ORIGINAL ARTICLE
doi: 10.2176/nmc.oa2012-0330
Neurol Med Chir (Tokyo) 53, 839–846, 2013
Online October 21, 2013

Acetyl Salicylic Acid Locally Enhances Functional Recovery after Sciatic Nerve Transection in Rat

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Abstract

Local effect of acetyl salicylic acid (ASA) on peripheral nerve regeneration was studied using a rat sciatic nerve transection model. Forty-five male healthy White Wistar rats were divided into three experimental groups (*n* = 15), randomly: Sham-operation (SHAM), control (SIL), and ASA-treated (SIL/ASA) groups. In SHAM group after anesthesia left sciatic nerve was exposed through a gluteal muscle incision and after homeostasis the muscle was sutured. In SIL group the left sciatic nerve was exposed the same way and transected proximal to tibio-peroneal bifurcation leaving a 10-mm gap. Proximal and distal stumps were each inserted into a silicone tube and filled with 10 µl phosphate buffered solution. In SIL/ASA group defect was bridged using a silicone tube filled with 10 µl acetyl salisylic acid (0.1 mg/ml). Each group was subdivided into three subgroups of five animals each and were studied 4, 8, and 12 weeks after surgery. Data were analyzed statistically by factorial analysis of variance (ANOVA) and the Bonferroni test for pair-wise comparisons. Functional study confirmed faster and better recovery of regenerated axons in SIL/ASA than in SIL group ($p < 0.05$). Gastrocnemius muscle mass in SIL/ASA was significantly more than in SIL group. Morphometric indices of regenerated fibers showed that the number and diameter of the myelinated fibers in SIL/ASA were significantly higher than in control group. In immuohistochemistry, location of reactions to S-100 in SIL/ASA was clearly more positive than in SIL group. Response to local treatment of ASA demonstrates that it influences and improves functional recovery of peripheral nerve regeneration.

Key words: peripheral nerve repair, local, acetyl salicylic acid, silicone tube

Introduction

Reconstructive surgical procedures are required following traumatic or iatrogenic damage to peripheral nerves or after excision of primitive neoplasms. Experimental studies and clinical reports indicate that insertion of a conduit could be an interesting alternative to direct end-to-end suturing of nerve stumps or interposition of an autograft.[18,20] A widely accepted method by most surgeons is bridging the defect with an autologous donor nerve. Different graft equivalents have also been applied to bridge the nerve stump and regulated through the interaction of a variety of protein and cell signals.[7]

It has been reported that using silicone tubes in bridging of nerve defects could be promising because it is inert and does not induce extensive scarring or degeneration after implantation.[5] The advantages like no donor morbidity, availability, affordability, and no foreign reactions make silicone rubber chamber an attractive alternative to other standard grafts.[14] It has been demonstrated that silicone rubber tubes are well tolerated in humans even after 3 years of implantation.[24] Silicone chambers are used as a standard experimental model to study the nerve regeneration process.[2]

The exact physiological and molecular signals involved in inducing the regenerative process are largely unknown. Induction of transcription factors, adhesion molecules, growth-associated proteins, and structural components are required for axonal elongation and intracellular signaling molecules that control cell cycle and differentiation. These seem to play a key role in nerve regeneration process.[15]

Received September 22, 2012; Accepted November 26, 2012

The inflammatory process and its mediators have been implicated in the regulation of both the axonal degenerative and regenerative processes after injury.[12,16,19] Cyclins and the cyclin-dependent kinases (CDKs) play a crucial role in regulating the cell cycle progression in all eukaryotic organisms.[21] CDK-5 is a member of this CDK family of serine/threonine kinases. CDK-5 along with its activators, p35 and p39, is predominantly expressed in post-mitotic neurons.[11] It seems to be involved in active reorganization of the actin cytoskeleton during neurite outgrowth.[13] Enhanced CDK-5 activity and expression of p35 are associated with differentiation of cultured neuronal cells as well as accelerated neurite outgrowth.[22] A high expression of CDK-5 and p35 in regenerating nerves is reported to result in reduction in CDK-5 activity and retardation of nerve regeneration due to inhibition of CDK-5 activity, through CDK-5 inhibitors roscovitine and olomoucine.[17] Nonsteroidal anti-inflammatory agent acetyl salicylic acid (ASA), in addition to its well-known inhibitory action on cyclooxygenases, affects several cellular signaling pathways involved in regulation of cellular proliferation and differentiation.[26] It has been identified that ASA induces p35 synthesis and activates CDK-5.[15] ASA has shown a neuroprotective effect in an *in vitro* model of neuronal ischemia reperfusion injury.[26] Its effect on peripheral nerve injuries is reported to be accelerative in a nerve crush model in mice.[23]

Gastrointestinal (GI) effects ranging from relatively mild dyspepsia to potentially lethal GI bleeding and perforated ulcers of chronic administration of NSAIDs are well-known adverse effects of these compounds.[3] In order to avoid systemic adverse effect of NSAIDs in the process of nerve repair, the present study was conducted to evaluate possible local effect of ASA on peripheral nerve regeneration in rat sciatic nerve transection model. Assessment of the nerve regeneration was based on functional (Walking Track Analysis), muscle mass measurement, histomorphometric, and immuohistochemical (Schwann cell detection by S-100 expression) criteria 4, 8, and 12 weeks after surgery.

Materials and Methods

I. Animals and study design

Forty-five male White Wistar rats (*Rattus norvegicus*) weighing approximately 290 g and 3 months age were divided into three experimental groups (n = 15), randomly: Sham-operation (SHAM), control (SIL), and ASA-treated (SIL/ASA) group. Each group was further subdivided into three subgroups of five animals each. A random number generator was used to create a list of random numbers. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages (50 × 40 × 20 cm) with an ambient temperature of 23 ± 3° C, stable air humidity, and a natural day/night cycle. The animals were handled on a regular daily basis for 2 weeks prior to the study in order to acclimatize them with testing area and experiments. This could minimize anxiety-related testing inaccuracies.[25] The rats had free access to standard rodent laboratory food and tap water.

II. Grafting procedure

Animals were anesthetized by intraperitoneal administration of ketamine 5%, 90 mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5 mg/kg (Rompun 2%; Bayer, Leverkusen, Germany). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain.[27] The University Research Council approved all experiments.

Following surgical preparation in SHAM group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with Vicryl (Ethicon, Norderstedt, Germany) 4/0 sutures and the skin with 3/0 nylon (Dafilon, B/Braun Melsungen AG, Melsungen, Germany). The rats were observed on a heating pad during recovery. In SIL group, the left sciatic nerve was exposed the same way, transected proximal to the tibio-peroneal bifurcation where a 7-mm segment was excised, leaving a gap about 10 mm due to retraction of the nerve ends. The proximal and distal stumps were each inserted 2 mm into the conduit and two 10/0 nylon sutures were placed at each end of the cuff to fix the tube in place and leave a 10-mm gap between the stumps. The inner and outer diameters of the silicone tube were 2 mm and 6 mm. The silicone tube was filled with 10 μl phosphate buffered solution. In treatment group (SIL/ASA) the defect was bridged using a silicone tube filled with 10 μl ASA (0.1 mg/ml). All surgical procedures were carried out by the same surgeon, using a sterile microsurgical technique. After surgery, animals were housed in groups of five per cage under the same conditions mentioned above. No drugs were administered during postoperative period.

The animals of each group were anesthetized by intraperitoneal administration of ketamine-xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) 4, 8, and 12 weeks after surgery.

III. Functional assessment of nerve regeneration

Walking track analysis was performed 4, 8, and 12 weeks after surgery based on Bain et al.[1] 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) in each animal was calculated by the following formula:

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

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

IV. Muscle mass measurement

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after killing the animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. All measurements were made by two independent observers unaware of the analyzed group.

V. Histological preparation and quantitative morphometric studies

Graft middle cables of SHAM, SIL, and SIL/ASA groups were harvested and fixed in 2.5% glutaraldehyde. The nerves were post-fixed in OsO_4 (2%, 2h), dehydrated through an ethanol series and embedded in Epon. Semi-thin transverse (5 µm) sections, prepared from mid-point of the silicone tube, were next stained with toluidine blue and examined under light microscopy. Morphometrical analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, Maryland, USA). Equal opportunity, systematic random sampling, and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related, and fiber-size related biases.[10]

VI. Immunohistochemical analysis

For immunohistochemical studies, anti-S-100 (1:200, DAKO, Carpinteria, California, USA) was used. Anti-S-100 is a marker for myelin sheath. Specimens were post-fixed with 4% paraformaldehyde for 2h and embedded in paraffin. Before immunohistochemistry, the nerve sections were dewaxed and rehydrated in phosphate buffered saline (PBS) (pH = 7.4). The nerve sections were then incubated with 0.6% hydrogen peroxide for 30 min. To block nonspecific immunoreactions the sections were incubated with normal swine serum (1:50, DAKO). Then sections were incubated in S-100 protein antibody solution for 1h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labeled secondary antibody was applied for 1h. After that all sections were incubated with 3,3´-diaminobenzidine tetrahydrochloride chromogene substrate solution (DAKO) for 10 min. The results of immunohistochemistry were examined under a light microscope.

VII. Statistical analysis

Experimental results were expressed as means ± standard deviation (SD). Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, Illinois, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial analysis of variance (ANOVA) with two between-subject factors. Dunnett's test for pairwise comparisons was used to examine the effect of time and treatments. The data were presented as mean ± SD and differences were considered significant when $p < 0.05$.

Results

I. Recovery of sciatic nerve function

Figure 1 shows SFI values in experimental groups. Prior to surgery, SFI values in all groups were near zero. After the nerve axotomy, the mean SFI

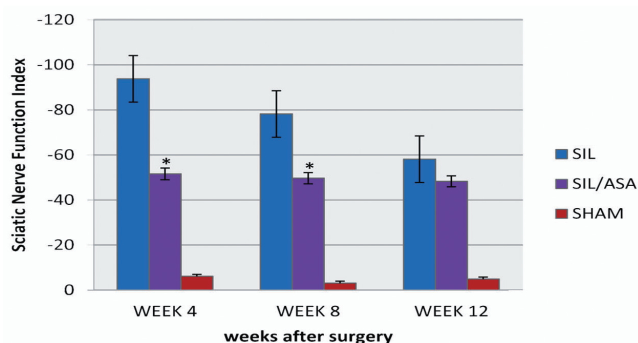

Fig. 1 Diagrammatic representation of effects on the sciatic nerve function index (SFI). Local application of acetyl salicylic acid (ASA) gave better results in functional recovery of the sciatic nerve than in control (SIL) group. Data are presented as mean ± standard deviation. *p < 0.05 versus SIL group.

decreased to −100 due to the complete loss of sciatic nerve function in all animals. This did not occur in animals of SHAM group. Because no deleterious effects were inflicted on their sciatic nerves and the nerves were only manipulated. Four weeks after surgery the mean SFI was −51. 64 ± −3.45 in SIL/ASA group, compared to −93.82 ± −3.24 in SIL group. Eight weeks after surgery an improvement in SFI was observed in animals of SIL/ASA group, −49.72 ± −3.69, that was significantly higher than SIL, −78.2 ± −4.37, animals ($p < 0.05$). After 12 weeks, animals of group SIL/ASA achieved a mean value for SFI of −48.35 ± −2.59, whereas in SIL group a mean value of −57.1 ± 4.10 was found. Statistical analyses revealed that the recovery of nerve function was significantly faster in SIL/ASA than SIL ($p < 0.05$) and, ASA locally promoted functional recovery.

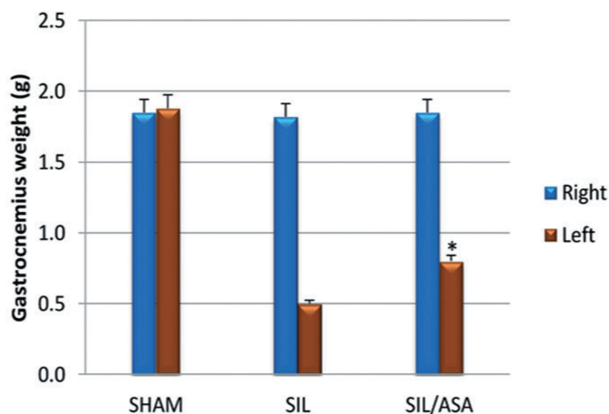

Fig. 2 Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean ± standard deviation. *p < 0.05 versus control (SIL) group.

II. Muscle mass measurement

The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of SIL/ASA and SIL groups ($p < 0.05$). The results showed that in SIL/ASA group muscle weight ratio was bigger than SIL group and weight loss of the gastrocnemius muscle was ameliorated by local administration of ASA (Fig. 2).

III. Histological and morphometric findings

Table 1 shows quantitative morphometric analyses of regenerated nerves for each of the experimental groups. Statistical analysis by means of a one-way ANOVA test showed that 4 weeks after surgery, SIL/ASA group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness compared to SIL animals ($p < 0.05$). Although animals in SIL/ASA group presented earlier regeneration patterns, the morphometric indices after 12 weeks were not significantly different between SIL/ASA and SIL groups ($p > 0.05$). Using factorial ANOVA analysis with two between-subject factors (Group × Time), in SIL/ASA group axon diameters did not show significant difference between 8 and 12 weeks ($p > 0.05$). Increase in mean thickness of myelin sheath did not show statistical difference between 8 and 12 weeks inside SIL/ASA and SIL groups ($p > 0.05$) (Figs. 3 and 4).

IV. 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. 5). In both groups, the

Table 1 Morphometric analyses of regenerative nerves for each of the experimental groups: values are given as mean ± standard deviation

	SHAM			SIL			SIL/ASA		
Weeks	4	8	12	4	8	12	4	8	12
N	8124 ± 385	8379 ± 446	8028 ± 404	1654 ± 301*	3026 ± 285*	3674 ± 272	3508 ± 251	3619 ± 238	3919 ± 277
D	12.01 ± 0.01	11.93 ± 0.17	12.06 ± 0.23	3.57 ± 0.83*	8.03 ± 0.21*	8.45 ± 0.72*	8.97 ± 0.71	9.67 ± 0.42	9.97 ± 0.68
d	7.03 ± 0.02	6.97 ± 0.39	7.06 ± 0.46	2.68 ± 0.53*	4.21 ± 0.38*	4.78 ± 0.34*	5.36 ± 0.19	5.41 ± 0.45	5.78± 0.48
T	2.56 ± 0.01	2.48 ± 0.02	2.53 ± 0.01	0.52 ± 0.07*	2.06 ± 0.41	2.21 ± 0.24	2.07 ± 0.18	2.21 ± 0.23	2.40 ± 0.32
G-ratio	0.58 ± 0.02	0.57 ± 0.02	0.59 ± 0.03	0.54 ± 0.03*	0.52 ± 0.02	0.57 ± 0.03	0.53 ± 0.12	0.55 ± 0.18	0.56 ± 0.14

*Results were significantly different from those of celecoxib-treated animals ($p < 0.05$). ASA-treated group, d: diameter of axon (μm), D: diameter of fibers (μm), N: number of fibers, SHAM: Sham-operation group, SIL: control group, SIL/ASA: T: thickness of myelin sheath (μm).

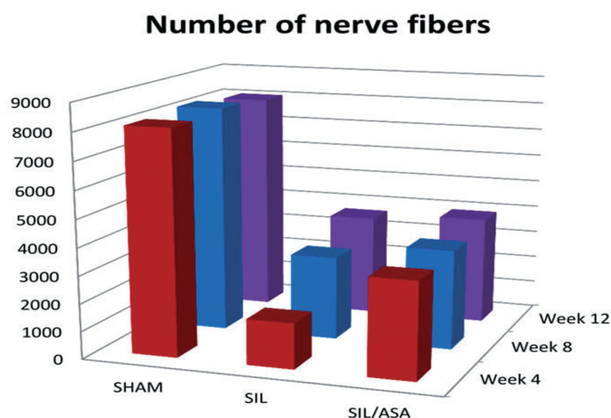

Fig. 3 The graph shows the quantitative results of fiber counting. The mean number of nerve fibers in Sham-operation (SHAM) group was nearly 8177 ± 411 (mean ± standard deviation). Both groups of control (SIL) and ASA-treated (SIL/ASA) showed the lower number of fibers than the SHAM group even at the end of the study.

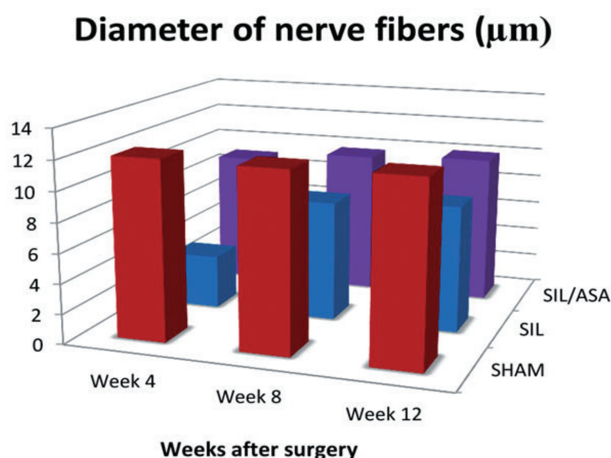

Fig. 4 The graph shows the quantitative results of mean diameter of nerve fibers. The mean diameter of nerve fibers in Sham-operation (SHAM) group was nearly 11.6 ± 0.13 (mean ± standard deviation). Both groups of control (SIL) and ASA-treated (SIL/ASA) showed the lower mean diameter of nerve fibers than the SHAM group even at the end of the study.

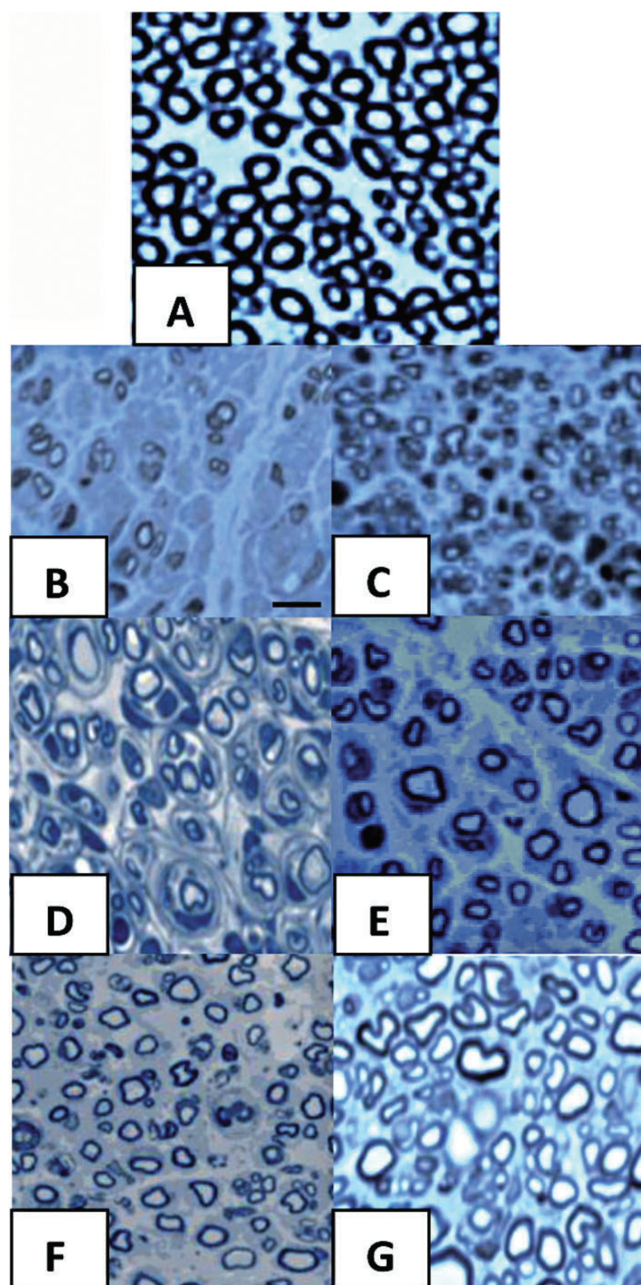

Fig. 5 Light micrograph of representative cross-section taken from (A) midpoint of normal sciatic nerve (Sham-operation, SHAM), (B) middle point of control (SIL) and (C) ASA-treated (SIL/ASA) group 4 weeks after surgery. D: Middle point of SIL and (E) SIL/ASA group 8 weeks after surgery. F: Middle cable of inside-out vein graft (IOVG) and (G) SIL/ASA group 12 weeks after surgery. Toluidine blue staining. Scale bar: 10 μm.

expression of S-100 and the findings resembled those of the histological evaluations.

Discussion

Development of agents and methods of repair that speed axonal regeneration remains a concern because of severity of sciatic nerve injuries and subsequent disabilities. Entubulation neurorrhaphy is an excellent alternative to short interposition nerve grafts.[6] In the present study we used silicone as a scaffold for keeping the delivered drug *in situ*. Selection of an appropriate method to

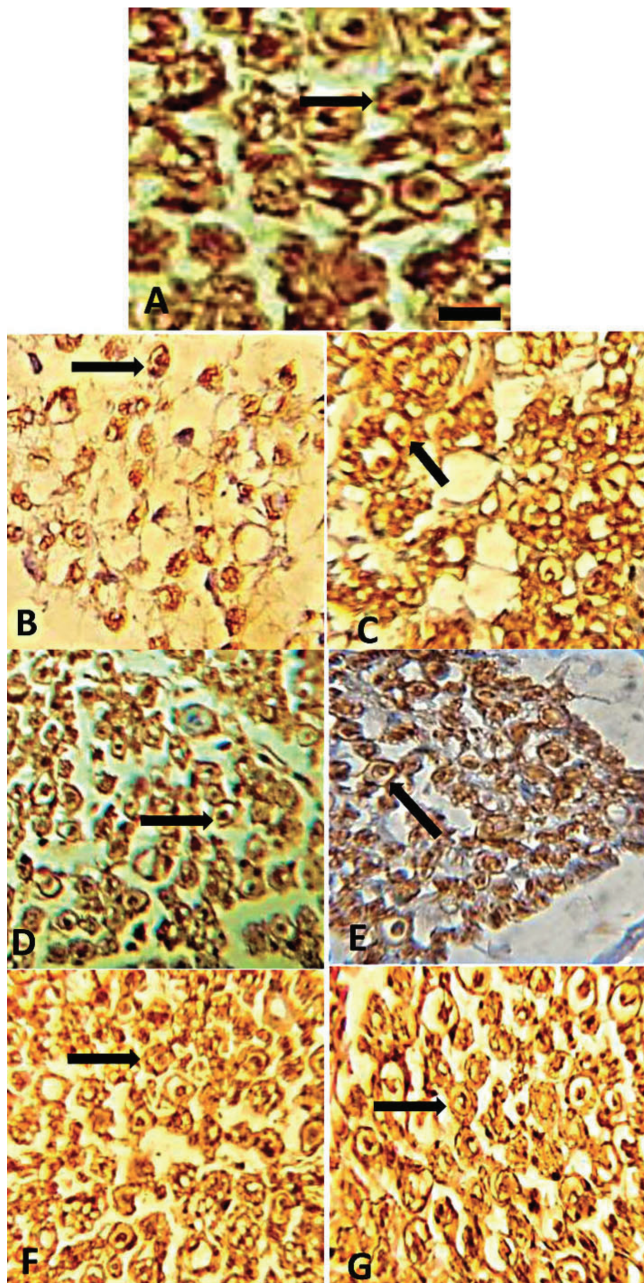

Fig. 6 Immunohistochemical analysis of the regenerated nerves. Representative cross-section taken from (A) midpoint of normal sciatic nerve (Sham-operation, SHAM), (B) middle point of control (SIL) and (C) ASA-treated (SIL/ASA) group 4 weeks after surgery. D: Middle point of SIL and (E) SIL/ASA group 8 weeks after surgery. F: Middle cable of IOVG and (G) SIL/ASA group 12 weeks after surgery. 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 ASA-treated nerve compared to that of the SIL 4 and 8 weeks after surgery. Scale bar: 10 μm.

evaluate functional recovery of nerve regeneration is extremely influential. Walking is a coordinated activity involving sensory input, motor response, and cortical integration.[4] Therefore, walking track analysis (SFI) is a comprehensive test. The results of the present study showed that ASA when loaded in a silicone tube ended up a faster functional recovery of the sciatic nerve.

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.[8,13] In the present study, 12 weeks after surgery the muscle mass was found in both experimental groups. However, SIL/ASA group showed significantly greater ratios of the mean gastrocnemius muscle weight than SIL group indicating indirect evidence of successful end organ reinnervation.

In the histological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant difference between SIL/ASA and SIL groups indicating beneficial effect of topical ASA on the nerve regeneration. However, there was no significant difference in functional and morphometric indices in animals of SIL/ASA group within the study period. Regarding better functional and morphometric indices in group SIL/ASA versus group SIL at week 4, it could be stated that local administration of ASA only accelerated the process of nerve regeneration and its topical application was time saving.

In immunohistochemistry the expression of axon and myelin sheath special proteins was evident in both groups which indicated the normal histological structure. The location of reactions to S-100 in SIL/ASA group was clearly more positive than SIL group further implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of regenerated nerve fibers.

ASA is widely used as analgesic, antipyretic, and anti-inflammatory drug and exerts these effects through inhibition of cyclooxygenases.[26] Novel cyclooxygenase-independent actions of ASA like inhibition of excitatory amino acid release, NF-kappa beta (Nfkb) translocation to the nucleus, and expression of inducible nitric acid synthase (iNOS) following cerebral ischemia are projecting ASA as a promising neuroprotective agent for treating stroke.[26] Our results show that ASA, at anti-inflammatory dose, significantly accelerates functional recovery following peripheral nerve transection. ASA at 50 mg/kg/day dose has shown marginally higher functional recovery; however, it was not significant in comparison to normal saline treatment.[23] Hence, the neuroprotective action of ASA following peripheral

nerve injuries appears to be dose-dependent. Even though our study shows the topical neuroprotective action of ASA in peripheral nerve injuries, data regarding the molecular mechanisms leading to the neuroprotective action are still lacking. Based on the previous reports describing the role of CDK-5 in nerve regeneration[17] and effect of ASA on CDK-5,[26] it may be assumed that ASA promotes nerve regeneration following peripheral nerve injury through activation of CDK-5. ASA also affects prostaglandin synthesis, iNOS expression, Nfkb translocation, and mitogen-activated protein (MAP) kinase pathway which can modulate nerve regeneration following peripheral nerve injury. Hence, understanding the molecular pathways leading to the neuroprotective action of ASA is necessary.[3]

One of the main problems in the usage of NSAIDs is the side effects. Adverse effects of chronic systemic administration of NSAIDs have been well dealt with in the literature.[9] The ability of ASA to accelerate nerve regeneration *in vivo* is reported to be promising in systemic administration in rat.[3] Thus, we decided to test if topical administration of ASA would result in comparable improvement in regeneration of sciatic nerve transection models through a tubulized 10-mm gap.

ASA accelerates axonal regrowth with systemic administration.[3] However, a topical formulation that provides the same neurotrophic stimulus would be ideal because of less systemic toxicity. Entubulation neurorrhaphy using ASA loaded silicone as an *in situ* delivery system of ASA in bridging the defects could be considered as an excellent alternative to short interposition nerve grafts. Using silicone tubes in bridging of nerve defects could be promising because it is inert and does not induce extensive scarring or degeneration after implantation.

In the present study the beneficial effect of the one-time administration of ASA after nerve injury is revealed. The one-time local application of ASA recovered the nerve function up to the half of preoperative function that could be because of anti-inflammatory effect of the drug on the preliminary phase of the nerve repair. Nowadays, slow and continuous topical release of drugs utilizing new materials is possible. Hence, a comparison of one-time topical administration of ASA and continuous oral ASA intake remains to become investigated.

Conclusion

In conclusion, in the present study ASA applied topically at the time of sciatic nerve repair using silicone conduit neurorrhaphy demonstrated promising results in nerve regeneration. Thus, dose–response studies should be conducted for ASA to determine the combination of graft and the compound that achieve maximal efficacy in nerve transection models.

Acknowledgments

The authors like to thank Mr. Mohammad Hasan Matin, Department of Clinical Sciences, and Haji Jaafary, Urmia Pathobiology Center, for their expert technical help. They also express their special appreciations to Dr. Bahram Dalir-Naghadeh for his contribution in statistical analyses.

Conflicts of Interest Disclosure

We have no conflicts of interest to disclose.
No funding source is contributed to this research.

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