SUPPLEMENT

Murat Uygun, Assistant Professor¹
Osman Y. Yavuz, Associate Professor¹
İsmail Uraş, Associate Professor¹
İbrahim G. Duman, Assistant Professor²
Hacer Haltaş, Associate Professor³
Mahmut Kömürcü, Professor¹

¹Turgut Özal University Faculty of Medicine,
Department of Orthopaedics and
Traumatology
²Mustafa Kemal University Faculty of
Medicine
³Turgut Özal University Faculty of Medicine,
Department of Pathology

Effect of Ankaferd Blood Stopper on muscle healing

Abstract

Purpose: Ankaferd Blood Stopper (ABS), which is a standardized mixture of herbal extracts obtained from five plants, has been proven as an efficient hemostatic agent and is still used in emergency situations. It is not known exactly if decreased bleeding has positive or negative effects on muscle healing and fibrosis, so the purpose of this study was to test the effect of ABS on muscle healing and morphology.

Methods: A total of 66 outbred Wistar rats were divided into three control and three experimental subgroups. In the experimental groups, ABS was sprayed on the cut surface of the soleus. In the control groups, a saline solution was sprayed on the cut surface of the soleus. Subgroups were euthanized after 2 weeks, 3 weeks and 4 weeks, respectively. In each subgroup, eight rats were used for the biomechanical study to determine muscle healing and three rats were used for the histopathological investigation.

Results: Although muscle strength in the control groups was lower than that of the experimental groups in early weeks, no differences were found between the control and the experimental groups at 4 weeks.

Conclusions: ABS has no negative effect on muscle healing. We also observed that ABS accelerated muscle healing compared to the control group. ABS could be used in hemostasis of open fractures and elective orthopedic surgeries.

Clin Invest Med 2016; 39 (6): S187-S191.

Correspondence to:

Murat Uygun
Alparslan Türkeş Bulvarı No: 57 06510 Emek
Ankara/Turkey
E-mail: muratuygun71@gmail.com

InAnkaferd Blood Stopper (ABS, Trend Teknoloji Ilac AS, Istanbul, Turkey) is a standardized mixture of herbal extracts obtained from five plants (*Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum* and *Urtica dioica*) [1]. The basic mechanism of action of ABS is the formation of an encapsulated protein network that provides the grounds for the red blood cell aggregation [2]. Additionally, ABS affects the levels of various critical transcription factors active in the erythrocyte protein profile [3] and ABS also mediates endothelial cells [4]. ABS is currently approved for use in external and dental surgical bleeding [5]. The efficacy and safety of ABS as a hemostatic agent in dental surgery have been well documented, as well as its wound healing effectiveness [6]. ABS is also used as a hemostatic agent in acute intractable bleeding and surgery [7-10]. Hematoma is known to take an active role in wound healing [8], and preventing hematoma may have a negative effect on wound healing. Reduced hematoma may also decrease fibrosis. The aim of this study was to test the effect of ABS on muscle healing and morphology.

Materials and Methods

After the Animal Ethics Committee approved the study, the study was initiated. A total of 66 outbred Wistar rats (mean weight: 250-300 g; mean age: 2 months) were used. The rats were kept in laboratory animal cages with plastic bottoms and sides and a wire cage cover on top. The rats were fed pellet-type laboratory animal feed. The animals were divided into control and experimental groups. Each group was then divided into three subgroups (11 rats) according to the duration of the experiment for a total of six groups. One subgroup was euthanized after 2 weeks, one subgroup was euthanized after 3 weeks, and one subgroup was euthanized after 4 weeks.

Following sedation with xylazine (10 mg/kg s.c.) under intraperitoneal anesthesia (ketamine, 80 mg/kg), all animals were restrained in a supine position and shaved, and a 2 cm longitudinal incision was made on the cruris under sterile conditions. The soleus muscle was cut with a scalpel just distal to the center of the muscle. In the experimental groups, ABS was sprayed (1 ml to each cut surface) using a syringe on the actively bleeding cut surface of the soleus immediately after excision. In the control groups, a saline solution was sprayed (1 ml to each cut surface) using a syringe on the actively bleeding cut surface of the soleus immediately after excision. The wound was closed without primary repair. Paracetamol (50 mg/kg, oral) was given postoperatively for analgesia. Rats were followed for wound, feeding, behavior, activity and body weight.

The groups were euthanized 2, 3, and 4 weeks after the index operation. Under intraperitoneal anesthesia (ketamine, 80 mg/kg) all animals were euthanized with cervical dislocation. In each subgroup, eight rats were used for the biomechanical study and three rats were used for the histopathological investigation.

Muscle healing was determined by measuring the resistance of the muscle to stretching. After the specimens were prepared, they were placed in a ZWICK/Z100 tensile testing machine (Zwick Roell AG, Ulm, Germany). All authors participated in the biomechanical testing of the specimens. After being informed about the study, the staff of the laboratory provided help during the testing and recording of the results.

A histopathological examination of three samples in each group was performed. Sample tissues were fixed in 10% neutral buffered formalin, dehydrated with graded ethanol, and embedded in paraffin. Tissue sections 5 µm thick were stained with hematoxylin and eosin (H&E). Evaluation and scoring were performed with a light microscope.

Statistical analysis was performed with SPSS 21 (SPSS Inc., Chicago, IL). The Mann-Whitney U test was used to compare the biomechanical test results for the groups. We also compared histopathological findings by using a chi-square test. The level of statistical significance was set at $p < 0.05$.

Results

Muscle healing was determined by measuring the resistance of the muscle to stretching. The mean peak force was 11.12 ± 5.69 N (5-20 N) in group I (the 2-week experimental group), 12.37 ± 5.87 N (5-21 N) in group II (the 3-week experimental group), and 13.5 ± 3.42 N (9-18 N) in group III (the 4-week experimental group). The mean peak force was 7.75 ± 2.91 N (3-13 N) in group IV (the 2-week control group), 7.75 ± 1.9 N (4-10 N) in group V (the 3-week control group), and 12.25 ± 3.61 N (6-16 N) in group VI (the 4-week control group). At the end of 4 weeks, we did not find any difference between the control group (VI) and the experimental group (III) in terms of muscle strength. Muscle strength in the 2- and 3-week experimental groups (I and II) was not statistically different from that of the 4-week experimental group (III). Muscle strength in the 2- and 3-week control groups (IV and V) was lower than that of the 2- and 3-week experimental groups (I and II) and the 4-week control group (VI) (Figure 1).

The histopathological examination did not show differences between the control and experimental groups in terms of necrosis; however, we observed that in 3- and 4-week

FIGURE 1. Demonstration of peak force of subgroups in boxplot form.

experimental groups, there was more fibrosis and inflammation (Figure 2).

Discussion

The basic mechanism of action has been found previously to be the formation of an encapsulated protein network that provides ground for the red blood cell aggregation [2]. Crucial proteomic components of the ABS-induced erythroid-protein network have been revealed by Demiralp *et al.* [2]. Essential erythroid proteins (spectrin alpha, actin-depolymerization factor, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, mitochondrial NADP [+] dependent malic enzyme) and the required adenosine triphosphate (ATP) bioenergy are included in the protein library of ABS. ABS also up-regulates the GATA/FOG transcription system affecting erythroid functions [2,3]. Additionally, ABS affects the levels of various critical transcription factors active in the erythrocyte protein profile [3]. ABS also mediates endothelial cells [4]. ABS has dual diverse dynamic reversible actions on EPCR and PAI-1 inside the vascular endothelial cells [4]. Based on these findings, sudden anti-hemorrhagic efficacy of ABS has been

FIGURE 2. Light micrographs (H&EX100) of incisional wound bed. Mild edema and inflammation, marked fibrosis between muscle fibers (control group, 2nd week) (a). Moderate edema and inflammation, mild fibrosis in muscle tissue (Ankaferd, 2nd week) (b). Mild inflammation and fibrosis in muscle tissue (control group, 3rd week) (c). Intense edema and inflammation, fibrosis (Ankaferd, 3rd week) (d). Mild edema and inflammation and marked fibrosis in muscle tissue (control group, 4th week) (e). Intense edema and inflammation, fibrosis (Ankaferd, 4th week) (f).

attributed to its immediate enhancement of expression of pro-hemostatic PAI-1 and down-regulated anti-coagulant EPCR, resulting in the unique hemostatic effects of ABS [4].

The effectiveness of ABS has been proven as an efficient hemostatic agent and is still used in emergency situations. Topical application of ABS in an experimental major arterial vessel injury model reduced bleeding time and blood loss [8]. Also ABS seems to be an effective hemostatic agent for patients undergoing total thyroidectomy [9] and adenoidectomy [10].

The clinical appearance of muscle injury depends on the hematoma that develops [11]. After muscle injury, the early granulation tissue and biomechanical strength of the injured area are provided by the hematoma. In addition, the first signals in the injured area arise from the hematoma [12]. It is not known exactly if decreased bleeding has positive or negative effects on muscle healing.

Open fractures, which are often seen in orthopedic emergencies, are frequently accompanied by muscle injury. If it is shown that ABS has no adverse effects on muscle healing, the agent could be used in hemostasis of open fractures. ABS could also be used in elective orthopedic surgeries to prevent side effects of tourniquet use, and better visibility during surgical dissection could be achieved.

We observed that ABS had no adverse effect on muscle healing since the muscle strength expressed with the peak muscle force did not differ between the experimental group and the control group at the end of the 4th week. We attribute this situation to the fact that the inflammatory reaction was amplified with various substances released by satellite cells and necrotized parts of the myofibers [12]. In addition, granulation tissue may also be derived from the fibroblasts that originate in the myogenic cells [12]. We also observed that muscle strength in the experimental group peaked earlier than in the control group. We think that this situation is due to the short destruction phase [11] since hematoma was decreased.

Conclusion

According to the findings, there were more inflammation and fibrosis in the experimental group. We attribute this finding to the body's reaction to ABS. We observed that muscle healing in the experimental group was as sufficient as in the control group. We also observed that ABS accelerated muscle healing compared to the control group. These observations indicate that ABS has no negative effect on muscle healing.

Acknowledgements

Kobay DHL A.Ş. Animal Ethics Committee, approved the study in March 18th 2013.

Surgical procedures and animal care were performed in Kobay DHL A.Ş. Ankara/Turkey, biomechanical tests were performed in mechanical laboratory of Tosyalı Holding Hatay/Turkey and histological examinations were performed in Turgut Özal University Ankara/Turkey.

Financial Support

We received financial support for this study from Project Office of Turgut Özal University. Each author certifies that he or she has no commercial associations (eg, consultancies, stock ownership, equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article.

References

1. Goker H, Haznedaroglu IC, Ercetin S, Kirazli S, Akman U, Ozturk Y, Firat HC. Haemostatic actions of the folkloric medicinal plant extract Ankaferd Blood Stopper. *J Int Med Res.* 2008;36(1):163-170.
2. Demiralp DO, Haznedaroglu IC, Akar N. Functional proteomic analysis of Ankaferd Blood Stopper. *Turkish Journal of Hematology* 2010;27:70-77.
3. Karabiyik A, Yilmaz, E., Gulec, S., Haznedaroglu, I.C., Akar, N. Dual diverse dynamic reversible actions of Ankaferd on EPCR and PAI-1 inside vascular endothelial cells with and without LPS. *Turk J Hematol* 2011. DOI: 10.5152/tjh.2011.41.
4. Yilmaz E, Gulec S, Torun D, Haznedaroglu IC, Akar N. The effects of of Ankaferd Blood Stopper on transcription factors in HUVEC and erythrocyte protein profile. *Turk J Hematol* 2011. DOI:10.5152/tjh.2011.39.
5. Sevil S, Haznedaroglu IC, Kurt M, Onal IK, Aktas A, Kurt OK, Goker H, Ozdemir O, Kirazli S, Firat HC. Safety and efficacy of Ankaferd Blood Stopper in dental surgery. *Int J Hematol Oncol.* 2010;20(1):1-5.
6. Baykul T, Alanoglu EG, Kocer G. Use of Ankaferd Blood Stopper as a hemostatic agent: a clinical experience. *J Contemp Dent Pract.* 2010;11(1):E088-94.
7. Beyazit Y, Kurt M, Kekilli M, Goker H, Haznedaroglu IC. Evaluation of hemostatic effects of Ankaferd as an alternative medicine. *Altern Med Rev.* 2010;15(4):329-336.
8. Ulus TA, Turan NN, Özyalçın S, Aydoğ G, Ulus F, Göker H, Haznedaroglu IC. Surgical and histopathological

- effects of topical ankaferd hemostat on major arterial vessel injury in relation to the enhanced intra-arterial blood pressure. *Turk J Hematol* 2011;28:206-212.
- Borrione P, Gianfrancesco AD, Pereira MT, Pigozzi F. Platelet-rich plasma in muscle healing. *Am J Phys Med Rehabil*. 2010;89(10):854-861.
9. Guler M, Maralcan G, Kul S, Baskonus I, Yilmaz M. The efficacy of Ankaferd Blood Stopper for the management of bleeding following total thyroidectomy. *J Invest Surg*. 2011;24(5):205-10. doi: 10.3109/08941939.2011.586092.
 10. Iynen I, Bozkus F, San I, Alatas N. The hemostatic efficacy of Ankaferd Blood Stopper in adenoidectomy. *Int J Pediatr Otorhinolaryngol*. 2011 Oct;75(10):1292-5. doi: 10.1016/j.ijporl.2011.07.014. Epub 2011 Aug 2.
 11. Järvinen TA, Järvinen M, Kalimo H. Regeneration of injured skeletal muscle after the injury. *Muscles Ligaments Tendons J*. 2014;3(4):337-345.
 12. Järvinen TA1, Järvinen TL, Kääriäinen M, Kalimo H, Järvinen M. Muscle injuries: biology and treatment. *Am J Sports Med*. 2005;33(5):745-764.