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Local administration of prostaglandin E1 combined with silicone chamber improves peripheral nerve regeneration

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ABSTRACT

The aim of this study was to assess the effect of locally administered prostaglandin E1 on peripheral nerve regeneration and functional recovery. Sixty male healthy white Wistar rats were divided into four experimental groups ($n = 15$), randomly: In transected group (TC), left sciatic nerve was transected and stumps were fixed in the adjacent muscle. In treatment group defect was bridged using silicone graft (SIL/PE) filled with 10 μ L prostaglandin E1. In silicone graft group (SIL), the graft was filled with phosphate-buffered saline alone. In sham-operated group (SHAM), sciatic nerve was exposed and manipulated. Each group was subdivided into three subgroups of five animals each and regenerated nerve fibers were studied 4, 8 and 12 weeks after surgery. Behavioral testing, sciatic nerve functional study, gastrocnemius muscle mass and morphometric indices confirmed faster recovery of regenerated axons in SIL/PE than SIL group ($p < 0.05$). In immunohistochemistry, location of reactions to S-100 in SIL/PE was clearly more positive than that in SIL group. When loaded in a silicone graft, prostaglandin E1 improved functional recovery and morphometric indices of sciatic nerve. Local application of prostaglandin E1 improved functional recovery and morphometric indices of sciatic nerve.

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1. Introduction

Where there is considerable nerve tissue loss, peripheral nerve regeneration is still a concern in regenerative medicine.¹ When an axon is crushed or severed, changes occur on both sides of the lesion.² Where a gap is present between the severed ends of the nerve, proliferating Schwann cells emerge from the stumps (mainly the distal stump) and form a series of nucleated cellular cords (the bands of Bungner) which bridge the interval.³

Following nerve transection Wallerian degeneration occurs which is a sequential pattern of axonal degeneration, myelin degradation and supporting glial cell proliferation. During this process, various events take place, including blood-nerve barrier dysfunction, endoneurial space reorganization, and most importantly the induction of an intense inflammatory response, constituted by inflammatory mediator release and production.^{4,5}

Axonal degeneration recruits this response activating Schwann cells and macrophages that proliferate and activate, clearing myelin debris and producing cytokines that perpetuate an inflammatory

state. Axonal regeneration is then regulated by the interactions between all the involved cell types and by cytokines, chemokines, growth factors, and other inflammatory mediators.⁵ All these events culminate in the promotion of an environment suitable for subsequent regeneration, repair, and axon regrowth. Arachidonic acid and its metabolites are known to modulate neuronal function and survival. There is also evidence that arachidonic acid derivatives, such as prostaglandins, are centrally involved in Wallerian degeneration and in axonal regeneration.⁵

Regarding the association that exists between arachidonic acid derivatives and nerve degeneration and regeneration, the therapeutic modulation of this pathway emerges as a novel strategy aimed at increased motor, sensory, and structural recovery after nerve injury.⁶

Aimed to study local effects of prostaglandin E1 (PGE 1) on peripheral nerve regeneration, the present study was designed to attempt to determine if topical PGE 1 do in fact reduce dysfunction after small gap nerve transection injury in the rat sciatic nerve transection model. Assessment of nerve regeneration was based on behavioral, functional (walking track analysis), histomorphometrical and immunohistochemical (Schwann cell detection by S100 expression) assessment at 4, 8, and 12 weeks after surgery.

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2. Materials and methods

2.1. Study design and animals

Sixty male Wistar rats weighing approximately 290 g were divided into four experimental groups ($n = 20$), randomly: sham-operation group as normal control (SHAM), transected control (TC), silicone graft (SIL) and prostaglandin E1 treated group (SIL/PE). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 weeks after surgery. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of $(23 \pm 3) ^\circ\text{C}$, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups.

2.2. Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine–xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.⁷ The University Research Council approved all experiments.

Following surgical preparation in the sham-operation group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. In TC group, the left sciatic nerve was transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a 10 mm gap due to retraction of nerve ends. Proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture. No graft was interposed between the stumps. In the SIL group, a 7 mm nerve segment was resected to produce a 10 mm nerve gap after retraction of the nerve transected ends. The gap was bridged using a silicone graft (Polyerubb Industries, Ahmedabad – 380023, Gujarat, India), entubulating 2 mm of the nerve stump at each end. The graft was 14 mm in length, 2 mm in inner diameter and 2 mm in thickness. A subtle retraction of 1 mm was already expected. Two 10/0 nylon sutures were used to anchor the graft to the epineurium at each end. In prostaglandin E1 treated group (SIL/PE) the graft was filled with 10 μl prostaglandin E1 (100 ng/mL) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) 4, 8 and 12 weeks after surgery.

2.3. Behavioral testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function.⁸ Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries.⁹ Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination.^{8,9} BBB recordings were performed by a trained observer who was blinded to the experimental design. The animals were observed and assessed within a course of a 4-min exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks.

2.4. Functional assessment of reinnervation

2.4.1. Sciatic functional index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others.¹⁰ The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

$$\text{SFI} = -38.3 \times (\text{EPL} - \text{NPL})/\text{NPL} + 109.5 \times (\text{ETS} - \text{NTS})/\text{NTS} + 13.3 \times (\text{EIT} - \text{NIT})/\text{NIT} - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

2.4.2. Static sciatic index (SSI)

SSI is a time-saving digitized static footprint analysis described by others.¹¹ A good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others.¹¹ The SSI is a time-saving and easy technique for accurate functional

assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:

$$\text{SSI} = [(108.44 \times \text{TSF}) + (31.85 \times \text{ITSF})] - 5.49$$

where:

$$\begin{aligned}\text{TSF} &= (\text{ETS} - \text{NTS})/\text{NTS} \\ \text{ITSF} &= (\text{EIT} - \text{NIT})/\text{NIT}\end{aligned}$$

Like SFI, an index score of 0 was considered normal and an index of -100 indicated total impairment. When no footprints were measurable, the index score of -100 was given.³¹

2.5. Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance.

2.6. Histological preparation and morphometric studies

Nerve mid-substance in SIL group, nerve mid-substance in prostaglandin E1 treated group, midpoint of normal sciatic nerve (SHAM) and regenerated mid substance of TC group were harvested and fixed with glutaraldehyde 2.5%. They were post fixed in OsO_4 (2%, 2 h), dehydrated through an ethanol series and embedded in Epon. The nerves were cut in $5 \mu\text{m}$ in the middle, stained with toluidine blue and examined under light microscopy. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional disector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases.¹²

2.7. Immunohistochemical analysis

In this study, anti-S-100 (1:200, DAKO) was used as marker for myelin sheath. Specimens prior to immunohistochemistry were post fixed with 4% paraformaldehyde for 2 h and embedded in paraffin. Then the nerve sections were dewaxed and rehydrated in PBS (pH = 7.4). They were incubated by 0.6% hydrogen peroxide for half an hour to neutralize endogenous peroxide. After that the sections were incubated with normal swine serum (1: 50, DAKO, Germany) for blocking of non-specific immunoreactions and then were incubated in S-100 protein antibody solution for 1 h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1 h. Horseradish peroxidase-labeled secondary antibody (1:100 swine anti-rabbit diluted in 5% normal rat serum) was applied for 1 h. All sections were then incubated with diaminobenzidine tetrahydrochloride chromogen (DAB, DAKO) substrate solution for 10 min. The sections were mounted in corbit balsam with coverslip. The results of immunohistochemistry were examined under a light microscope.

2.8. Statistical analysis

The results were expressed as means \pm SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., ChicSILO, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were set at $P < 0.05$.

3. Results

3.1. BBB recovery

In order to assess hind limb recovery the open field locomotor was used. Fig. 1 shows BBB scores compared to the baseline. All experimental groups, except for sham, showed the greatest degree of functional deficit one week after surgery. The prostaglandin E1 treated group showed significant improvement in locomotion of the operated limb compared to the SIL group during the study period ($P < 0.05$).

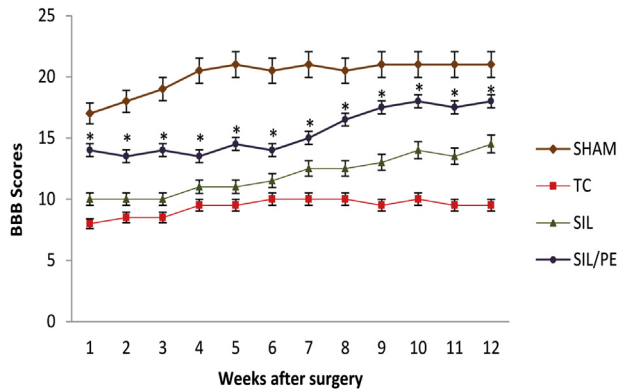


Fig. 1. BBB score for all experimental groups. Topical administration of PGE1 with silicone grafting gave better scores than in SIL group. Standard error at each data point is shown with bars. * $P < 0.05$ vs SIL group.

3.2. Recovery of sciatic nerve function

3.2.1. SFI outcome

Fig. 2 shows sciatic function index (SFI) values in experimental groups. Prior to surgery, SFI values in both groups were near zero. After the nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. At the end of the study period, animals of PGE 1 treated group achieved a mean value for SFI of -41.3 ± -2.45 whereas in SIL group a mean value of -58 ± -2.42 was found. The statistical analyses revealed that the recovery of nerve function was significantly ($P < 0.05$) different between SIL and SIL/PE groups and application of PGE 1 in silicone conduit significantly improved functional recovery in the course of time.

3.2.2. SSI outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Fig. 3).

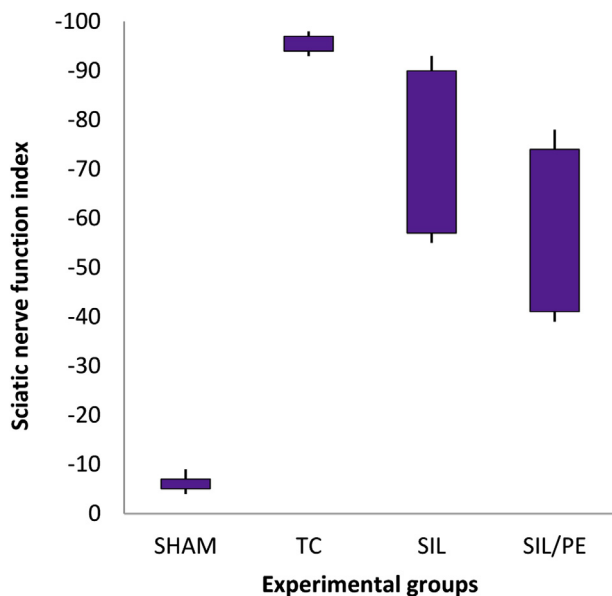


Fig. 2. Box-and-whisker plots of sciatic nerve function index values in each experimental group during the study period. Topical administration of PGE1 with silicone grafting gave better results in functional recovery of the sciatic nerve than in SIL group. Topical administration of PGE 1 with silicone grafting gave better results in functional recovery of the sciatic nerve than in SIL group ($P < 0.05$).

Static sciatic index (SSI)

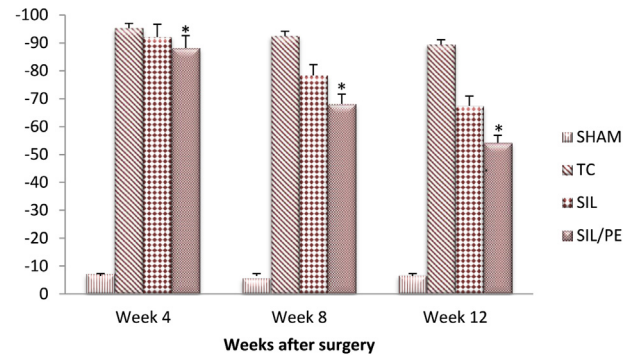


Fig. 3. Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Topical administration of PGE 1 with silicone grafting gave better results in functional recovery of the sciatic nerve than in SIL group. Data are presented as mean \pm SD. * $P < 0.05$ vs SIL group.

Changes in SSI were significant at weeks 4, 8 and 12 weeks of recovery ($P < 0.05$). The contrasts indicated SSI values at week 12 to differ significantly from those obtained from control, a trend also noticed for SFI ($P < 0.05$).

3.3. Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured at the end of the study period. There was a statistically significant difference between the muscle weight ratios of the SIL/PE and SIL groups ($P < 0.05$). The results showed that in the prostaglandin E1 treated group, the muscle weight ratio was larger than in the SIL group, and weight loss in the gastrocnemius muscle was ameliorated by prostaglandin E1 topical administration (Fig. 4).

3.4. Histological and morphometric findings

Figs. 5–7 show the quantitative morphometric analyses of regenerated nerves for each of the experimental groups. The prostaglandin E1 treated group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness 4 weeks after surgery, compared to SIL animals ($P < 0.05$). Sham-operation group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to SIL/PE and SIL groups animals. In case of myelin thickness there was no significant difference between SIL/PE and SIL groups, morphometrically ($P > 0.05$).

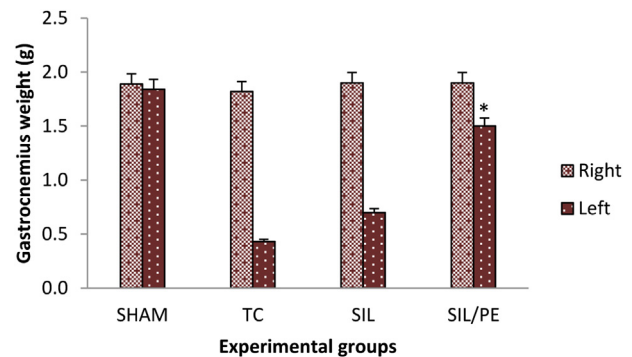


Fig. 4. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean \pm SD. * $P < 0.05$ vs SIL group.

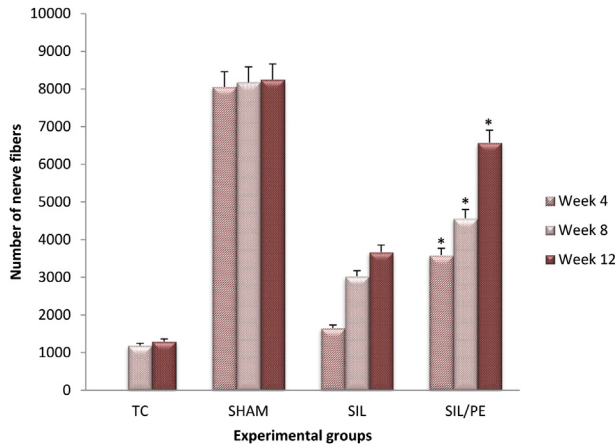


Fig. 5. The graph shows the quantitative results of fiber counting. The mean number of nerve fibers in SHAM group was nearly 8276 ± 189 (mean \pm SD). Both groups of SIL and SIL/PE showed the lower number of fibers than the sham-operated group even at the end of the study period. Data are presented as mean \pm SD. * $P < 0.05$ vs SIL group.

3.5. Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 8). In both SIL/PE and SIL groups, the expression of S-100 and the findings resembled those of the histological evaluations.

4. Discussion

Entubulation neurotization is an excellent alternative to short interposition nerve grafts.¹³ In the present study silicone tube was used as a scaffold for keeping the delivered drug *in situ*. Selection of an appropriate method to evaluate functional recovery of nerve regeneration is extremely influential. In this study, we did not perform nerve conduction tests because electrophysiological studies have poor correlation with functional indices.¹⁴ Nerve conduction velocity and peak action potential amplitude do not evaluate total nerve function but a fraction of nerve fibers

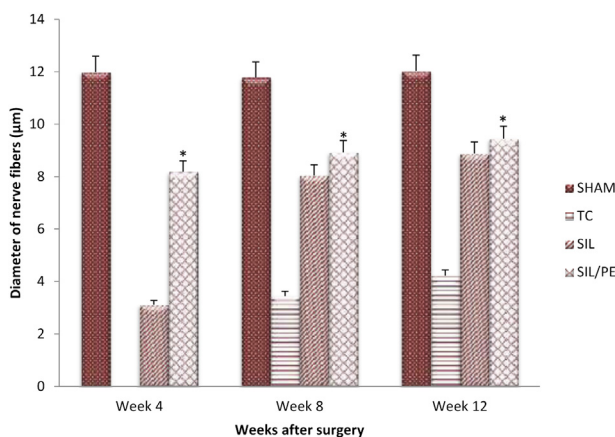


Fig. 6. The graph shows the quantitative results of mean diameter of nerves fibers. The mean diameter of nerve fibers in SHAM group was nearly 12.5 ± 0.19 (mean \pm SD). Both groups of SIL and SIL/PE showed the lower mean diameter of nerve fibers than the sham-operated group even at the end of the study. Data are presented as mean \pm SD. * $P < 0.05$ vs SIL group.

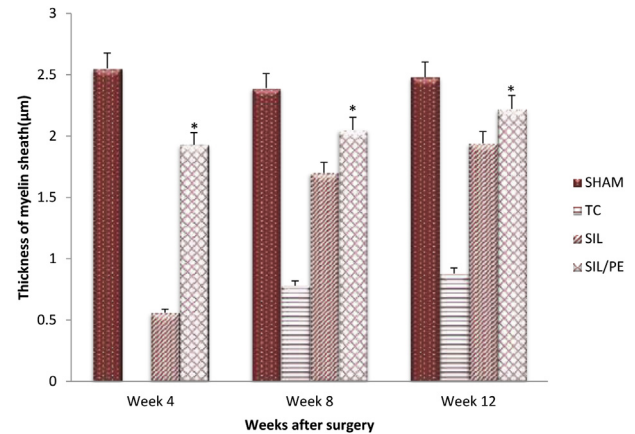


Fig. 7. The graph shows the quantitative results of mean thickness of myelin sheath. The mean thickness of myelin sheath in SHAM group was nearly 2.3 ± 0.06 (mean \pm SD). Both groups of SIL and SIL/PE showed the lower mean diameter of axons than the sham operated group even at the end of the study period.

population. Compound action potential is derived from extrinsic direct nerve excitation and does not correlate with proper central or peripheral connections.¹⁵ Castaneda et al.,¹⁶ suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that prostaglandine treated animals had been improved in locomotion of the operated limb compared to the control group during the study period. Walking track analysis and static sciatic index has frequently been used to reliably determine functional recovery following nerve repair in rat models.^{10,11,17} The present study again showed similar results taken from both SFI and SSI assessments. Walking is a coordinated activity involving sensory input, motor response and cortical integration.¹⁸ Therefore, walking track analysis, sciatic function index, is a comprehensive test. As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation.^{19,20} In the present study 12 weeks after surgery the muscle mass was found in both experimental groups. However, SIL/PGE group showed significantly greater ratios of the mean gastrocnemius muscle weight than SIL group indicating indirect evidence of successful end organ reinnervation.

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor.^{15,21,22} Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling^{23,24} do not necessarily predict the reestablishment of motor and sensory functions.^{16,25,26} Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery.²² Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment.

A wide variety of materials have been used to produce nerve guides, including non-biodegradable and biodegradable materials. Because of its inert and elastic properties, the silicon tube was one of the first and most frequently used to bridge the transected nerves.²⁷

After nerve injury, increased cyclooxygenase expression induces the production of PG in nerve terminals and nonneural cells in the surrounding areas. This process is known to initiate hyperalgesia and neuropathic pain.^{28,29} Prostaglandins are produced in important

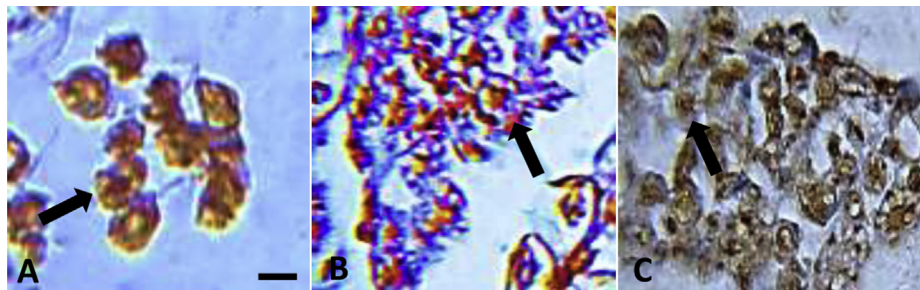


Fig. 8. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from (A) middle cable TC, (B) SIL/PE and (C) SIL. There is clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in prostaglandin E1 treated nerve compared to that of the SIL. Scale bar: 10 μ m.

quantities, and for prolonged time periods, both directly by injured nerves and macrophages in response to soluble factors produced by injured nerves.³⁰ It has been shown that prostanoid receptors, effectors of biological actions of prostaglandins, are expressed in Schwann cells and could modulate Schwann cell function *in vivo*.³¹ Moreover, it has been shown that prostaglandin E2 could modulate microglial migration and function.³² Prostaglandins have also been shown that interact with nerve growth factor in the regulation of inflammatory responses and degeneration following injury.³³ After nerve injury prostaglandins play a crucial role in nerve degeneration and regeneration. These are vasoactive molecules and their action in blood-flow homeostasis and inflammation during nerve injury could be important. Prostaglandin E1 diminishes peripheral nerve ischemia-reperfusion injury, probably through such a mechanism.³⁴ Following nerve crush injury, PG E1 treatment has resulted in reduced injury, increased repair rates and the upregulation of vascular endothelial growth factor.³⁵ Schratzberger et al.³⁶ showed that vascular endothelial growth factor gene transfer significantly increased nerve blood flow as well as the amount of vasculature in nerves, suggesting that the induction of local angiogenesis ameliorates experimental neuropathy. One mechanism that PGE1 can promote the repair of peripheral nerve with crushing lesion, could be via increasing the expression of vascular endothelial growth factor.³⁵ It has been reported by clinical and experimental studies that vasoactive treatment can alleviate the effects of lesions in peripheral nerves.^{37,38}

The induction of neuronal apoptosis is a recognized phenomenon after nerve injury that contributes to the physiopathology of nerve degeneration. The administration of PGE1 inhibits neuronal apoptosis in the spinal cord after sciatic nerve constriction injury, independently of changes in local blood flow.³⁹ This suggests that neuroprotective mechanism of PG E1 is not solely dependent on its vasoactive properties. Apart from hyperalgesia and blood-flow regulation, prostaglandins also reported to contribute to the molecular and cellular process of nerve degeneration and regeneration.⁶

Further studies on sciatic nerve crush injury also showed that PGE1 both reduced apoptosis and improved neuronal regeneration.⁴⁰ Prostaglandins are known to modulate the upregulation of heat-shock protein-70 expression, a protein response known to participate in the maintenance of neuron survival after nerve injury.^{41,42} After nerve injury, macrophages migrate into the area and initiate degenerative and regenerative processes.

Even though our preliminary study shows the neuroprotective action of local prostaglandin E1 in peripheral nerve injuries, data regarding the molecular mechanisms leading to the neuroprotective action is limited and the exact mechanisms are just being uncovered. We have not given the histological and molecular evidence for neuroprotective action of prostaglandin E1. This may be considered as a limitation to our study. Therefore, the authors stress that the aim of the current investigation was to evaluate a

single local dose and clinical treatment potential of prostaglandin E1 on nerve regeneration. Mechanism of neuroprotective action remains to be investigated.

In conclusion, the present study demonstrated that local application of prostaglandin E1 could accelerate functional recovery after transection of sciatic nerve. It is available and easily performed. Thus, dose–response studies should be conducted for prostaglandin E1 to determine the combination of the graft and the compound that achieve maximal efficacy in nerve transection models.

Ethical approval

None.

Funding

None.

Author contribution

Alireza Najafpour: Study design and writing.

Rahim Mohammadi: Data collection.

Darab Faraji: Data collection.

Keyvan Amini: Data analysis.

Conflicts of interest

None.

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