RESEARCH ARTICLE

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The Effects of Local Application of Ankaferd Blood Stopper on Bone Regeneration of Defects in Rat Tibias

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Objective: Ankaferd Blood Stopper (ABS) is a historical hemostatic material that is a mixture of five medicinal plant extracts. The local application of ABS is believed to increase bone healing due to its anti-inflammatory and antioxidant capacity. In this experimental study of rats, the effects of local ABS on bone healing in a tibia defect model were examined histopathologically.

Materials and Methods: Sixty female Sprague-Dawley rats were randomly divided into two groups of 30 animals each. One cylindrical bone defect with a diameter of 4 mm and a depth of 4 mm was created in the right tibia of each rat under general anesthesia. As much ABS as the defect could take was applied locally in the rats in the experimental group. No additional procedure was applied in the controls. At 2, 4, and 8 weeks postoperative, the rats were sacrificed with carbon dioxide ventilation, and bone samples were taken from their right tibias. The samples were evaluated histopathologically for fibrosis and new bone formation.

Results: The results showed that ABS application did not increase the regeneration of bone tissue in the defect areas during the bone healing period, although this was not statistically significant ($p>0.05$). No statistically significant difference was observed between the control and ABS groups in terms of fibrosis ($p>0.05$).

Conclusion: This herbal product may not have positive effects on bone tissue healing when used locally. Further studies are needed to evaluate the possible benefits and side effects of ABS on bone tissue.

Key Words: *Ankaferd blood stopper, local application, herbal medicines, bone healing, bone regeneration*

Ankaferd Kanama Durdurucu'nun Lokal Uygulanmasının Sıçan Tibiya Kemik Defektlerinde Kemik Rejenerasyonuna Etkisi

Amaç: Ankaferd Blood Stopper (ABS), beş şifalı bitki ekstraktının karışımından oluşan tarihi bir hemostatik malzemedir. ABS'nin lokal uygulamasının, antiinflamatuar ve antioksidan kapasitesinden dolayı kemik iyileşmesini arttırdığına inanılmaktadır. Sıçan üzerinde yapılan bu deneysel çalışmada, tibia kemiği defekti modelinde lokal ABS'nin kemik iyileşmesi üzerindeki etkileri histopatolojik olarak incelenmiştir.

Gereç ve Yöntem: Altmış dişi Sprague-Dawley sıçan rastgele her birinde 30 hayvan bulunan iki gruba ayrıldı. Genel anestezi altında her sıçanın sağ tibiasında 4 mm çapında ve 4 mm derinliğinde silindir şeklinde kemik defekti oluşturuldu. Deney grubundaki sıçanlara defektin alabileceği kadar ABS lokal olarak uygulandı. Kontrollerde ek bir işlem uygulanmadı. Postoperatif 2., 4. ve 8. haftalarda sıçanlar karbondioksit ventilasyonu ile sakrifiye edildi ve sağ tibiya kemikleri alındı. Tibiya kemikleri fibrozis ve yeni kemik oluşumu açısından histopatolojik olarak değerlendirildi.

Bulgular: ABS uygulamasının kemik iyileşmesi döneminde defekt alanlarındaki kemik dokusunun yenilenmesini istatistiksel olarak anlamlı olacak şekilde artırmadığını gösterdi ($p>0.05$). Kontrol ve ABS grupları arasında fibrozis açısından istatistiksel olarak anlamlı bir fark gözlenmedi ($p>0.05$).

Sonuç: Bu bitkisel ürün lokal olarak kullanıldığında kemik dokusu iyileşmesi üzerinde olumlu etkiler göstermeyebilir. ABS'nin kemik dokusu üzerindeki olası fayda ve yan etkilerini değerlendirmek için daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler*:* *Ankaferd kanama durudurucu, lokal uygulama, bitkisel ilaçlar, kemik iyileşmesi, kemik rejenerasyonu*

Introduction

Oral and maxillofacial bone defects resulting from conditions such as congenital bone disorders, trauma, tumors, and bone loss due to advanced periodontitis can be treated with reconstructive surgery. Small bone defects may heal on their own, while various materials may be required to treat large bone defects (1). The aim of bone defect treatment in oral and maxillofacial surgery is to repair and regenerate bone tissue (2).

Ankaferd Blood Stopper® (ABS) (Immune Cosmetics Pharmaceutical Co. Ltd., Istanbul, Turkey) is a type of herbal medicine produced from a mixture of herbal extracts, such as *Urtica dioica*, *Thymus vulgaris*, *Vitis vinifera*, *Alpinia officinarum*, and *Glycyrrhiza glabra* (3,4). ABS's ability to form a clot in less than a second and stabilize

physiologically collected erythrocytes is its biggest advantage over other hemostatic agents (5). According to mass spectroscopy analysis, ABS contains many antioxidant compounds, including tryptophan, lycopene, thymol, enoxolone, tertiary butylhydroquinone, tomatine, vitamin E derivatives, tocotrienol, and galangin (6). ABS activates a network of proteins by collecting protein molecules, especially fibrinogen, in bleeding areas. The cells involved in the formation of the protein network are erythrocytes and platelets. Studies on ABS have reported that this material does not have an inhibitor effect on any coagulation factors during the formation of the hemostatic plug and that this non-inhibitor effect occurs as a result of a purely physiological process (7-9). Additionally, ABS has been reported to have anti-inflammatory, antioxidant, antimicrobial, and antineoplastic effects, to help the bone-healing process, and to accelerate wound healing (8-18). In addition to its hemostatic effect, ABS has been reported to accelerate early bone healing and formation. The anti-inflammatory and antioxidative effects of ABS are also believed to contribute to its regenerative properties in bone tissue (3,9). İşler et al. reported in a study that more new bone formation occurred and less necrosis was observed in defects treated with ABS. İşler et al. suggested that the higher bone formation in ABS-applied subjects compared to controls may be related to the increased healing rate and decreased inflammation associated with the antioxidant activity of ABS components (10).

The aim of this study was to histopathologically evaluate whether local ABS application to the defect area during surgery has any effect on bone defect regeneration in experimental bone defects created in rat tibias.

Materials and Methods

Research and Publication Ethics: Approval to carry out this study was received from Fırat University's Animal Experiments Local Ethics Committee. This study was conducted at Fırat University Experimental Research Center, Elazig, Turkey (Protocol Number: 396358, Date: June 15, 2020). The rats required to carry out the study were obtained from Fırat University Experimental Research Center. During the experimental period, all instructions and rules regarding experimental animal studies specified in the Declaration of Helsinki were followed.

The experimental setup was carried out with 60 female Sprague-Dawley rats in the same estrus period; the rats were divided into two groups of 30; experimental and control groups. For each rat in the control defect group (n=30), one cylindrical defect of 4 mm in diameter and 4 mm in length was surgically created in the corticocancellous bone of the metaphyseal part of the right tibia of each rat (1). No other application was performed in this group during the experimental period. For each rat in the ABS defect group (n=30), one cylindrical defect of 4 mm in diameter and 4 mm in length was surgically created in the corticocancellous bone of the metaphyseal part of the right tibia (1). Following the creation of the defects by surgical methods, ABS was applied locally to all bone sockets. At the end of the 2nd, 4th, and 8th weeks after surgical application, 10 rats were sacrificed in each group.

Surgical Procedures and Histological Analysis: Before the creation of the defects, all of the rats were left unfed for 8 h. All surgical applications were performed under general anesthesia. As general anesthetics, 10 mg/kg xylazine hydrochloride (Rompun®, Bayer, Germany) and 50 mg/kg ketamine hydrochloride (Ketasol®, Richter Pharma, Austria) were used. After shaving the operation area, antisepsis was provided using a povidone iodine solution. A 1.5 cm surgical incision was made on the tibial crest in the surgical area, and a periosteal elevator was used to elevate the soft tissue in the corticocancellous bone area where the defect was to be created. A bone defect was created in the corticocancellous bone in the metaphyseal part of the right tibia with a surgical drill under sterile serum perfusion. Cylindrical defects of 4 mm in diameter and 4 mm in length were surgically created in the metaphyseal parts of the right tibial bones of the rats in both the control and ABS groups. After the defects were created in the rats in the ABS group, as much ABS as the socket could take was applied locally. After the surgical application, all the soft tissue and skin were sutured at the original positions (5/0 vicryl, Ethicon, Inc., USA). For infection and pain control, intramuscular antibiotics (45 mg/kg penicillin), and analgesic (0.2 mg/kg tramadol hydrochloride) were administrated postoperatively for 3 days. Ten rats each from the control and ABS groups were sacrificed with carbon dioxide ventilation (with gas flow of 30–70% vol/min / 5-10 minutes) at the 2nd, 4th, and 8th weeks of the study, and the tibial bone tissue containing the defect was taken. Bone blocks containing the defects in the right tibia bones of the rats were removed, decalcified, and subjected to histological analysis.

Histological analyses were performed at Fırat University Faculty of Medicine Department of Pathology. After hematoxylin eosin staining, the histological samples were evaluated under a light microscope. When scoring fibrosis, the density of the fibrosis within the defect was evaluated semiquantitatively as follows: no fibrosis formation, 0; mild fibrosis intensity, 1; moderate density of fibrosis, 2; and extensive density of fibrosis, 3. Newly regenerated bone (NRB) tissue was scored as follows: no bone formation, 0; mild visible bone formation, 1; moderate density of bone formation, 2; and extensive density of bone formation, 3. Images of all histological sections were taken with a digital camera connected to a light microscope (Olympus Bx51; Olympus Corporation, Tokyo, Japan) and recorded in a computer environment. Imaging software (Olympus DP71; Olympus Corporation) was used to perform histomorphometric analyses (2).

Statistical Analysis: IBM SPSS Statistics version 22 was used to evaluate the data obtained in this study. Whether the data conformed to normal distribution was evaluated with the Kolmogorov–Smirnov test. In

evaluating data that did show normal distribution, a one-way analysis of variance was used to compare between groups, and Tukey's honestly significant difference test was used to determine the group causing the difference. Significance was evaluated at $p<0.05$.

Results

An examination of the fibrosis values revealed that the control and ABS data for the 2nd week were statistically higher than that for for all other time periods ($p<0.05$). The control and ABS fibrosis data for the 4th week were higher than that for the 8th week ($p<0.05$), and the fibrosis level for the 4th week was found to be lower than that for the 2nd week in both the control and ABS rats ($p<0.05$). When the fibrosis values at the 2nd, 4th, and 8th weeks were examined in pairwise comparisons between the control and ABS groups, no statistical difference was detected between the two groups ($p>0.05$) (Table 1). An examination of NRB determined that the values of the control and ABS groups for the 2nd week were statistically lower than those for for all other weeks ($p<0.05$). When the NBR data for the 4th week were examined, the values for both the control and ABS groups were higher than those for the 2nd week ($p<0.05$) but lower than those for the 8th week ($p<0.05$). When the NRB values at the 2nd, 4th, and 8th weeks were examined in pairwise comparisons between the control and ABS groups, no statistically significant difference was found between the groups ($p>0.05$) (Table 1) (Figure 1A, B, C, D; Figure 2A, B, C, D; Figure 3A, B, C, D).

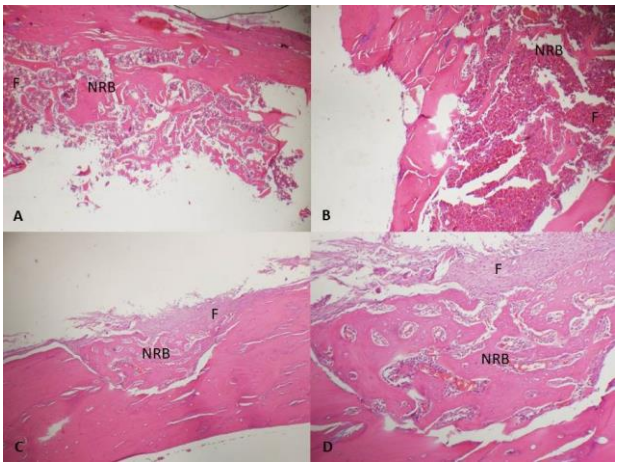

Figure 1. Histopathological sections of the control group; A:(2x), B: (4x) and ABS group; C: (2x), D: (4x) group after two (2) weeks from surgical defect application. *Newly Regenerated Bone (NRB) formation of the defect area. Fibrosis (F) 2x:Magnified 20 times, 4x: Magnified 40 times. Figure 1C is a 20x magnified view of the defect area, while Figure 1D is a 40x magnified view of the same defect area.

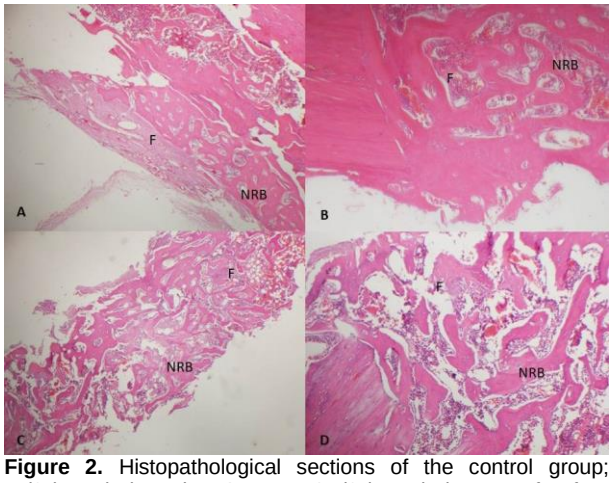

Figure 2. Histopathological sections of the control group; A:(2x), B: (4x) and ABS group; C: (2x), D: (4x) group after four (4) weeks from surgical defect application. *Newly Regenerated Bone (NRB) formation of the defect area. Fibrosis (F) {2x: Magnified 20 times, 4x: Magnified 40 times.

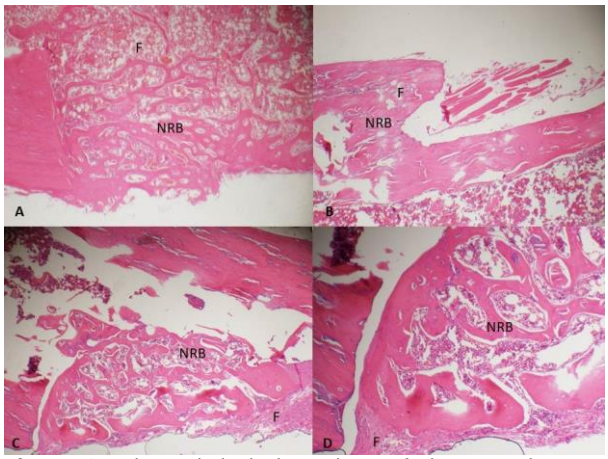

Figure 3. Histopathological sections of the control group; A:(2x), B: (4x) and ABS group; C: (2x), D: (4x) group after eight (8) weeks from surgical defect application. *Newly Regenerated Bone (NRB) formation of the defect area. Fibrosis (F2x: Magnified 20 times, 4x: Magnified 40 times.

Discussion

ABS is a plant extract and medicinal product used for hemostatic purposes in traditional Turkish medicine. This experimental in vivo study was designed with the hypothesis that some properties of locally applied ABS could help heal bone defects. ABS consists of a mixture of various plants, including *V. vinifera*, *G. glabra, T. vulgaris*, *A. officinarum*, and *U. dioica*. These plant extracts have various positive effects on hematological parameters, neovascularization with cytokine-induction, vascular functions, cellular proliferation, and antitumor, antiplatelet, antioxidant, anti-inflammatory, anti-thrombin and anti-atherosclerotic activities. Each component that makes up this mixture has its own characteristics (9, 17-26).

Goker et al. (17) histopathologically examined the effects of ABS on healing in bone defects in diabetic animals and reported that ABS forms an encapsulated protein network that creates binding sites that allow red blood cell collection. Bulut et al. reported that ABS in both diabetic and nondiabetic rats increased bone tissue healing. In addition, Bulut et al. (18) reported that ABS, which is used locally in bone defects, does not cause an inflammatory reaction but instead reduces inflammation and necrosis in early bone healing and increases new bone formation. Based on these results, the researchers emphasized that ABS could be used safely in oral and maxillofacial surgical procedures in patients with wound-healing disorders. According to the histological data obtained in the current study, locally used ABS did not cause any foreign body reaction in the ABS and control groups.

In an in vivo study on rat tibias, Isler et al. reported that ABS decreased inflammation, cell death, and fibrous tissue formation and increased new bone formation in the early bone healing period and that no foreign body reaction was observed against ABS (9). In addition to these studies, Ezirganlı et al. (27) reported that the local application of ABS had a positive effect on bone healing in an ovariectomized rat model in an experimental in vivo study. In addition, the researchers stated that further experimental animal and clinical studies focusing on ABS may be needed to determine the therapeutic dose of ABS. Turgut et al. (28) reported in an experimental animal study that ABS provided positive effects on bone healing, while bone wax impaired bone regeneration. Based on scintigraphic, histochemical, and immunohistochemical analyses, researchers reported that local application of ABS has positive effects on bone regeneration and can stop bleeding quickly. In a study on rats, Tanik et al. evaluated the healing of 7 mm defects created in the calvarium by applying a graft and local ABS (29). According to histological and bone mineral density data, the researchers determined that both graft + ABS and ABS alone had positive effects on wound healing and bone formation in nondiabetic rats (29). Healthy rats were used in the current study, and no diabetic or osteoporotic rats were used. The fact that the bone healing and fibrosis values obtained in this study for the experimental groups are not statistically different from the controls does not support the mentioned studies. In a clinical study of individuals with chronic periodontitis, Pamuk et al. (30) reported that local ABS application with autogenous cortical bone grafts could stimulate angiogenesis and vascular endothelial cell function, thus increasing the soft tissue healing of periodontal defects, preventing gingival recession, and increasing clinical attachment gain. In the histological and immunohistochemical results obtained from a study comparing the effectiveness of ABS and enamel matrix derivatives in the treatment of fenestration defects in rats, Guler et al. (31) reported that ABS can contribute to bone healing in periodontal defects. As indicators of bone healing in periodontal defects, increases in clinical attachment levels and soft tissue healing have been reported in patients receiving local ABS. However, the data obtained in this study do not support the studies of Pamuk et al. (30) and Guler et al. (31) because, although bone healing was higher in subjects treated with ABS compared to controls at a numerical level, the data were not statistically significant. In addition, fibrosis, which can be used as another indicator of defect healing, was not found to be statistically significant.

Some studies in the literature have investigated bone healing and regeneration of bone grafts with ABS use (29, 31). However, in the current study, we thought that the regenerative properties of ABS applied to defects created in the bone could be determined more accurately and objectively, as the most common use of ABS in oral and maxillofacial surgery is local application to stop bleeding. Additionally, we thought that the risk of infection in the area where the graft was applied and the difficulty of applying the graft would compromise the objectivity of the study.

The current study has some limitations. First, the relationship between ABS and bone metabolism could not be revealed at the molecular level; only histopathological methods was used, immunohistochemistry and ELISA were not used. Second, in vivo studies can be used to estimate the physiological and pathological conditions of the subject in humans. Third, bone healing was not evaluated in the long term in this study. Fourth, long bones such as tibia and femur have different osteogenic properties compared to other bones of the body and therefore, other bones of the body such as mandible and maxilla may respond differently to ABS application (32).

In conclusion, based on the limited results of this study, the local application of ABS to bone defects cannot be confirmed to contribute to the healing of bone tissue. Further studies will help elucidate the mechanism of ABS and bone tissue healing.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict Of Interest: The authors declare that there is no conflict of interest.

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