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Effects of Topically Applied Contractubex® on Epidural Fibrosis and Axonal Regeneration in Injured Rat Sciatic Nerve

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ABSTRACT

AIM: This study aimed to investigate the effects of Contractubex® (Cx) on peripheral nerve regeneration and scar formation.

MATERIAL and METHODS: A surgical procedure involving sciatic nerve incision in 24 adult male Sprague–Dawley rats followed by epineural suturing was performed. In weeks 4 and 12 following surgery, macroscopic, histological, functional, and electromyographic examinations of the sciatic nerve were conducted.

RESULTS: No significant difference was found between the Cx group and the control group in terms of sciatic function index (SFI) and distal latency results at week 4 ($p>0.05$). However, significant improvements in the Cx group were observed in SFI amplitudes and nerve action potentials at week 12 ($p<0.001$ and $p<0.001$, respectively). Significant improvements were found in the amplitudes of nerve action potentials in the treatment group after weeks 4 and 12 ($p<0.05$ and $p<0.001$, respectively). Macroscopically and histopathologically, epidural fibrosis decreased ($p<0.05$ and $p<0.001$, respectively). For both measurement times, the treatment group had significantly higher numbers of axons (week 4, $p<0.05$; week 12, $p<0.001$), and the treatment group had better results regarding its axon area (weeks 4 and 12, $p<0.001$) and myelin thickness (weeks 4 and 12, $p<0.05$).

CONCLUSION: Cx, which is applied topically in peripheral nerve injury, affects axonal regeneration and axonal maturation positively and reduces the functional loss.

KEYWORDS: Contractubex, Peripheral nerve, Scarring

ABBREVIATIONS: PN: Peripheral nerve, **PF:** Perineural fibrosis, **Cx:** Contractubex, **SFI:** Sciatic function index, **EMG:** Electromyography, **ScFI:** Scar tissue formation index, **NF-κB:** Nuclear factor kappa B, **GF:** Growth factor, **H_2O_2:** Hydrogen peroxide

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INTRODUCTION

Peripheral nerves (PNs) are highly defenseless against trauma (5). This risk of injury starts at birth and increases constantly in our daily lives depending on various reasons, such as chemical, traumatic, and thermal factors. Despite treatments, it is not always possible to relieve the excruciating pain or accompanying functional losses caused by severe neuropathy (22,34).

Regardless of the cause of PN injuries, functional losses that qualitatively and quantitatively disrupt the quality of life may develop depending on the extent of damage (e.g., neuropraxia, axonotmesis, and neurotmesis) (5,22,34). The functional recovery process after axonal regeneration is directly proportional to the length of the damaged nerve (30,31,44). Chronically, atrophy develops in related muscles, and this outcome is irreversible (30,31). Additionally, the inflammatory process at the site of PN injury induces fibroblastic activity. This histopathological process is known as perineural fibrosis (PF) and results in axonal regeneration inhibition and neuroma formation that lasts for 6–12 months (14). In this context, since the classification of PN injury was based on the observational results of Seddon in the mid-19th century to the present, the pathophysiological mechanisms of axonal regeneration have been clarified, albeit relatively (5,43). End-to-end and end-to-side anastomosis techniques and anastomosis techniques that align the proximal and distal ends within a graft, especially in those with neural tissue loss, have been applied in complete cuts created in PNs (16,20). The relevant surgical techniques have been reinforced with neurotrophic, neuroprotective, anti-inflammatory, antifibrotic, and fibrinolytic agents that can be administered intravenously (i.v.), intraperitoneally (i.p.), or topically in parallel with physiological, pharmacological, and biochemical developments (1,21,26). In the literature, some studies have covered extreme practices such as the use of X-rays, amniotic membrane, amniotic fluid, and contents of glioblastoma cysts to achieve optimal recovery (15,35,37).

In this study, we used Contractubex (Cx; Merz Pharmaceuticals GmbH, Frankfurt, Germany), which consists of *Allium cepa* extract (10% aqueous), heparin (50 U), and allantoin (1%). Cx is a topical gel that is prevalently used to prevent scar development (keloid and hypertrophic skar) in various dermatology and surgical clinics. As Cx has antioxidant, anti-inflammatory, and antifibrotic effects, studies have examined its effects on PF in post-laminectomy and sciatic nerve injury models (3,22,33,36,50). In the present study, by using Cx in a PN injury model, we aimed to generate an antifibrotic effect with the help of the *Allium cepa* (cepae) extract, increase perfusion in the damaged neural tissue with the help of its heparin component, and induce an anti-inflammatory effect with its allantoin component. Therefore, we investigated the effects of Cx, which contains a balanced combination of cepae extract, allantoin, and heparin, on axonal regeneration, PF, and functional recovery.

MATERIAL and METHODS

This study was conducted with the approval of the Animal Experiments Local Ethics Committee at Gazi University (17.09.2013-157-20767). The animals used in this study included 24 adult male Sprague–Dawley rats weighing 300–350 g. The rats were divided into the control group (group 1, n=12) and treatment group (group 2, n=12). During the experiments, the rats with ear tags were put into separate cages in groups of four and housed under standard laboratory conditions at room temperature (22°C) and a photoperiod of 12-/12-h light/dark, and they had free access to food and water.

Surgical Procedure

Anesthesia was induced i.p. using xylazine (5 mg/kg; Bioveta, Ankara, Turkey) and ketamine hydrochloride (25 mg/kg, Ketalar, Pfizer, Istanbul, Turkey), and spontaneous respiration was provided. The anesthetized rats were fixed in the prone decubitus position. The right hind legs of all rats were shaved, and the skin of the surgical site was disinfected with a 10% Betadine solution (Glividon®, Bikar İlaç San., Istanbul, Turkey). The right sciatic nerve of each rat was exposed by blunt dissection and cut approximately 1 cm above the trifurcation with micro scissors. Then, using 8-0 monofilament polypropylene sutures (Ethicon/Prolene, Edinburgh, UK), the primary epineural anastomosis method was performed for repair in a way that would match the loose nerve ends together. Cx (0.4 mL) was applied topically to the anastomosis site in the treatment group, whereas normal saline (0.4 mL) was applied topically in the control group. The muscle and cutaneous–subcutaneous layers of all rats in both groups were closed by preserving their anatomical plans. The surgical operations were performed by the same surgeon (RÖ) using micro-scale surgical instruments and a surgical microscope (Zeiss Opmi 9-FC, Oberkochen, Germany). After the procedure, each rat received i.p. injection of 10 mg/kg cefazolin sodium (Sefazol; Mustafa Nevzat Ilac AS, Istanbul, Turkey), and a topical antibiotic agent was applied to the incision sites (Neo-Caf spray, Intervet, Apulia, Italy). No postoperative complications, such as mortality, neurological deficit, or wound site infections, occurred in either group. By using an electromyography (EMG) device at the end of weeks 4 and 12 after the surgery, the functional and electrophysiological recovery of the rats was examined by a neurologist who was blinded to the group allocation. After the functional examination, the rats were sacrificed, and their right sciatic nerves were removed using blunt or sharp dissection to include the repair site. Following the macroscopic examination during this procedure, the tissues were transferred to appropriate pathology containers containing 10% buffered formalin solution for histopathological examinations. Functional outcome assessments and histopathological and electrophysiological examinations were made by observers who were blinded to the intervention.

Functional Evaluation

Walking Track Analysis

In experimental studies on sciatic nerve injuries, the sciatic functional index (SFI) is the test that best shows nerve recovery and is used most frequently (19,23). SFI is calculated by measuring the length of the limb in addition to measuring

the distance between the digits of the first and third limbs and the distance between the digits of the second and fourth limbs (Figure 1). As in other studies, we calculated SFI preoperatively and postoperatively at weeks 4 and 12 (19,23,37). The measurements and SFI calculations were made by a neurologist who was blinded to the group allocation.

Electrophysiological Evaluation

At the end of weeks 4 and 12 postoperatively, EMG was performed for the electrophysiological examination of axonal regeneration. After anesthesia induction by i.p. injection of 30 mg/kg sodium thiopental, the rats in both groups were placed in the prone position and their extremities fixed on a table. Electrodes were placed in appropriate anatomical localizations. After ensuring that the distance between the two electrodes was longer than 2 cm, by providing repetitive stimuli at increasing intensities (0.04 ms), the best plantar flexion responses were recorded (Figure 2). The measurements were made by a neurologist who was blinded to the group allocation.

Macroscopic Evaluation of Wound Healing and Nerve Adherence

At the end of weeks 4 and 12, following functional examinations (EMG and SFI), the rats were anesthetized again, and scar formations at the incision site and anastomotic line were macroscopically graded. The procedure was performed on six rats randomly selected from each group. By opening the muscle regions again through microdissection, the adherence of the sciatic nerve to the surrounding tissues and whether it could be easily separated from these tissues were carefully examined in each step. Skin closure, muscle and fascia closure, nerve adherence to the surrounding muscle cavity tissue, and separability of the nerves were assessed using the numerical grading system reported by Petersen et al. (40).

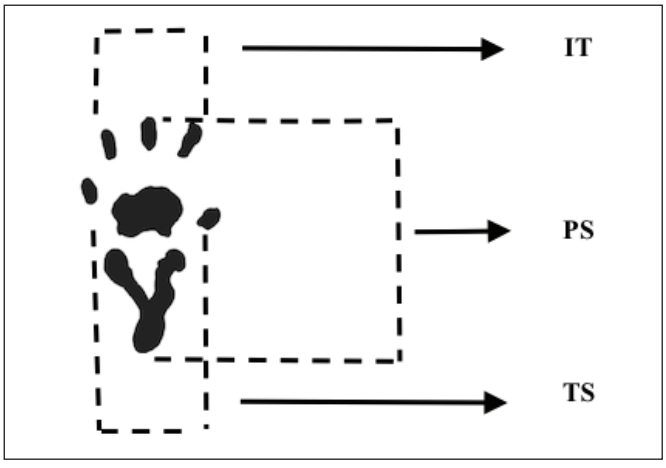

Figure 1: Appearance of the right footprint and measurements of the Sciatic Function Index (SFI) measurements (right footprint). **PL:** Print length, **TS:** Toe spread, **IT:** Intermediate toe spread.

Histological and Ultrastructural (Stereological) Evaluation

Evaluation of Axonal Organization

After the surgical procedure for axonal organization, serial longitudinal sections were taken together with the anastomosis region of the nerve (length of 6 mm, thickness of 5 µm) and stained with hematoxylin and eosin. The longitudinal organization of the regenerative nerve was evaluated using the scale described by Brown et al. (Table I) (4).

Evaluation of Axonal Maturation

Postoperatively, sections were taken from the anastomosis site for axon maturation. Nerve sections taken were fixed in 2.5% glutaraldehyde (4 h) and washed twice with phosphate buffer (pH 7.4). Tissues were then fixed, this time with 1% osmium tetraoxide (2 h), and dried with different ethanol concentrations (3 × 70%, 3 × 80%, 3 × 90%, and 3 × 100%). The tissues were then embedded in the Epon Embedding Kit (Fluka GmbH, Switzerland) (60°C, 48 h). Semi-thin sections were taken with an ultramicrotome (Super Nova Reichert-Yung, Austria) and stained with toluidine blue (38).

Tablo I: Evaluation of the Longitudinal Organization of the Regenerative Nerve (4)

Score	Histopathological findings
1	Failure (indicative of no axonal continuity from the proximal to the distal ends)
2	Poor organization of the repair site
3	Fair organization of the repair site
4	Good organization of the repair site
5	Excellent organization of the repair site (indicative of no difference between the repair site and the normal condition)

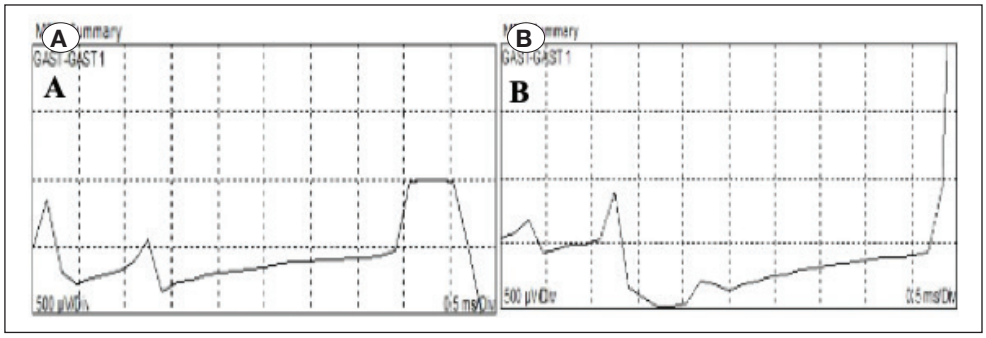

Figure 2: Sample results of EMG of the control **(A)** and treatment (**B)** groups at the end of the 4th and 12th weeks postoperatively.

We conducted stereological analysis faithfully based on previous studies (24,28,38). Axons were counted using a random sampling method with a stereological workstation (mbf/Bioscience, Qimaging digital camera, Leica light microscope, DM400B, mbf/Bioscience, Stereo investigator, version 9). The morphological features (such as size, direction, and localization) of axons in each nerve section were sampled with equal probability (6,38). At this point, while counting the axons, the area of the frame (30 × 30 µm) and stepping range (70 µm × 70 µm) were kept constant, allowing sampling of all nerves (6,38,41,52). All these processes were performed for the stereological analyses of myelin thickness and axon cross-sectional area. A two-dimensional isotropic uniform random nucleator was used to estimate the cross-sectional axon area and myelin layer thickness using an oil immersion lens (100×, NA 1.40) (17,18).

Evaluation of PF

For each group, six right sciatic nerves were examined for PF until the end of week 4 after injury (n=12). All rats underwent the same procedures. The entire 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-µm-thick transverse sections, and stained with Masson–Trichrome for the evaluation of epineural fibrosis. The perineural scar tissue formation index (ScFI) was calculated by dividing the thickness of the scar tissue by the thickness of the nerve tissue (37,40).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Quantitative data are presented as mean ± SD and median (interquartile range, Q1–Q3). In addition to the analysis of the longitudinal organization at the repair site, the normality of the distribution of the data was tested for each continuous variable before analyzing the SFI and histological measurement values, longitudinal organization of the repair site, number of axons and fibers, and EMG results. As a result of the normality test, to compare two independent groups, the parametric independent-samples t-test was used to analyze normally distributed data, and the non-parametric Mann–Whitney U test was used to analyze non-normally distributed data. The level of significance was accepted as $p<0.05$.

RESULTS

Functional Evaluation

Walking Track Analysis

According to the SFI results, no significant difference was found in the values obtained at the end of week 4 between the treatment and control groups ($p>0.05$, Table II). On the contrary, at the end of week 12, the SFI values in the treatment group were significantly better than those in the control group ($p<0.001$, Table II).

Electrophysiological Findings

The amplitudes and distal latencies of the nerve action potentials obtained from the EMG records at weeks 4 and 12 postoperatively were statistically analyzed. In the first records obtained at 4 weeks postoperatively, no significant difference was found between the distal latency values of the two groups ($p>0.05$, Table II). However, in the Cx-treated group, a significant improvement was found in the distal latency values obtained 12 weeks postoperatively ($p<0.05$, Table II). Regarding the amplitudes of the nerve action potentials, significant improvements were found in the treatment group at the end of weeks 4 and 12 ($p<0.05$ and $p<0.001$, respectively, Table II).

Macroscopic Evaluation of Wound Healing and Nerve Adherence

No signs of infection or inflammatory response were observed in either group. In the analysis conducted based on the results of the evaluations according to the classification by Peterson et al., when the skin and muscular fascia closure levels were compared between the groups for both measurement times after surgery, no significant difference was identified ($p>0.05$, Table III). Nerve adherence was significantly lower in the

Table II: Distal Latency, Amplitude Levels, Sciatic Function Index Scores Based on the Groups and Sacrifice Times

	Control Group	Treatment Group	p
Distal latency			
4th week	0.23 (0.2-0.3)	0.21 (0.2-0.3)	p>0.05
12th week	0.20* (0.15-0.25)	0.13* (0.10-0.18)	p<0.05*
Amplitude			
4th week	338.33* (310-370)	385.00* (330-410)	p<0.05*
12th week	410.00** (370-450)	500.00** (460-540)	p<0.001***
Sciatic Function Index			
4th week	-78.73 ± 2.13	-76.61 ± 1.94	p>0.05
12th week	-64.57 ± 1.64	-48.96 ± 2.98	p<0.001***

**p<0.05, **p<0.01, ***p<0.001. The difference between groups was analyzed using the Mann-Whitney U test and independent-samples t-test.*

treatment group than in the control group at week 4 (p<0.05, Table III). However, no significant difference in macroscopic examination results was found between the two groups at week 12 (p>0.05, Table III).

Histological and Ultrastructural (Stereological) Evaluation

Evaluation of Axonal Organization

The histopathological examinations did not reveal any inflammatory response to the topical application of Cx. In the examination of the sections obtained at week 4, the axonal organization levels at the eperineural anastomosis site were 2.17 (1–3) in the control group and 3.00 (2–5) in the treatment group, whereas these values were 2.33 (1–3) in the control group and 3.50 (3–5) in the treatment group at week 12. Although no significant difference was noted between the control and treatment groups for either measurement time in terms of axonal organization (p>0.05, Table IV), the values of the treatment group were noticeably better (Figure 3).

Table III: Skin, Muscle, Fascia Closure and Nerve Adhesion Scores Based on the Groups and Sacrifice Times

	Control Group	Treatment Group	p
Skin, muscle, fascia closure			
4th week	1 (1-1)	1 (1-1)	-
12th week	1 (1-1)	1 (1-1)	-
Nerve adhesion-separability			
4th week	2.50 (2-3)	1.33 (1-2)	p<0.05*
12th week	2.33 (1-3)	1.33 (1-2)	p>0.05

**p<0.05, NS: p>0.05. The difference between the groups was analyzed using Mann-Whitney U test.*

Evaluation of Axonal Maturation

In both measurements (at the end of weeks 4 and 12), the numbers of axons in the treatment group were significantly higher than those in the control group (week 4, p<0.05; week 12, p<0.001, Table IV). Additionally, for both measurements, the treatment group had significantly and noticeably better axonal area (weeks 4 and 12, p<0.001, Table IV) and myelin thickness than the control group (weeks 4 and 12, p<0.05, Table IV).

Evaluation of PF

In the sections obtained from the control group at the end of week 4, a very thick layer of connective tissue was observed around the nerves. By contrast, in the sections obtained from the treatment group, a thin layer of connective tissue surrounded the nerves (Figure 4). The calculations and analyses of the ScFI values of the groups revealed that topical application of Cx led to a significant reduction in the mass of fibrotic tissue forming around the nerves (p<0.001, Table IV, Figure 4).

Table IV: Numbers of Axons, Axon Areas, Axonal Organization Values and Myelin Thicknesses Based on the Groups and Sacrifice Times

	Control Group	Treatment Group	p-value
Number of axons			
4th week	5250.67 * (2440.67-6542.05)	9550.45 * (5209.34-14737.98)	p<0.05*
12th week	8353.54 ** (5488.59-11534.15)	12944.06 ** (11744.46-14019.95)	p<0.001***
Axon area			
4th week	8.63 ** (7.64-9.68)	11.85 ** (9.79-14.78)	p<0.001***
12th week	13.35 ** (11.83-14.15)	16.05 ** (14.50-17.95)	p<0.001***
5-point score of axonal organization			
4th week	2.17 (1-3)	3.00 (2-5)	p>0.05
12th week	2.33 (1-3)	3.50 (3-5)	p>0.05
Myelin thickness			
4th week	0.78 * (0.70-0.82)	0.90 * (0.77-1.00)	p<0.05*
12th week	0.95 * (0.90-1.07)	1.23 * (0.98-1.51)	p<0.05*
Scar Formation Index			
4th week	2.17 ** (1-3)	3.00 ** (2-5)	p<0.001***

**p<0.05, **p<0.01, ***p<0.001. The difference between the groups was analyzed using Mann-Whitney U test.*

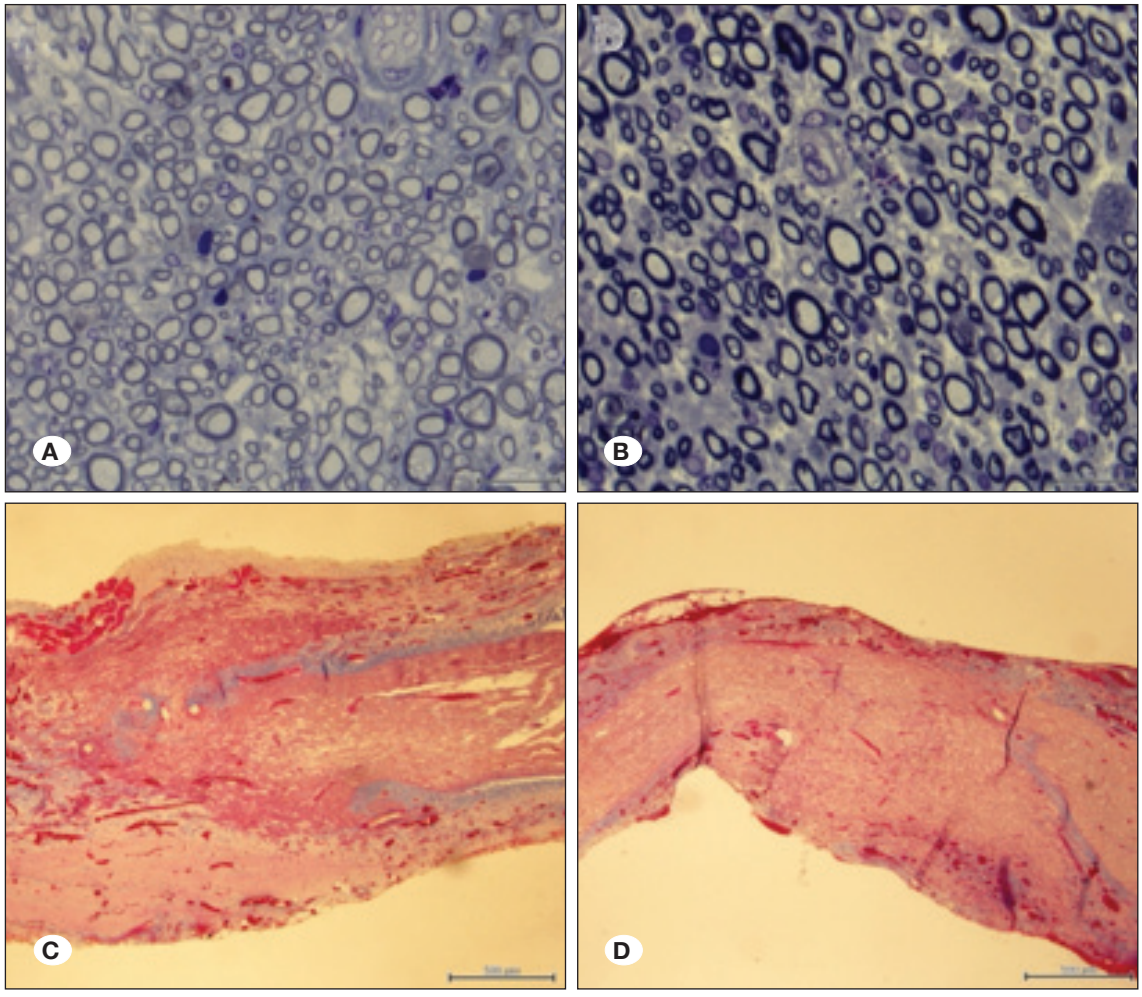

Figure 3: Images of the axonal maturation (Toluidine blue, ×100, Scale Bar 20μm) and organization (Masson–Trichrome, ×4, scale bar 500 μm) of the rats sacrificed at the end of week 12 after the surgery. Weak maturation **(A)** and organization **(C)** in the control group; better maturation **(B)** and organization **(D)** in the treatment group.

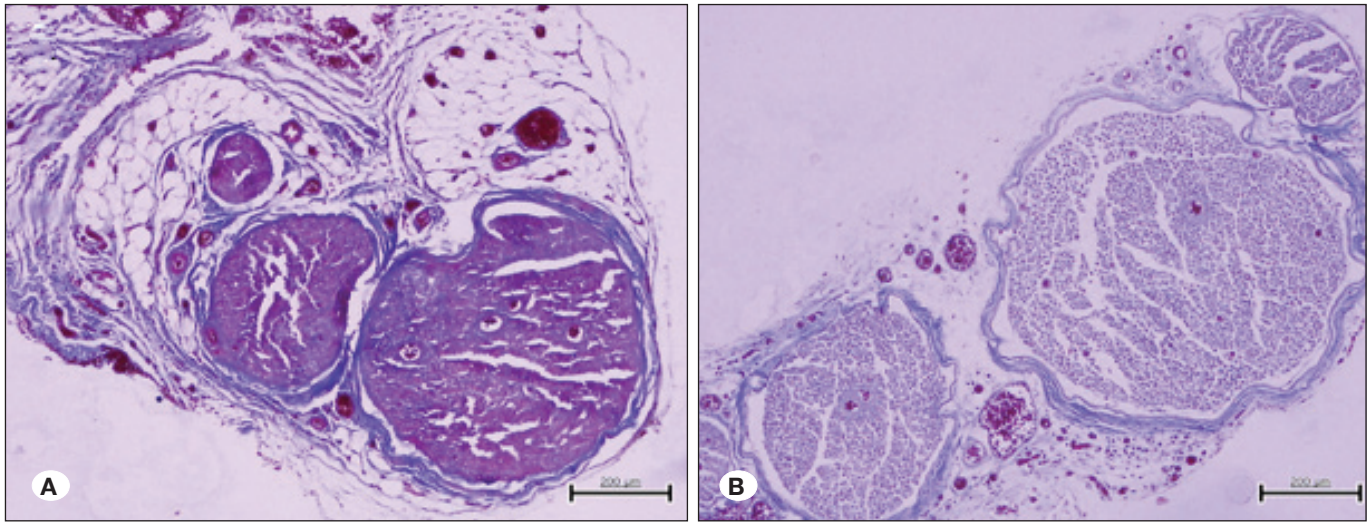

Figure 4: Images of the scar formation (Masson–Trichrome, ×4, scale bar 500 μm) of the rats sacrificed at the end of week 4 after surgery. Highly thick scar tissue belonging to a rat in the control group **(A)** and very thick, lucent scar tissue membrane barely surrounding the nerve in the treatment group **(B)**.

DISCUSSION

The preservation of axonal connections is extremely important for the structural integrity of PNs (48). The complete or partial interruption of these connections is the underlying cause of PN injuries with a traumatic nature, such as rupture, compression, laceration, chemical, thermal, and electrocution injuries (44,48). This study investigated the effects of Cx on antifibrotic and axonal regenerations in a rat sciatic nerve injury model and examined the electrophysiological and histomorphological aspects of functional recovery. The functional (week 4, $p<0.05$; week 12, $p<0.001$, Table II) and histomorphological (week 4, $p<0.05$; week 12, $p<0.001$, Table 4) results of our study demonstrated that while Cx did not show an adverse effect on wound healing, it reduced PF and neuroma formation, and most probably due to this activity, it increased axonal regeneration.

Following trauma, scar tissue development in all tissues starting with the skin is a physiological process (25,42). Fibrosis, which is caused by hemorrhage and hematoma formation in tissues surrounding PNs, plays a significant role in the onset of peripheral neuropathy (25,27). Fibrosis developing at the nerve repair site causes the formation of a neuroma. PF and neuroma create a mechanical barrier to axonal regeneration while causing traction and ischemia in neural tissues because of adherence in surrounding tissues (30,31,44). This process results in Wallerian degeneration, atrophy in the related muscle mass, and irreversible nerve damage (30,31). Although studies focusing on preventing PF that develops in PNs have been comprehensive enough, unfortunately, results regarding the ideal functional recovery and an uninterrupted histomorphological appearance from the proximal to the distal have not been obtained yet (11,35,53).

Cx became available for the first time in the late twentieth century, and it has become prevalent today for its usage in scar treatment (8). Several experimental studies have investigated separately the pharmacological effects of its main three components (cepae extract, allantoin, and heparin) (7,13). Considering the effects of Cx and its area of usage (as an antifibrotic agent), its most important ingredients are flavonoids obtained from onion extract (quercetin and kaempferol). Although the effect of flavonoids on the molecular level is not yet completely known, it is believed that they reduce scar formation through the inhibition of fibroblasts (10). In an experimental post-laminectomy model where Cx was topically applied, Ozay et al. investigated the effects of Cx on epidural fibrosis (EF) development and recovery and showed that Cx not only prevented EF formation but also reduced the levels of EF that had already formed (36). Based on the results of histomorphological examinations, they argued that this effect mainly resulted from a reduction in the number of fibroblasts (36). Similarly, in another experimental post-laminectomy model used by Temiz et al., Cx prevented EF formation by suppressing (acute and chronic) inflammation (50). Moreover, Bozkurt et al. used a similar model that combined topical application of Cx with 5% benzothiazole and revealed that its potential effects (induced) on EF (3). In their PN injury model, Kahraman and Kahveci applied onto a polytetrafluoroethylene mesh (MotifMesh™) following repair by primary suturing and stated that Cx increased functional recovery in the sciatic nerve (22). In our study, based on both macroscopic and histomorphological examinations after PN injury, Cx that was topically administered without any supplementary substance or supportive tissue significantly reduced PF formation ($p<0.05$, Table III; $p<0.001$, Table IV). Moreover, it did not induce a chemical or inflammatory reaction in the neural and surrounding tissues ($p<0.001$, Table IV, Figure 4).

"Extractum cepae," which is one of the components of Cx, is obtained from *Allium cepae*, and it suppresses inflammation and cell proliferation with the help of the flavonoids (53). Extractum cepae contains many vitamins, iron, and trace elements. Vitamin A, which is abundantly found in this mixture, has not only epithelium-protective effects but also anti-inflammatory and antiallergic activities. Likewise, in an experimental study, it had a noticeable antibacterial activity; it increased wound construction and reduced it in the epithelialization period in an excision wound model (46). Accordingly, while extractum cepae stimulates the first stage of wound healing, it prevents scar tissue formation, thanks to its anti-inflammatory, anti-fibroblastic, and antibacterial activities without causing any side effects (9,12,54).

Allantoin ($C_4H_6N_4O_3$) is found in *Symphytum* species (*S. officinale* and *S. cordatum*) known as comfrey and plants in the Boraginaceae family that usually grow in temperate and subtropical regions (49). Also known as 5-ureidohydantoin or glyoxyldiureide, allantoin is a metabolic component of uric acid oxidation products in organisms, and it is natural and safe (47,49). In addition to its keratolytic effect that helps in the dissolution of the stratum corneum that holds cells together and increases skin smoothness, it has antioxidant and anti-inflammatory effects (49). Patented drugs that contain allantoin are used for complementary therapies in the treatment of several dermatological diseases, such as dermatitis, psoriasis, ichthyosis, and burns and healing of wounds (2). Inflammatory agents that emerge with Wallerian degeneration after PN injury play a role in not only inflammation but also management of apoptotic and necrotic processes (49). The nuclear factor kappa B (NF-κB), which is a significant mediator between the chemical regulators of the primary response, is a protein complex that has a role in the regulation of cytokines including interleukin-1 and tumor necrosis factor-alpha. In the presence of oxidative stress triggered by PN injury, hydrogen peroxidase is stimulated, and the levels of hydrogen peroxide (H_2O_2), which is a second messenger for many growth factors, rise (47). As a reactive oxygen species, H_2O_2 can also induce inflammation through the activation of the transcription factor NF-κB (51). In the experimental model of induced PN injury, Delibas et al. reported that allantoin increased the levels of H_2O_2 by inducing NF-κB activation; thus, it strengthened axonal regeneration (9). The results of our study also revealed that Cx, which contains allantoin, increased axonal regeneration (week 4, $p<0.05$; week 12, $p<0.001$, Table IV) and provided beneficial effects on axonal maturation (weeks 4 and 12, $p<0.001$, Table IV).

Heparin disrupts the clotting cascade via plasminogen activation, prevents inflammation resulting in fibrosis by reducing the secretion of tumor growth factor beta and speeds up fibrinolysis (29,45). Studies have claimed that it prevents ischemia that induces fibrosis formation with its ultimately anticoagulant effect. Several experimental and clinical studies have examined the antifibrotic effects of different forms of heparin (29,32,39,45). Furthermore, it may be considered that it will have beneficial effects on intraneural angiogenesis and improve nerve regeneration by increasing the synthesis of vascular endothelial growth factor, which is a potential neurotrophic agent (29,45). We also think it affected the results of our study through similar mechanisms, and in addition to preventing fibrosis formation in the early period, it increased axonal regeneration in the long term by the neovascularization that was triggered ($p<0.001$, Table IV, Figure 4).

Kahraman and Kahveci determined that the combination of Cx and polytetrafluoroethylene mesh (MotifMesh™) did not affect adversely wound healing and it improved SFI results; in contrast to these findings, it increases epidural scar tissues (22). While the discordance in the study by Kahraman and Kahveci may be associated with their combination of Cx with polytetrafluoroethylene mesh, it may be also related to their failure to investigate the effects of the combination using adequate and reliable histomorphological analyses (in their study, PF severity was evaluated with visual examination alone) (22). Clearly, histomorphological examinations accompanied by microscopic morphometric analyses will increase the reliability of data determining PF severity. Stereological tests have been used prevalently in the morphological analysis of regeneration in recent studies investigating PN injury models (4,9). This is because measurable data such as the number of axons in PNs, their axonal organization, and myelin thickness are highly important in determining the severity of injuries. Random sampling in stereological analyses can obtain fast, effective, and reliable results about these morphological data. Thus, in this study, primary anastomosis was performed following PN injury, and stereological–ultrastructural morphometric analysis methods (number of axons, axon area, and myelin thickness) were utilized to evaluate axonal regeneration and maturation in the transverse sections obtained from the distal stump of the sciatic nerve following the topical treatment. PF was evaluated both macroscopically and microscopically (conservative methods), whereas axonal organization was microscopically examined using a five-point scale. Although both sets of measurements (weeks 4 and 12) regarding axonal organization revealed no significant difference between the control and treatment groups ($p>0.05$, Table IV), the values of the treatment group were noticeably better (Figure 3). Nonetheless, the results of the treatment group were significantly better than those of the control group in terms of the number of axons (week 4, $p<0.05$; week 12, $p<0.001$, Table IV), axon diameter (weeks 4 and 12, $p<0.001$, Table IV) and myelin thickness (weeks 4 and 12, $p<0.05$, Table IV). In the histomorphological sense, these results suggested that Cx affected the myelin thickness and axon size of the sciatic nerve positively and resulted in the functional recovery of the damaged nerve.

In this study, the functional recovery of the damaged sciatic nerve was also evaluated using electrophysiological analyses. Burcu et al. observed in the EMG results of their experimental sciatic nerve injury models that allantoin increased the amplitudes of the damaged sciatic nerve, but it did not increase its latency (9). Based on the SFI results of their study, they found a significant difference between the allantoin group and the control group. They explained the lack of an increase in their latency values despite the increase in myelin thickness levels was not significant. Moreover, in their experimental sciatic nerve injury model, Kahraman et al. did not find a significant difference regarding EMG results between the group that received Cx and polytetrafluoroethylene mesh (MotifMesh™) and the other groups at the end of week 8, and they observed a significant improvement in SFI results in the former (22). In our study, while no significant difference in SFI values was found at week 4 between the treatment and control groups ($p>0.05$, Table II), the SFI values of the treatment group were significantly better than those of the control group at week 12 ($p<0.001$, Table II). Likewise, while the EMG results of our study were not significantly different between the treatment and control groups at week 4 regarding distal latency ($p>0.05$, Table II), a significant improvement was found in the distal latency values of the treatment group at week 12 ($p<0.05$, Table II). Regarding the amplitudes of the nerve action potentials, the treatment group showed significant improvements in both measurements (week 4, $p<0.05$l; week 12, $p<0.001$, Table II).

Limitations

The results of this study revealed that Cx reduced EF noticeably and strengthened axonal regeneration. However, it is not possible to identify which component of the mixture consisting of three active components contributed to these results or the contribution degree of each component. To solve this problem, further studies with groups where cepae extract, allantoin, and heparin are applied separately and in combination are warranted. Additionally, with studies that included biochemical and physiological parameters, it may be possible to determine the effect mechanisms of this agent.

CONCLUSION

The results of our study, which were strengthened by our comprehensive histomorphological and electrophysiological analyses, showed that topical application of Cx after PN injury affected axonal regeneration and maturation positively and reduced the resulting functional loss. Studies using Cx and considering its effect on both the nervous system and tissues outside the nervous system have reached similar results and reported that Cx can be used safely to prevent postoperative PF. Our study similarly revealed that Cx can be an option in PN surgery, not only in primary anastomosis performed in the early period following injury, but also in the long run involving procedures that require revision such as neuroma excision and graft use.

AUTHORSHIP CONTRIBUTION

Study conception and design: RÖ, EK, MSB
Data collection: RÖ, AA
Analysis and interpretation of results: RÖ, AA, MSB, UDA, OBBE
Draft manuscript preparation: RÖ, EK
Critical revision of the article: TA, SH, ZS
Other (study supervision, fundings, materials, etc...): RÖ, TA, CA
All authors (RÖ, EK, MSB, AA, UDA, OBBE, TA, SH, CA, ZS) reviewed the results and approved the final version of the manuscript.

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