Effect of bleeding on nerve regeneration and epineural scar formation in rat sciatic nerves: an experimental study

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Objective: Epineural scar formation is one of the most significant negative factors affecting surgical repair after peripheral nerve injury. The scar tissue mechanically hinders axonal regeneration and causes adhesions between nerves and surrounding tissues. A hemostatic agent Ankaferd Blood Stopper (ABS; İmmun Gıda İlaç Kozmetik San. ve Tic. Ltd. Şti., Istanbul, Turkey) has not been previously used. Decreasing the postoperative bleeding and adhesions between nerve and surrounding tissues will prevent the formation of scar tissue, as well as corresponding compressive neuropathy and/or deceleration of axonal regeneration. The purpose of this experimental study was to investigate the effects of bleeding on nerve healing and scar tissue after repair of peripheral nerve injuries.

Methods: The right sciatic nerve of 30 Sprague-Dawley male rats (weighing 260–330 g) was cut 1.5 cm proximal to the trifurcation and repaired primarily with 8/0 sutures using epineural technique. The rats were then divided into 3 groups. Saline was applied in Group 1 (n=10), ABS in Group 2 (n=10), and heparin in Group 3 (n=10) for 5 minutes to the repair site and surrounding tissues. In each group, electrophysiological measurements were performed with electromyography (EMG) at postoperative week 12. Magnetic resonance diffusion tensor imaging was used at week 12. Macroscopical and histopathological evaluations were conducted after sacrificing the rats at week 24 with total excision of the repaired sciatic nerves and surrounding tissues.

Results: The ABS and saline groups showed better healing than the heparin group. The ABS and saline groups were better in the histopathologic evaluations, but there was no statistically significant difference between the 2 groups.

Conclusion: Statistically significant differences were not found between the 3 groups. Significant results may be obtained with larger studies.

Keywords: Ankaferd Blood Stopper; bleeding; EMG; epineural scar.
Peripheral nerve injuries are encountered in different forms, from sharp instrument injuries to long-standing compression neuropathies and iatrogenic injuries.[1,2] Ideal repair is primary end-to-end suturing of the cut nerve edges as soon as possible following injury.[1–5] However, reconstructive surgical procedures may also be required after traumatic losses or losses as a result of tumor excision.[6] Autogenic nerve grafting is the currently preferred procedure in such repairs.[7,8] Whether primarily or reconstructively performed, one of the most important factors affecting postoperative peripheral nerve regeneration and clinical outcome is uncontrollable scar formation surrounding the repair area.[9,10] Postoperatively formed epineural scar constitutes a mechanical barrier, complicating axons advancing distally to proper fascicles and leading to conduction block.[10,11] In the case of extraneural scar formation, the scar leads the nerve to adhere to the adjacent tissues, preventing normal longitudinal sliding movement of the nerve.[12–14]

When the nerve is stretched for an extended period of time, additional damage may occur in the repaired nerve.[2,4,15] Various pharmacological agents are used to reduce epineural and extraneural scar formation, including aprotinin, ADCON-T/N (a carbohydrate polymer gel), cis-hydroxyproline (cis-hipro), estrogen-progesterone, methylprednisolone-acetate, and antibody to transforming growth factor beta (TGF-β). The pharmacological agent used to decrease the scar formation in the peripheral nerve surgery should be inexpensive, readily available, with no toxic effect to the applied tissues in the applied dosage, and have a localized effect.[4,16–19] In this study, Ankaferd Blood Stopper (ABS; İmmun Gıda İlaç Kozmetik San. ve Tic. Ltd. Şti., Istanbul, Turkey), which is believed to possess these qualities, was utilized. Bleeding in the surgical area is decreased by ABS. Heparin, which increases bleeding, was used for comparison. In this study, the effect of application on the surgical area superficially for 5 minutes of physiological saline, ABS, and heparin on nerve regeneration and epineural-extraneural scar formation after immediate primary repair of the sciatic nerves of rats was examined.

**Materials and methods**

Thirty Sprague-Dawley type male rats weighing 260–330 g were studied. The right sciatic nerve and the tibial and peroneal components of each rat were separated by blunt dissection. The sciatic nerve was cleanly cut approximately 1.5 cm above the trifurcation and was repaired primarily by epineural technique with 8/0 atraumatic nylon suture material, placing 4 sutures at an angle of 90° to each other (Figure 2b).

Nerves were severed in all groups and underwent epineural repair.

The test solutions were applied to the repaired nerve and the surrounding tissue superficially for 5 minutes.
to the different groups as listed below, after which the entire operative field was washed with isotonic solution and the tissues were closed anatomically by 4/0 chromic catgut sutures.

Group 1: Physiological saline, 0.2 ml (Figure 3a).
Group 2: Heparin 0.002 ml, (Figures 3b, c).
Group 3: ABS, 0.2 ml (Figure 3d).

Before the evaluations were conducted at postoperative Week 12, 1 rat from the heparin group and 1 rat from the physiological saline group died. No new rats were added to the study. The study proceeded with 9 rats in the heparin group, 9 rats in the physiological saline group, and 10 rats in the ABS group.

In our study, nerve conduction speed was evaluated by EMG examination at Week 12. Operated right side and healthy control side measurements were performed on all rats with the same Keypoint EMG device (Medtronic, Copenhagen, Denmark) (Figure 4).

At the end of postoperative Week 24, the sciatic nerve of the sacrificed rats was removed en bloc, including the repair line and the surrounding tissue. After being fixed with formalin 10%, the removed tissue was given a sample tracking number, and paraffin blocks were prepared. Sections were collected 1 cm proximally and 1 cm distally, including the repair line, and dyed with hematoxylin and eosin (H&E). Myelin staining was performed to observe myelination with luxol fast blue (LFB), and Masson’s trichrome (MTC) staining was performed to identify the collagen tissue. In addition, the presence of macrophages in the histological sections was determined using monoclonal antibody to CD68 cell markers (Clone-KLMI, Monoclonal, Ready-to-use, Prediluted; Thermo Shandon Neomarkers, Fremont, CA, USA).

SPSS software (version 17.00, SPSS Inc., Chicago, IL, USA) was used for statistical analyses, which included mean and standard deviation, together with Kruskal Wallis H test and Wilcoxon signed-rank test for non-parametric tests, as appropriate. The results were presented with 95% confidence interval, with a significance level of 5%.
Results

At postoperative Week 12, distal latency and conduction speed of the 3 groups were assessed on both the right (experimental) and the left (control) sides by EMG.

In terms of distal latency, there was a statistically significant difference between the experimental (right) leg and control (left) leg values in all groups (p<0.05). The least differences between control and experimental values were found in the physiological saline group, and the greatest differences were found in the heparin group (Table 1).

In terms of conduction speed, there was a significant difference (p<0.05) between the experimental (right) leg and control (left) leg values in all groups. The least differences were found in the ABS group, and the greatest were found in the heparin group (Table 2).

In terms of cellular organization in the repair area after H&E staining of the histopathological sections, the least normal organization was observed in the heparin group, and the most normal organization was observed in the ABS group. However, no significant difference was found amongst the 3 groups. In the assessment of collagen tissue density performed following MTC staining of the histopathological sections, the highest increase in collagen quantity was observed in the heparin group. However, no significant difference was found amongst the 3 groups (p>0.05). In assessment of the myelin loss rates following LFB staining of the histopathological sections, the highest myelin loss was found in the heparin group. However, no significant difference was found amongst the 3 groups (p>0.05). CD68 staining showed the least macrophage activity was found in the physiological saline group, but no significant difference was found amongst the 3 groups (p>0.05).

Histopathological sections were obtained to assess intramuscular fibrosis, and MTC stainings were performed on the sections. While the least fibrosis was observed to be in the ABS group, no significant difference was found amongst the 3 groups.

Discussion

Peripheral nerve injuries have been studied by numerous researchers to date and were first divided into 3 groups by Seddon as neuropraxia, axonotmesis and neurotmesis in 1943. In our study, by cutting the rat sciatic nerves, a neurotmesis type of damage was created. The suture material used for repair and the surgical technique applied are extremely important in terms of minimizing the development of fibrosis and scar formation in the repair area, thus obtaining a more functional healing. In addition to different suture methods, non-suture...
methods utilizing various tissue adhesives have been applied in experimental and clinical studies.[20,21] However, since the material used for the epineural suture itself is claimed to stimulate scar formation[10] the nerve repair performed in this study by epineural technique used 8/0atraumatic sutures.

Following a peripheral nerve injury, the ideal repair technique is to stitch the transected nerve ends end-to-end primarily as soon as possible after occurrence of the injury. ‘Primary nerve repair’ is performed within hours after the injury, while ‘delayed primary repair’ is performed within the first 7 days, while nerve repair performed more than 1 week after the injury is ‘secondary repair.’ Primary repair of ulnar and medial nerve transections has been shown to yield better results.[22–25] Therefore, primary nerve repair was performed in the present study. In traumatic nerve lesions where the nerve fiber diameter and myelin sheath thickness changed, nerve conduction speed defect is an inevitable consequence. The conduction speed of a nerve fiber is related to the diameter of the axon, and thicker fibers ensure faster conduction compared to thinner fibers. In myelinated nerve fibers, the myelin sheath serves as an insulator, and a linear relationship exists with the conduction speed, with thicker myelin sheaths having faster conduction speeds.[10,26] After peripheral nerve surgery, information about regeneration can be obtained by measuring the nerve conduction speed. Following a nerve injury, solitary nerve fiber begins to develop towards the regeneration unit, comprised of numerous nerve fibers. If a favorable number of fibers reach appropriate sensory/motor targets in an appropriate time period, functional healing occurs. However, clinical and experimental nerve repair deemed successful does not always correspond to fully functional healing.[27,28] Even when a large number of fibers are damaged in a nerve, there still could be several fibers which transmit effectively. For this reason, the nerve conduction speed forms an estimate of the most rapid and possibly healthiest fibers but not the total nerve function.[27,29] Nerve conduction speed recorded in our study differed even in the same group, confirming that nerve conduction speed is not an indicator of total nerve function.

If the distance between the proximal and distal edges of a nerve is more than several millimeters or the gap between the edges is filled by fibrous tissue, the probability of healing is quite low.[26] The regenerating axon advances within the scar at an average of 0.25 mm per day. The scar formation between the proximal and distal edges of the nerve physically obstructs forward growth of the axon and gives rise to branching, diverging, regrowth, or termination of the nerve growth cone. Following severing of the nerve and coaptation developed under favorable conditions, only one-sixth or one-seventh of the budding axons are estimated to reach the distal stump and continue growing distally. Healing of the sutured traumatic peripheral nerve damage depends on the balance between Schwann cell regeneration and scar formation.[10,26,30–32]

The regeneration buds formed on the proximal ends of the cut axons stem from the most distal healthy Ranvier node.[30] Those which reach the terminal plate survive, while the others regress and are phagocytosed. It is crucial to which target organ the growing axons will reach. The regenerated axons might be normal in number, but once they reach an unfavorable target organ, an insufficient functional response is obtained.[33] The sensory endplates might survive for an extended time after denervation. Sunderland reported a satisfactory degree of re-excitation after 1 year of denervation.[32] Regarding motor endplates, in human biopsy studies, the presence of intermediate fibrosis and atrophy of the muscles after a 3-month period of denervation and severe fibrosis after 11 months have been demonstrated.[34] Overall, results of several studies of nerves indicate that unless the cut ends were brought together, the axons are unable to repopulate the target organ. The goal of peripheral surgery is to bring the proximal and the distal nerve ends together by an appropriate technique.

Cytokines such as transforming growth factor beta (TGFβ) and basic fibroblast growth factor (bFGF) have been established to have a significant role in collagen formation and Schwann cell activity by fibroblasts during the process of peripheral nerve regeneration.[19,35,36] In experimental studies, it has been reported that epineural fibroblasts were the cells responsible for collagen formation. Application of neutralizing antibodies for the growth factors in a traumatized nerve lesion prevents excessive collagen formation, thus permitting axonal regeneration. Increased scar formation and wound healing, depending on the increased collagen synthesis, were reported to be significantly accelerated as a consequence of systemic or topical TGF-B1 application.[37]

Pleasure et al.[17] deemed that collagen accumulation after experimental nerve sectioning was especially significant when it occurred in the distal end of the cut nerve. If scar formation can be inhibited, development of the axonal extensions and the regeneration process will be accelerated.[17] Siironen et al.[38] demonstrated immunohistochemically that fibroblasts account for the formation of type I collagen in nerve regeneration. Collagen formation and accumulation takes place in the damaged
tissue in response to a traumatic incident. Fibroblasts aggregate in the damaged area and produce collagen after a severe nerve trauma. After the injury, the connective tissue cells in the distal stump alter their appearance and function. Extensive variations occur in collagen types and fibronectin distribution in the endoneurium and perineurium. The scar tissue of the damaged nerve is composed mainly of type I and to a lesser extent type III collagen accumulation.

As previously reported, various histopathological changes occur during the regeneration process after peripheral nerve injury. In our study, we microscopically evaluated the regeneration and histopathological changes of the repaired nerves at the end of postoperative Week 24. From histopathological evaluation, we found the worst reconstitution in the repair area to be in the heparin group and the best reconstitution to be in the ABS group. The highest increase of collagen tissue density and the most myelin loss were observed in the heparin group. The least macrophage activity was seen in the physiological saline group, as evaluated by histopathological sections obtained from the repair area.

Although ABS has been used in numerous surgical processes, as well as experimental and clinical studies, it has not been used in peripheral nerve surgery to date.

The more severe the trauma, the more fibroblasts aggregate in the damage area, with, consequently, more scar tissue in the damaged area. Epineural fibrosis also increases in pathological conditions such as vascular tissue damage, hematoma, infection, and ischemia.

ABS is a natural product used as a hemostatic agent. It is an admixture of extracts of *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*, herbs which influence the endothelium, blood cells, angiogenesis, cell growth, and mediators. While ABS is used safely, the mechanism of action is not fully understood yet. It was first used as local hemostatic agent on superficial skin incisions and dental bleeding in Turkey. The effect occurs within seconds, and a protein coat of aggregating erythrocytes is formed. However, this coat does not affect coagulation factors and thrombocytes, and ultimately, the erythrocytes are used to create a plug. Anti-inflammatory and anti-neoplastic effects are also demonstrated in vitro. In a study conducted with rats, duration and volume of bleeding have been shown to decrease significantly with ABS in the amputated leg of the rats, whether Warfarin was administered before the amputation or not. It has also been reported to be effective in stopping bleeding due to solitary rectal ulcers. In a different experimental study, immunohistochemical and histopathological effects of ABS on aortic bleeding patterns and successful outcomes were demonstrated in a rat model. In a study by Ergenoglu et al., successful outcomes were reported when ABS was used to stop sternal bleeding in cardiac surgery.

In conclusion, we found that ABS use decreases bleeding, reduces epineural/extraneural scar formation, and affects nerve regeneration positively. Our findings suggest that epineural scar formation may be reduced by ABS use after peripheral surgery. While the outcomes were significant in each treatment vs non-treatment comparison in individual groups, there were no significant differences in group-to-group comparisons. Studies conducted with a larger number of subjects may be needed to demonstrate significant differences.

**Conflicts of Interest:** No conflicts declared.

**References**

