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Effects of insulin-like growth factor–I and platelet-rich plasma on sciatic nerve crush injury in a rat model

Laboratory investigation

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Object. Local administration of insulin-like growth factor–I (IGF-I) has been shown to increase the rate of axon regeneration in crush-injured and freeze-injured rat sciatic nerves. Local administration of platelet-rich plasma (PRP) has been also shown to have a measurable effect on facial nerve regeneration after transection in a rat model. The objective of the study was to compare the effects of locally administered IGF-I and PRP on the parameters of the Sciatic Function Index (SFI), sensory function (SF), axon count, and myelin thickness/axon diameter ratio (G-ratio) in a rat model of crush-injured sciatic nerves.

Methods. The right sciatic nerve of Wistar albino rats (24 animals) was crushed using a Yasargil-Phynox aneurysm clip for 45 minutes. All animals were randomly divided into 3 groups: Group 1 (control group) was treated with saline, Group 2 was treated with IGF-I, and Group 3 was treated with PRP. Injections were performed using the tissue expander's injection port with a connecting tube directed at the crush-injured site. Functional recovery was assessed with improvement in the SFI. Recovery of sensory function was using the pinch test. Histopathological examination was performed 3 months after the injury.

Results. The SFI showed an improved functional recovery in the IGF-I–treated animals (Group 2) compared with the saline-treated animals (Group 1) 30 days after the injury. In IGF-I–treated rats, sensory function returned to the baseline level significantly faster than in saline-treated and PRP-treated rats as shown in values between SF-2 and SF-7. The G-ratios were found to be significantly higher in both experimental groups than in the control group.

Conclusions. This study suggests that the application of IGF-I to the crush-injured site may expedite the functional recovery of paralyzed muscle by increasing the rate of axon regeneration. *(DOI: 10.3171/2010.9.JNS091928)*

Key Words • **crush injury** • **insulin-like growth factor–I** • **platelet-rich plasma** • **rat**

The 70–amino-acid polypeptide hormone IGF is a normal component of the plasma and is transported by IGF-binding proteins.[14,34,48] IGF-I has been shown to increase axon outgrowth in cultured neuroblastoma cells and myenteric plexus neurons. Insulin, IGF-I, and IGF-II are mitogenic to the myelinating Schwann cells in cultured rat sciatic nerve segments.[13,27,31,46] By studying co-cultured dorsal root ganglion neurons with Schwann cells, Cheng et al.[5] found that axon myelination would not occur in the absence of IGF-I. Locally infused IGF-I has been shown to increase the rate of axon regeneration after sciatic nerve crush or freeze injury.[22,40]

Platelets contain various growth factors, such as PDGF, TGF-β, PF4, VEFG, EGF, PDEGF, ECGF, and IGF. When platelets are activated, they release these factors, which play important biological roles in various conditions. These growth factors act locally to recruit undifferentiated cells to the site of injury, trigger mitosis in these cells, and induce angiogenesis. Application of platelet-rich plasma (PRP) provides the nerve with a medium enriched with platelet growth factors to improve regeneration.[1,12,16,25,29]

The objective of the present study was to compare the effect of IGF-I and PRP on crush-injured sciatic nerves in rats.

This article contains some figures that are displayed in color online but in black and white in the print edition.

Abbreviations used in this paper: AC = axon count; ECGF = epithelial cell growth factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; G-ratio = myelin thickness/axon diameter ratio; IGF = insulin-like growth factor; PDEGF = platelet-derived endothelial growth factor; PDGF = platelet-derived growth factor; PF4 = platelet factor 4; PRP = platelet-rich plasma; SF = sensory function; SFI = Sciatic Functional Index; TGF = transforming growth factor; VEFG = vascular endothelial growth factor.

Methods

Animals

Wistar albino rats weighing 200–240 g were purchased from the Institute for Experimental Medicine of Istanbul University and kept on standard laboratory diet and tap water. All procedures were reviewed and approved by the Ethics Committee for Animal Experimentation of Istanbul University.

Clip Features

The Aesculap Yasargil-Phynox aneurysm clip, manufactured from a high-grade Phynox cobalt-chrome alloy for permanent occlusion of cerebral aneurysms, was used to perform the sciatic nerve crush injuries. The closing force of the clip, which is determined by a computerized, electronic gauged–scale, is measured at a point at one-third of the blade length. We used an FE-752 K clip with a blade length of 11.0 mm.

Preparation of PRP

Eight additional rats were used to obtain blood for PRP. These animals were anesthetized using intraperitoneal injections of thiopental sodium (50 mg/kg) and their blood was collected by cardiac puncture.[2]

The blood was transferred to siliconized tubes containing 3.8% sodium citrate at a blood/citrate ratio of 9:1 and was centrifuged at 220 G for 20 minutes (Hettich Zentrifugen). The blood sublayer was removed, and the platelet-rich fraction was used for a second centrifugation at 480 G for 20 minutes. The pellet from the second centrifugation was saved and diluted with supernatant until the platelet concentration became 1.5×10^{12} platelets/L. Collected PRP was stored at −80°C for further use.

For platelet activation, the buffy coat containing the PRP was mixed in a 1:0.15 ratio with 100 U/ml thrombin (Sigma Aldrich) in 10% CaCl.

Injection and Surgical Procedure

After the induction of general anesthesia by thiopental sodium (50 mg/kg, administered by intraperitoneal injection), the right hindlimb was shaved and swabbed with antiseptic solution (Betadine). One longitudinal cutaneous incision was made in the back of the thigh. Dissection was carried out along a plane separating the hamstring and gluteal muscles to expose the sciatic nerve. Careful blunt dissection was performed to isolate the sciatic nerve from the surrounding connective tissue over a length of 2–2.5 cm.

The right sciatic nerve was crushed for a total of 45 minutes using a Yasargil-Phynox aneurysm clip. An expander's injection port with connecting tube was placed on a subcutaneously prepared dorsal pouch. The connecting tube's tip was tied with a suture in the adjacent muscle to mark the crushed site and to apply the injection materials to it. The wound was closed with a 3-0 Ethicon silk suture and the rats were allowed to recover. After recovery from anesthesia, the animals were housed individually and given food and water ad libitum.

Injections of saline, IGF-I, or PRP were administered to animals in the respective groups on the operation day and on the 3rd, 5th, 7th, 9th, 11th, 13th, and 15th postoperative days via the tissue expander's injection port with connecting tube.

Experimental Protocol

Twenty-four rats were randomly assigned to 3 groups (8 animals per group). While all 24 animals underwent the right sciatic nerve crush injury procedure as described above, the left sciatic nerves of the rats were left intact. Every 48 hours, starting on the day of the injury and continuing for 15 days, the animals were injected with 0.125 ml saline (Group 1), 15 μg of IGF-I (BioVision) in 0.125 ml vehicle (Group 2), or 0.125 ml PRP (Group 3) via the tissue expander's injection port.

The experimental protocol is shown in Table 1.

Three months after the crush injury, all rats in each group were anesthetized (with thiopental sodium, 50 mg/kg, administered intraperitoneally), tissue samples were taken and the rats were killed by decapitation soon after the procedure.

Evaluation Tests

Walking Pattern Analysis. Recovery of motor function was monitored by analysis of the free-walking pattern. The overall index of sciatic nerve function was used as a parameter to evaluate the recovery of coordinated motor function of the injured hind paw. The test procedure is as follows: The paws of both hindlimbs were dipped in ink, and the rats were allowed to walk on a corridor (80 cm long, 7 cm wide, and inclining 10°) covered by a photocopy paper. The Sciatic Functional Index (SFI) was calculated from the paw prints, using the formula developed by Bain et al.[3] An SFI of 0 is normal, whereas an SFI of −100 means total impairment. The walking patterns were analyzed 1 day before the operation (SFI-0), 1 day after the operation (SFI-1), and 1, 2, and 3 months postoperatively (SFI-2, -3, -4).

Sensory Function. Recovery of sensory function was analyzed using the pinch test. The lateral side of the rat's paw was pinched using the same aneurysm clip. Animals showing a withdrawal response to pinching were noted. Pinch tests were performed 1 day before the operation (SF-0) and on the day following the operation (SF-1) and were repeated at 2-week intervals for 12 weeks postoperatively (SF-2, SF-3, SF-4, SF-5, SF-6, SF-7).

Histological Evaluation. Distal parts of the crushed site of the right sciatic nerves and intact left sciatic nerves were sampled for every group. Samples were fixed with 3.6% glutaraldehyde in 0.1 mol/L Sørensen's phosphate buffer (pH = 7.3) and postfixed with 1% osmium tetraoxide in the same buffered solution; after dehydration through a graded series of ethanol solutions, they were embedded in epoxy resin (Fluka, Sigma Aldrich). The specimens were then serially cross-sectioned (20 cross-sections for each specimen) at 0.5-μm thickness on an ultra microtome. All sections were stained with thionin and were examined and photographed by means of a light microscope (Olympus BH2 RFCA). Axon counts for all

TABLE 1: Experimental protocol*

Group	Saline (days x dose)	IGF-I (days x dose)	PRP (days x dose)	No. of Rats
Group 1	8 x 0.125 ml	—	—	8
Group 2	—	8 x 15 μg (0.125 ml)	—	8
Group 3	—	—	8 x 0.125 ml	8

* Saline, IGF-I, and PRP were administered via the tissue expander's injection port every 48 hours starting on the day of injury and continuing for 15 days thereafter.

cross-sections were obtained with the aid of a digital counter in 6 random fields (1 center and 5 peripheral) at a magnification of 40.

Average sciatic nerve myelin thickness/axon diameter ratios (G-ratios) were obtained with Clemex-Lite Version 3.5.3.0 image analysis software.

All measurements were carried out in a blinded fashion.

Data Analysis. Data obtained from the free-walking pattern were expressed as mean SFIs per group. Data obtained from pinching tests were expressed as mean SFs per group and data obtained from histopathological examinations were expressed as G-ratios and axon counts.

Statistical analyses of SFI, G-ratio, and axon count values were done using Kruskal-Wallis variance analysis. A p value < 0.05 was considered statistically significant.

The Mann-Whitney U-test was applied with Bonferroni correction to the significant values between the groups. A p value < 0.016 was considered statistically significant.

The SF statistical analyses were done using the Fisher exact test. A p value < 0.05 was considered statistically significant.

Results

After surgery, all rats remained healthy throughout the study period.

Sciatic Functional Index

The SFI values are shown in Fig. 1. Before surgery, there were no significant differences in SFI values (SFI-0) between the experimental groups. The mean SFI values were −11.22, −13.37, and −6.04 for Groups 1, 2, and 3, respectively.

One day after the injury, the mean SFI values (SFI-1) were −93.3 in all experimental groups. Following nerve injury, the rats lost the ability to grip a wire screen with their hind paws and to walk normally, as indicated by a decrease in toe spread, intermediary toe spread, and an increase in the angle between hind feet. The print length of the animals in all experimental groups was considerably longer and toe spreading and intermediary toe spreading narrower on the crushed site.

One month after the operation, the SFI values (SFI-2) had improved relative to SFI-1 in all experimental groups. There was a significant difference between the mean values for the saline-treated rats (Group 1) and the IGF-I treated rats (Group 2, $p < 0.016$).

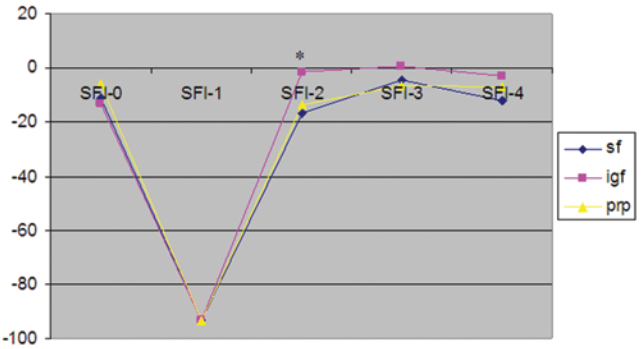

Fig. 1. Sciatic Functional Index values obtained the day before sciatic nerve injury (SFI-0), 1 day after injury (SFI-1), and 1, 2, and 3 months postinjury (SFI-2, SFI-3, and SFI-4, respectively). The *blue diamonds* indicate mean values from the saline-treated control group (Group 1, sf); the *magenta squares*, the IGF-I–treated rats (Group 2, igf); and the *yellow triangles*, the PRP-treated rats (Group 3, prp).

The ability of injured rats treated with IGF-I to grip an inverted screen returned to control levels significantly faster than that of saline-treated rats. Similarly, IGF-I–treated rats showed significantly better results in toe spread, intermediary toe spread, and angle values than did saline-treated rats.

There was no significant intergroup difference in SFI values at 2 (SFI-3) or 3 (SFI-4) months after the injury.

Sensory Function

The results of pinch tests performed before the crush injury were positive in all experimental groups (SF-0). When the same tests were performed following surgery, rats demonstrated loss of withdrawal ability, and in all experimental groups, SF-1 values deteriorated significantly in comparison with SF-0 ($p < 0.0002$).

In IGF-I–treated rats, withdrawal ability returned to baseline significantly faster than in saline-treated and PRP-treated rats at SF-2, SF-3, SF-4, SF-5, SF-6, and SF-7, as is shown in Fig. 2.

Nerve Histology

There was no statistically significant difference between any of the experimental groups in axon counts.

There was no statistically significant difference between the uninjured left sciatic nerve groups for G-ratio. There was a statistically significant difference only in Group 1 between uninjured left sciatic and injured right sciatic nerve groups for G-ratio, as is shown in Fig. 3 ($p < 0.05$).

In the comparison of the injured nerve groups, there was a statistically significant difference between Group 2 and the other 2 groups for G-ratio, as is shown in Fig. 4 ($p < 0.016$).

Representative cross-sections of sciatic nerve can be seen in Fig. 5. In Group 2, myelin loss and axonal degeneration were lower, compared with other groups. Myelin loss and axonal degeneration were apparently higher in the Group 1.

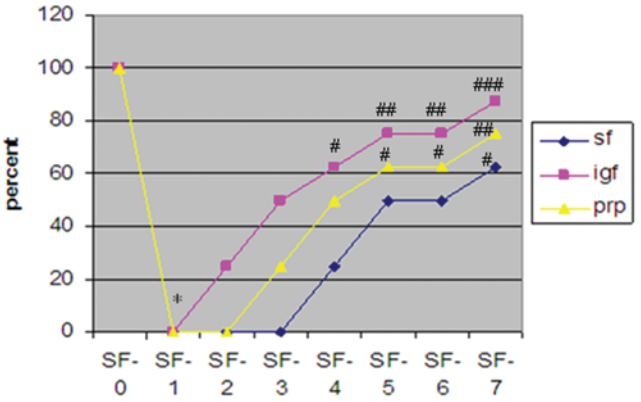

Fig. 2. Percentage of animals with a positive pinch test response (sensory function) 1 day before crush injury (SF-0), the day after crush injury (SF-1), and at 2-week intervals thereafter (SF-2 through SF-7) for 12 weeks postinjury. * $p < 0.0002$ for all groups.

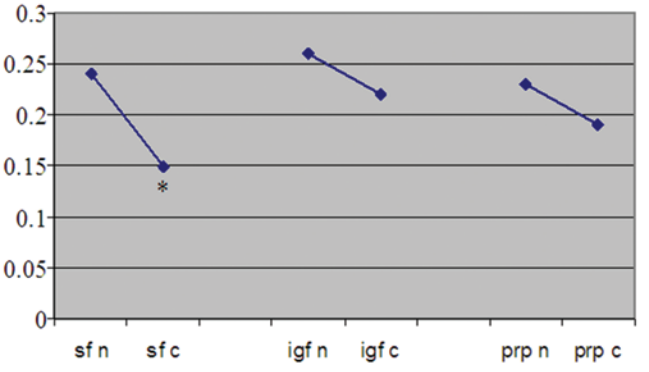

Fig. 3. Comparison of G-ratio values in uninjured (n) and injured (c) sciatic nerves in each of the 3 treatment groups.

Discussion

A crush injury occurs when tissue is subjected to a high degree of force or pressure, usually in being squeezed between 2 heavy objects. This injury can be described through an animal model. With this model, the usual method involves squeezing the nerve with smooth-tipped forceps or vessel clips, which induces a focal compression injury.[38,43]

In our study, standardization of the compression is provided by using an Aesculap Yasargil-Phynox aneurysm clip that produces a quantitative crush injury to the rat's sciatic nerve.

Following the clip application, compared with baseline values, SFI and SF values deteriorated, revealing functional motor and sensory damage of the sciatic nerves in the crush-injured rats. These results confirmed that our model was effective in inducing crush injuries.

In humans, compression injuries can be induced by fractures, hematomas, and compartment syndrome. Crush injuries occur when body tissues are severely traumatized, such as in motor vehicle accidents, falls, and gunshot wounds. These injuries frequently occur in the extremities. Peripheral nerve injuries may cause various clinical symptoms, ranging from paresthesia to total paralysis, depending on the cause, magnitude, and duration of the trauma. The pathophysiology of the nerve injury is not completely understood in crush injury, and it has been debated whether the compression-induced ischemia or the mechanical deformation of nerve fibers is the more significant etiologic factor. Although the role of ischemia has been stressed by some authors, the significance of the mechanical nerve fiber deformation has been emphasized by others.[28,30,35,39]

Evidence from animal studies on crush nerve injury has shown that it results from axonal interruption within intact Schwann cell basal laminal tubes. In these studies, after the injury, Schwann cells quickly invaded the injured region, and regenerating axons crossed the crushed area within a few days while the surviving basal laminal tubes guided them to their former peripheral connections.[39,43] It has been stated that Denny-Brown and Brenner (in 1944) and Richardson and Thomas (in 1979) had concluded that the mechanism of injury caused a combination of segmental demyelination, periaxonal and intramyelinic edema, and axonal interruption of nerves in their experimental studies.[11,32]

The basic process of nerve healing involves degeneration and regeneration. Wallerian degeneration is a process in which the damaged segment of a nerve is phagocytosed, beginning at the first intact node of Ranvier in peripheral nerves. The Schwann cell tubes are also phagocytosed to prevent obstruction of the regenerating axon. Many different growth factors and cytokines affect this process of degeneration and regeneration. Nerve regeneration is augmented by neurotrophic activity, which has long been known to be increased in lesioned nerves. IGF-I has sparked much interest among researchers because of its ability to stimulate the Wallerian degeneration and regeneration of axons.[17,39] By using a cross-facial nerve graft locally infused with IGF-I, Thanos et al.[42] obtained significantly increased axon regeneration across both coaptation sites, along with a more rapid return of orbicularis oculi muscle function, compared with placebo-infused grafts.

Investigating the effects of IGF-I in adult rats, Lutz et al.[23] found that 14 days of postoperative systemic administration did not improve motor nerve regeneration after peripheral nerve transection and repair. The age of the subject and the route of delivery may be important factors, since systemic administration in neonatal rats[45] and local infusions in adult rats[42] have demonstrated contradictory results. Examining the effects of 2 doses of local IGF-I infusion in a model of end-to-side nerve repair, Tiangco et al.[44] found that muscle function could be restored at a significantly faster rate than with placebo treatment. The IGF system also plays a role in repair following hypoxic-ischemic injury in many tissues. The functional outcome following proximal injury is often unsatisfactory because irreversible muscle atrophy may develop before reinnervation occurs. Because IGF-I has been shown to improve muscle regeneration after injury, it may help prevent muscle atrophy and secondary functional compromise after denervation. IGF-I is critical not only for Schwann cell attachment to and ensheathment of axons, but also for long-term myelination. No myelination occurs in the absence of IGF-I.[5,10,23,37,44,45]

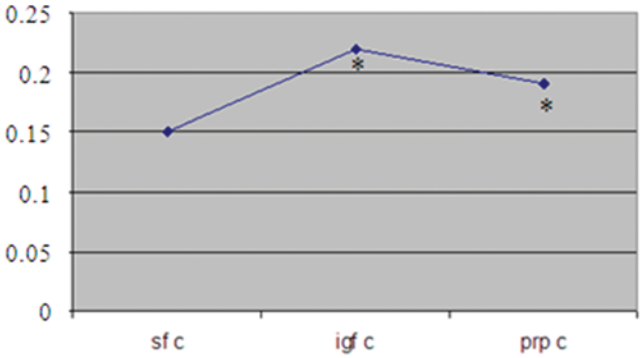

Fig. 4. Comparison of G-ratio values in the injured (c) sciatic nerves of each of the 3 treatment groups.

Local augmentation of IGF levels at the crush injury site may enhance axonal sprouting. By manipulating the inflammatory and early proliferative phases of nerve healing with locally administrated IGF-I, we aimed to increase the rate of axon regeneration through a crush injury model in this study.

Platelet-rich plasma (PRP) is a concentration of platelets and includes growth factors. Platelet-rich plasma is defined as a portion of the plasma fraction of blood having a platelet concentration above baseline. The regenerative potential of PRP depends on the levels of secretory growth factors that are released on platelet activation.[20,24,36,49] Numerous growth factors including PDGF, TGF-β, PF4, VEFG, EGF, PDEGF, ECGF, and IGF-I, secreted by the activated platelets, strongly influence many aspects of wound healing. Although with the exception of IGF-I, these are not classical neurotrophic factors, their effects on nerve regeneration have been comprehensively studied. There are experimental data indicating that VEGF can stimulate axonal outgrowth and enhance Schwann cell proliferation. TGF-β, PDGF, and FGF-II have been described as mitogens for rat Schwann cells. Improved nerve regeneration has also been identified with combined administration of PDGF and FGF-II.[8,9,19,33,41]

Studies have shown a 3- to 4-fold increase in growth factor concentration in PRP as compared with nonconcentrated blood.[20,24,37,49] Therefore, PRP has an effect on the process of remyelination of regenerating axons due to growth factors that are released from the platelets during activation. It may have an impact on the quality of healing, eventually leading to better functional recovery.

By releasing secretory proteins from their α-granules on activation, platelets set the pace of nerve healing. The enhancement of healing by the placement of a supraphysiological concentration of platelets at the site of tissue injury is probable, and this condition is created by using implantable osmotic pumps or ports to achieve reliable local concentrations.[20,36,44]

In our study, 3 months after the crush injury, partial improvements were seen in deteriorated motor and sensory functions in the control group. The improvement rates of SFI and SF values in the IGF-I–treated and PRP-treated groups differed significantly from those of the control group.

With respect to G-ratio values, the only significant difference between the values for the injured and uninjured nerves was in the Group 1 animals (the control group) at 3 months after the procedure. Between-groups comparison showed a statistically significant difference between the G-ratios in Group 1 and those in the other 2 groups. These results revealed that the nerve healing was faster in the IGF-I treated and PRP-treated groups (Groups 2 and 3) than in the control group (Group 1).
We did not observe any statistically significant difference in G-ratio values between the injured and uninjured sciatic nerves of the animals in Group 2 or Group 3 at the end of the 3rd month. The G-ratio values were found to be consistent with both SFI-2 and SF values.

Regeneration of a peripheral nerve occurs at a rate of approximately 1 mm/day. In injuries that are more proximal, improvement may not be obvious for many months. For that reason, surgery is postponed 2–5 months to await the spontaneous return of nerve function.[6,18,26] During this time, assessment of peripheral nerve injuries in these patients using MR neurography has the potential to confirm acute nerve injury as well as to monitor the recovery process. Magnetic resonance imaging is an excellent diagnostic tool for detection of peripheral nerve injuries, as it has the ability to directly reveal nerve injuries and display the abnormalities of denervated muscle. It is also used in monitoring the recovery process.[4,21] In their study, Cudlip et al.[7] demonstrated that quantitative assessment of nerve signals with MR neurography allowed the sequence of

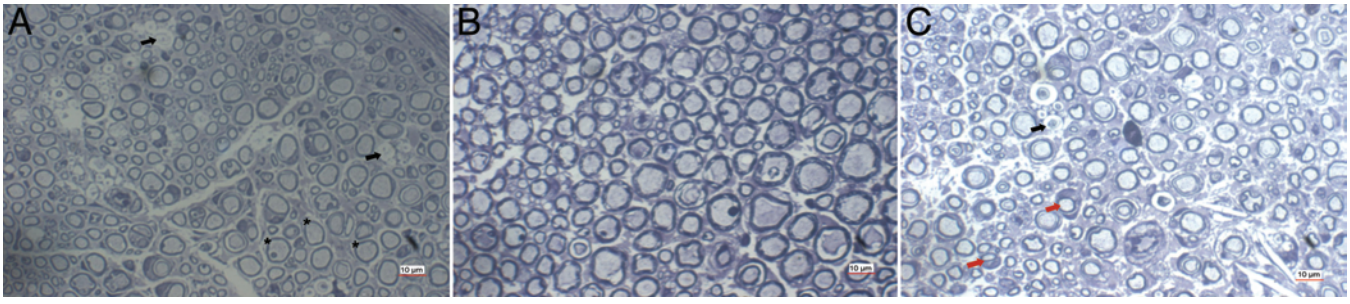

Fig. 5. Representative cross-sections of injured sciatic nerves. **A:** Specimen from a Group 1 rat, showing moderate reduction of myelinated fibers. All remaining axons are thinly myelinated. The *black arrows* indicate late stage Wallerian degeneration; the *asterisks* indicate thinly myelinated fibers. **B:** Specimen from a Group 2 rat, showing normal density of myelinated fibers. **C:** Specimen from a Group 3 rat, showing mild reduction of myelinated fibers. The *black arrow* indicates Schwann cell proliferation; the *red arrows*, regenerating fibers.

events following nerve crush injury to be followed in vivo and that a return toward a normal signal correlated with functional improvement. Hence, with MR imaging, the site of the injury can be detected and IGF-I or PRP containing an osmotic pump can be placed at the injured site. Thus, the direct injections of IGF-I or PRP to the injury site enhance the nerve healing and reduce the morbidity of the patient, and, as such, are compatible with the aim of our project.

Conclusions

Nowadays, studies on nerve regeneration have shifted away from surgical nerve repair to the biological manipulation of nerve regeneration at the cellular level. An understanding of molecular pathways and their physiological role under recent advances demonstrates that growth factors are an important part of the regeneration of the nervous system.[15,47] In our study, we showed the improvement in nerve regeneration with both IGF-I and PRP and aimed to contribute to the armamentarium of this topic.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Ergun, Emel. Acquisition of data: Ergun, Kotan, Zengin. Analysis and interpretation of data: Ergun, Gürsoy. Drafting the article: Ergun, Emel. Critically revising the article: Ergun, Parman. Reviewed final version of the manuscript and approved it for submission: all authors. Statistical analysis: Kotan, Zengin. Administrative/technical/material support: Ergun, Emel, Gürsoy, Parman, Zengin. Study supervision: Ergun.

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