF) contains various growth factors and extracellular matrix (ECM) proteins which are known as neurotrophic and neurite-promoting agents (Takahashi et al., 1990; Bodmer et al., 1991; Zumkeller et al., 1993; Weindel et al., 1994; Singer et al., 1999; Aronica et al., 2001; Karcher et al., 2006). Because GCF contains growth factors and extracellular matrix proteins, we hypothesized that GCF would augment peripheral nerve regeneration.

Material and Methods

Ethics statement

The experiments and animal care were conducted in strict accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), which can help protect animals used in the experiments. The experimental procedures were approved by the Animal Ethics Committee of Gazi University Faculty of Medicine, Turkey (G.U.E.T.-08-34).

Source of GCF

The GCF used in this study was obtained from a 60-year-old patient who underwent a surgical resection for a recently diagnosed primary glial tumor. The tumor was diagnosed as World Health Organization grade IV glioblastoma multiforme and had a cystic component. The patient signed the

written informed consent for participation in this study. The clinical procedure was approved by the Ethical Committee of Gazi University Faculty of Medicine, Turkey. During surgery, approximately 15 mL of cyst fluid was collected and centrifuged at $3,000 \times g$ for 10 minutes. The supernatant was preserved at -80°C for 1 week before use. Postoperative histopathological examination confirmed the diagnosis of glioblastoma multiforme.

Experimental groups

The study was conducted at the Animal Breeding and Experimental Research Laboratory Center of Gazi University, Turkey. Thirty-two female Sprague-Dawley rats weighing 200–300 g were used in this study. These rats were kept in a temperature-controlled ($22\text{--}25^{\circ}\text{C}$) environment with appropriate humidity, exposed to a 12-hour light/ dark cycle, and had free access to water and food. They were randomly assigned to two groups with 16 rats in each group. In the saline group ($n = 16$), rat models of sciatic nerve injury were established, an epineural anastomosis was performed as described below, and saline-soaked gelatin sponges (Spongostan™ Anal, Ethicon, Inc., Johnson & Johnson, USA) were wrapped onto the injured sciatic nerve. In the GCF group ($n = 16$), rat models of sciatic nerve injury were established and an anastomosis was performed as described below, and GCF-soaked gelatin sponges were wrapped onto the injured sciatic nerve (Figure 1).

Sciatic nerve injury models

Rats were anesthetized by intraperitoneal injection of 45 mg/kg ketamine (Ketalar, Parke Davis, Turkey) and 5 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey), after which they were allowed to breathe spontaneously. Temperatures were maintained at 37°C with a heating pad, and determined with a rectal digital thermometer (Digital Fever Thermometer, Becton-Dickinson, Franklin Lakes, NJ, USA). The local skin was disinfected with a povidone iodine mixture and shaved. Thereafter, the right sciatic nerve was exposed through a gluteal muscle splitting incision. The sciatic nerve was transected using a scalpel at 10 mm away from the sciatic foramen, immediately following which 8-0 monofilament polypropylene suture was used for an epineural repair of free nerve ends (Ethicon/Prolene, Edinburgh, UK) (Figure 1). Excessive tension at the repair site should not be applied. In the saline group, 0.4 mL of 0.9% NaCl was impregnated into absorbable $1 \times 2 \times 4 \text{ mm}^3$ gelatin sponges; in the GCF group, 0.4 mL of GCF was used. The sponges were wrapped tubularly at the anastomotic line. The skin was sutured primarily. All surgical procedures were performed by the same surgeon. The operating microscope (Zeiss OpMi 6; Carl Zeiss Surgical, Oberkochen, Germany) and microsurgical instruments were used.

Functional evaluation

A walking track analysis was used to preoperatively measure the sciatic function index (SFI). In addition, in the 4th week, all rats ($n = 32$) were subjected to walking track analysis. At

the 8th and 12th weeks, the remaining rats after the sacrifice at the 4th week ($n = 16$) were subjected to walking track analysis. A $10 \times 100 \text{ cm}^2$ wooden corridor was used to create walking tracks. One end of the structure opened to a dark compartment. Methylene blue was used during the experiment, whereby rat's hind limbs were dipped into the substance and they were allowed to move on a white piece of paper. This was done so that their footprints could be analyzed and the distance to the opposite foot, toe spreading, intermediate toe spreading, and footprint length, could be deduced. These indices were helpful in calculating the SFI (Hare et al., 1992; Kanaya et al., 1996).

Macroscopic evaluation of wound healing and nerve adherence

Macroscopic evaluation of wound healing and nerve adherence was evaluated at the 4th ($n = 16$) and 12th ($n = 16$) weeks after surgery. The rats were once again anesthetized and a microdissection was performed to examine the surgical area step-by-step. The sciatic nerves were examined after they were exposed once more. This was done to verify the degree of fibrous tissue enveloping the repair site. Muscle fascia closure, skin closures, nerve adherence to tissue from muscle cavity, and separability of nerves were all examined using a numerical grading system, as described by Petersen et al. (1996) (Table 1).

Histological and ultrastructural evaluation

Evaluation of epineural fibrosis

For each group, eight right sciatic nerves were examined for epineural fibrosis until the end of the 4th week after injury ($n = 16$). All rats underwent the same procedures. The whole sciatic nerve, along with the tissue attached to it (including the mended part), was severed, fixed in 10% formalin, embedded with paraffin, cut into $5\text{-}\mu\text{m}$ -thick transverse sections, and stained with Masson-Trichrome for evaluation of epineural fibrosis. The epineural scar tissue formation index was obtained by dividing the thickness of the scar tissue by how thick the nerve tissue was found to be (Petersen et al., 1996; Ozgenel, 2003; Ozay et al., 2007).



Figure 1 A photomicrograph showing rat epineural anastomosis after sciatic nerve transection. Arrow: Anastomosis line.

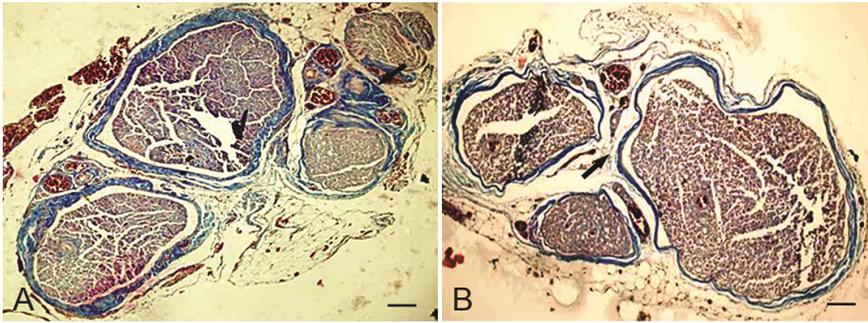


Figure 2 Presentation of epineurial fibrosis in rats with sciatic nerve injury after different treatments (Masson-Trichrome staining). The glioblastoma cyst fluid group (B) exhibited thinner bands of blue epineurial scar tissue than the saline group (A). Arrows: epineurial scar tissue. Scale bar: 100 μ m.

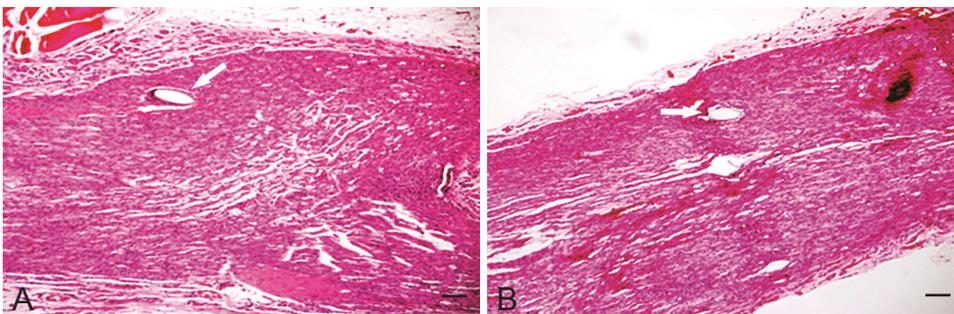


Figure 3 Histomorphologic appearance of axonal organization from the proximal to distal end of injured sciatic nerve in rats treated with glioblastoma cyst fluid at 12 week after injury (longitudinal sections stained with hematoxylin-eosin staining). The saline group exhibited grade 1 axonal organization (A). The glioblastoma cyst fluid group exhibited grade 4 axonal organization (B). White arrows: anastomosis line. Scale bar:100 μ m.

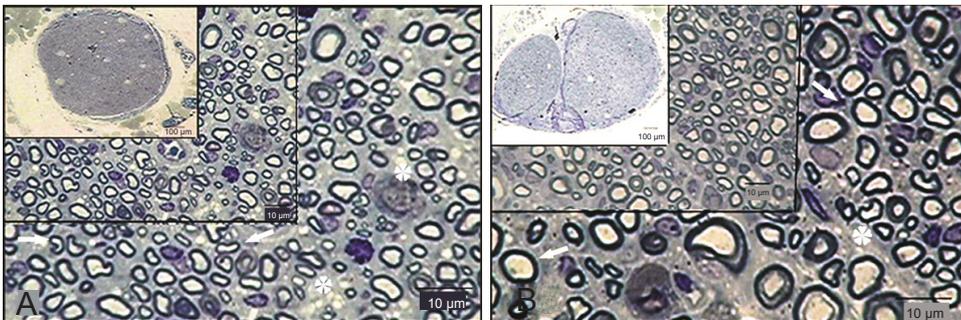


Figure 4 Histomorphologic appearance of axon maturation in rats with sciatic nerve injury following glioblastoma cyst fluid treatment (axial sections stained with Toluidine blue). Morphology of the axon and myelin sheaths were well preserved in the glioblastoma cyst fluid group (white arrow) (B), while myelin and axon shrinkage (white arrow) were observed in the saline group (A). Clover: interstitial space. Scale bars: 10 μ m-10 μ m-100 μ m.

Table 1 Numerical grading system used for macroscopic evaluation

Evaluation	Score	Definition
Skin and muscle fascia	1	Skin or muscle fascia entirely closed
	2	Skin or muscle fascia partially open
	3	Skin or muscle fascia completely open
Nerve adherence and nerve separability	1	No dissection or mild blunt dissection
	2	Some vigorous blunt dissection required
	3	Sharp dissection required

Table 2 Effect of glioblastoma cyst fluid (GCF) on sciatic nerve function in rats with sciatic nerve injury

Group	Time after peripheral nerve injury (week)		
	4	8	12
Saline	-81.84±1.48	-74.31±2.17	-66.32±2.14
GCF	-75.81±1.26**	-66.94±1.15**	-56.91±1.03**

Data are expressed as the mean \pm standard deviation (SD) ($n = 8$ rats at 8 and 12 weeks, and $n = 16$ rats at 4 weeks). ** $P < 0.01$, vs. saline group, Mann-Whitney U test.

Table 3 Effect of glioblastoma cyst fluid (GCF) on macroscopic results of rats with sciatic nerve injury

	Time after peripheral nerve injury (week)	
	4	12
Skin and muscle fascia closure		
Saline group	1	1
GCF group	1	1
Nerve adherence and separability		
Saline group	2.6±0.05	2.5±0.5
GCF group	1.8±0.06*	1.7±0.7*
Epineural scar tissue formation index		
Saline group	0.289±0.02	
GCF group	0.193±0.03**	

Data are expressed as the mean ± standard deviation (SD) with eight rats per time point. **P* < 0.05, ***P* < 0.01, vs. saline group, Mann-Whitney *U* test. Numerical grading system was summarized in Table 1.

Evaluation of axonal organization

Axonal organization was evaluated at the 4th (*n* = 16) and 12th (*n* = 16) weeks after surgery. Longitudinal serial sections at 5 μm thicknesses were obtained from a 6-mm length of the nerve, including the anastomosis site. The sections were stained with hematoxylin and eosin. The longitudinal organization of the regenerative nerve was evaluated using the following scale by Brown et al (1996): 1, failure (indicative of no axonal continuity from proximal to distal end); 2, poor organization of repair site; 3, fair organization of repair site; 4, good organization of repair site; 5, excellent organization of repair site (indicative of no difference between the repair site and the normal condition).

Evaluation of axonal maturation

Axonal maturation was evaluated at the 4th (*n* = 16) and 12th (*n* = 16) weeks after surgery. Sections taken from the region 4 mm distal to the anastomosis site were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The nerve samples were subsequently rinsed twice in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide for 2 hours, dehydrated through an ascending alcohol series, placed in propylene oxide for 16 minutes and then embedded with Epon for 48 hours (Fluka Chemie GmbH, Steinheim, Germany). For the purpose of embedding, a silicon embedding mold with 32 flat-shaped cavities with a depth of 5 mm each which are consecutively numbered. The mold helped orientate appropriately and pinpoint the angle at which the cuts were made for sciatic nerve sections. Semi-thin sections (1 μm) of the tissue located 4 mm distal to repair site were cut with an ultramicrotome (Super Nova Reichert-Yung, Wien, Austria) and stained with 1% toluidine blue.

A stereological analysis of the sciatic nerve was also performed as per the principles that were previously highlighted (Larsen, 1998; Kaplan et al., 2001). A manual stereological workstation that included a digital camera (COOLPIX5400; Nikon, Tokyo, Japan), a light microscope (Microphot-FX;

Table 4 Effect of glioblastoma cyst fluid (GCF) on nerve regeneration in injured sciatic nerve in rats

	Time after peripheral nerve injury (week)	
	4	12
Evaluation of axonal maturation		
Axon counts		
Saline group	7,061.444±442	11,655.875±433
GCF group	8,065.944±549	13,011.541±266*
Axon area (μm²)		
Saline group	3,229.442±128	6,945.0143±559
GCF group	3,648.764±222	9,824.0064±881*
Axon diameter (μm)		
Saline group	2.82±0.15	4.18±0.33
GCF group	2.97±0.21	6.09±0.23**
Fiber diameter (μm)		
Saline group	4.22±0.22	6.16±0.27
GCF group	4.58±0.32*	8.33±0.21**
Evaluation of axonal organization		
Saline group	2.25±0.7	2.75±0.7
GCF group	2.75±0.7	3.62±0.7*

The data are expressed as the mean ± standard deviation (SD) with eight rats per time point. **P* < 0.05, ***P* < 0.01, vs. saline group (Student's *t*-test for axon area and axon counts, and Mann-Whitney *U* test for axonal organization, axon diameter and fiber diameter). Scale of axonal organization was used which was described by Brown et al. (1996). Axon counts were obtained at the distal anastomotic suture line.

Nikon), and a manual dial indicator controlled specimen stage was used to measure axons. The total number of axons in the cross sections of the nerve was counted using the two-dimensional dissector technique to avoid unbiased evaluation. An area of 1,600 mm² made up the unbiased counting frame. A counting frame was put onto the monitor. The sample area was picked in a random and systematic uniform manner through the dial indicator-controlled specimen stage.

Meander sampling of each sectioned nerve profile was done through a systematic random 70 × 70 mm² step size. This helped make sure that all the points in the cross section of any nerve were evenly highlighted and that all axon profiles, without emphasis on their size, location, orientation, or shape were sampled with an equal probability. A stereological analyses of axon cross-sectional area, myelin thickness, nerve fiber, and axon diameters was conducted at another stereological workstation that consisted of a light microscope (Leica, Wetzlar, Germany), a microcator (Heidenhein Traunreut, Germany), a motorized specimen stage that was computer controlled (Prior Scientific, Cambridge, UK), a personal computer, a flash point 3D image capture card (Integral Technologies, Indianapolis, Indiana, USA), and a CCD digital camera. The Computer Assisted Stereological Toolbox (CAST)-GRID1 software program (Olympus, Copenhagen, Denmark) was used to help measure, control, and record area of a stereological nature. It also helped create digital images of all sections. The system helped create microscopic picture (60× objective with an NA of 1.40 at a final enlargement of 3,209×). This enlargement helped provide accurate

quantification and recognition of nerve fibers that were myelinated. A two-dimensional isotropic uniform random nucleator with a 60× oil objective with an NA of 1.40 and a final magnification of 3,209× helped measure and estimate axon area, axon diameter, nerve fiber and myelin sheath thickness. The meander sampling of each nerve profile was undertaken in successive 70 × 70 mm² systematic random steps.

Statistical analysis

Data were analyzed using SPSS11.5 software (SPSS, Chicago, IL, USA) and are expressed as the mean ± standard deviation (SD). Normality of the variables was assessed by the Shapiro-Wilk test. After confirming that the variables show a normal distribution, differences between the groups were tested using Student's *t*-test and Mann-Whitney *U* test. A value of $P < 0.05$ was considered statistically significant.

Results

Sciatic nerve function

Before modeling, the SFI of all rats was estimated to be zero for both groups. The SFI in the saline group was found to be extremely decreased from GCF in the 4th, 8th and 12th weeks after sciatic nerve injury (Table 2).

Macroscopic evaluation of wound healing and nerve adherence

Skin and muscle closure was performed in all of the animals, and no infections or inflammatory reactions were observed in any of the animals at both the 4th and 12th weeks after PNI. Treatment with GCF yielded statistically significant better nerve adherence and separability scores than treatment with saline at both the 4th and 12th weeks ($P = 0.028$ and $P = 0.039$, respectively) (Table 3).

Epineural fibrosis

In the saline group, the nerves were surrounded by thick bands of dense connective tissue. However, the band of connective tissue in the GCF group was different from that in the saline group (Figure 2). This quantification highlighted a statistically significant fall in the epineural scar tissue that surrounded the nerves in the GCF group at the 4th week after injury ($P = 0.001$) (Table 3).

Axonal organization

At the 4th week after injury, there was no significant difference in axonal organization between the saline and GCF groups ($P = 0.26$). At the 12th week after injury, axonal organization was better in the GCF group than in the saline group ($P = 0.036$) (Table 4, Figure 3).

Axonal maturation

At the 4th week after injury, there was no significant difference in axon counts between the saline and GCF groups ($P = 0.176$). However, at the 12th week after injury, axon counts in the GCF group were significantly greater than in the saline group ($P = 0.018$). At the 4th week after injury, no significant difference in axonal area was observed between the saline and GCF groups.

At the 12th week after injury, axonal area was significantly greater in the GCF group than in the saline group ($P = 0.015$). There was no significant difference in the mean axonal diameter between these two groups at the 4th week after injury ($P = 0.1$). At the 12th week after injury, axonal diameter in the GCF group was significantly greater than that in the saline group ($P = 0.001$). At the 4th and 12th weeks after injury, fiber diameter in the GCF group was significantly greater than that in the saline group ($P = 0.018$ and $P = 0.001$) (Figure 4). Histomorphological results are shown in Table 4.

Discussion

Different techniques and treatment options have been used to treat PNI, but no satisfactory results have been obtained. Axon to axon reconnection is considered an ideal approach for recovering close-to-normal nerve function after severe nerve injury (Terzis et al., 1997; Devesa et al., 2012); however, this approach is not currently possible. For this reason, treatment of PNI remains an area of interest in the fields of neurosurgery and other disciplines of medicine. After PNI, the complex process of nerve regeneration, which consists of axonal budding, growth and re-innervation, begins (Kanaya et al., 1996). Schwann cells play a significant role in regeneration of damaged peripheral nerves. The spread of neurites from proximal end of axotomized neurons is mediated by bands of Büngner that consist of Schwann cells and basal lamina (Frostick et al., 1998). Schwann cells synthesize and release nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) for axonal regeneration (Hansson et al., 1986; Meyer et al., 1992; McCallister et al., 2001). A number of neurotrophic factors, such as NGF, CNTF, BDNF, neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5), interleukin-1 (IL-1), -3 (IL-3), -6 (IL-6), and other factors, such as fibroblast growth factor (FGF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β), improve axonal regeneration (Lundborg, 2000). In addition, axonal regeneration requires ECM proteins such as laminin and fibronectin, which are both important neurite-promoting factors (Longo et al., 1984; Le Beau et al., 1988).

Glial tumor studies reported the secretion and presence of various neurotrophic factors that promote the proliferation, differentiation and vascularization of the tumor tissue (Schlegel et al., 1990; Bodmer et al., 1991; Brown et al., 1991; Weindel et al., 1994; Singer et al., 1999). Glial tumor tissue secretes NGF, TGF-α and -β, acidic and basic FGF, platelet-derived growth factor (PDGF), VEGF, IGF, BDNF and GDNF (Schlegel et al., 1990; Takahashi et al., 1990; Bodmer et al., 1991; Zunkler et al., 1993; Weindel et al., 1994; Stockhammer et al., 1995, 2000; Takano et al., 1996; Singer et al., 1999; Aronica et al., 2001; Karcher et al., 2006; Brown et al., 2008). In addition, ECM proteins that act as neurite-promoting agents, such as laminin, fibronectin, integrin, vitronectin, type-IV collagen and tenascin are synthesized by glioblastomas and promote tumor neovascularization, migration and invasion (Chintala

et al., 1996; Jallo et al., 1997; Mahesparan et al., 2003). VEGF, IGF-1 and -2 and ECM proteins have also been found in GCF (Zumkeller et al., 1993; Takano et al., 1996; Jallo et al., 1997; Stockhammer et al., 2000). Because glial tumors contain these neurotrophic and growth factors that may improve nerve regeneration, we hypothesized that GCF may have therapeutic effects in patients with PNI.

The histomorphological results of this study revealed regeneration of surgically transected sciatic nerves and functional recovery was improved by local application of GCF. Mean nerve fiber diameter, axon area, myelin sheath thickness, axon diameter, and axon counts were higher in the GCF group than in the saline group. Previous studies demonstrated that stereological analysis techniques could be used to reliably estimate histomorphological data (Larsen, 1998; Gundersen et al., 1999; Kaplan et al., 2001). Therefore, a stereological morphometric analysis was performed in this work.

Rats that were administered GCF exhibited significantly better SFI values than those administered saline. These striking results may be connected to the fact that GCF contains both neurotrophic and neurite-promoting factors. These results demonstrated that GCF application onto the injured sciatic nerve caused functional and histomorphological recovery.

The formation of the epineural scar is another problematic issue in patients with PNI. Previous studies demonstrated that GCF includes tenascin-c, TGF- β and IL-8 (Bodmer et al., 1991; Van Meir et al., 1992; Jallo et al., 1997). In particular, tenascin-c and TGF- β exert immunosuppressive effects by inhibiting T-lymphocyte proliferation and cytokine production (Jallo et al., 1997; Puente Navazo et al., 2001; Grütz, 2005; Sharma et al., 2005; Gomez and Kruse, 2006). The inhibition of T lymphocyte proliferation and cytokine production prevents further perineural scar formation and neuroma formation (Wang et al., 1997; Lee et al., 2000). The present findings highlighted that the use of GCF prevented epineural scar and neuroma formation on the anastomosis line.

In conclusion, histomorphological and functional analyses revealed that the topical application of GCF onto the injured sciatic nerve improves nerve regeneration and inhibits perineural fibrosis. Based on these promising results, GCF has potential for future clinical therapeutic application in patients with PNI.

Author contributions: RÖ, AA and BG conceived and designed this study. RÖ, AA and MÖT collected the data. RÖ, BG and YŞÇ contributed to paper writing. RÖ, AA, MÖT, BG, BE and YŞÇ provided critical revision of this paper. BG and BE were responsible for data analysis and interpretation. All authors approved the final version of this paper.

Conflicts of interest: None declared.

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