Effects of Ankaferd Blood Stopper® on Bone Regeneration in Rat Calvarial Defects

Ankaferd Blood Stopper®’in Sıçan Kalvaryal Defektlerindeki Kemik Rejenerasyonuna Etkileri

ABSTRACT Objective: The influence of Ankaferd Blood Stopper® (ABS) was experimentally investigated on calvarial defects in rats. Material and Methods: Fourteen Wistar-albino rats equally divided into two groups, control group (G1) and drug group (G2), were included in the study. In G1, only right frontal craniectomy was performed. In G2, 0.5 ml ABS was topically applied with a syringe after craniectomy. Surface areas of the defects were measured after four weeks, and comparative reconstruction images with were obtained, computed tomography (CT) as well. Blood samples were also collected to evaluate prothrombin time (PT), international normalized ratio (INR) and activated partial thromboplastin time (aPTT), and tissue samples were harvested for histologic examination. Results: Mean defect area in G1 (7.20 ± 2.62 mm²) was significantly larger than that in G2 (1.83 ± 1.65 mm²) (p< 0.001). Mean PT, INR and aPTT values were not different between the two groups (p> 0.05). Three dimensional (3D) CT analysis and histological examination revealed prominent osteogenesis in G2. Conclusion: We observed that surface area of the calvarial defects decreased significantly after ABS administration, and this observation was confirmed by 3D CT analysis and histology. Thus, ABS seems to have a potential of bone promoting effect; however, precise mechanisms underlying this osteogenic activity still remain unexplained.

Key Words: Bone regeneration; models; animal; osteogenesis; skull

ÖZET Amacı: Ankaferd Blood Stopper® (ABS)’in sıçanlardaki kalvaryal defeketlere etkisi deneniyel olarak araştırıldı. Gereç ve Yöntemler: Ondört Wistar-albino sıçan eşiği grubu; kontrol grubu (G1) ve ilaç grubu (G2) olarak bölünerek çalışmaya dahil edildi. G1’de, yalnızca sağ frontal kraneyotomi yapıldı. G2’de ise 0.5 ml ABS kranyeotomi sonrası toplikal olarak uygulandı. Defekt yüzey alanları dört hafta sonra ölçülü ve bilgisayarlı tomografi (BT) ile karşılaştırılarak değerlendirildi. Protrömbin zamanı (PT), internasional normalized ratio (INR) ve aktif parsiyel tromboplastin zamanı (aPTT) değerleri de alınarak histogram ile incelenmiştir. Bulgular: Ortalama defekt alan G1 grubundaki hayvanlarla (7.20 ± 2.62 mm²) G2’de kilerden (1.83 ± 1.65 mm²) anlamlı olarak daha büyüktü (p< 0.001). PT, INR ve aPTT değerleri ortalamasında (p> 0.05) farklı göstermedi. Boyutlu BT analizi ve histolojik incelemelerde G2’de belirgin osteogeniz saptandı. Sonuç: ABS uygulamasında sonra kalvaryal defeketlerin yüzey alanlarının anlamlı ölçüde azaldığı gözlandi ve bu gözlem üç boyutlu BT analizi ve histoloji ile doğrulandı. Sonuç olarak, ABS’nin kemik iyileşmesini hızlandıracak bir etkisi var gibi görünmektedir ancak bu osteogenetik aktifitenin kesin mekanizmaları çok iyi anlaşılmamıştır.

Anahtar Kelimeler: Kemik yenilmesi; modeller, hayvan; osteogenez; kafatası


Ankaferd Blood Stopper (ABS) has been used as a hemostatic agent in Turkish folkloric medicine. It is an herbal extract composed of five different plants, Thymus vulgaris (thyme), Glycyrrhiza glabra (liqui-
oric), Vitis vinifera (grape vine), Alpinia officinarum (blue ginger), and Urtica dioica (nettle), each having some effects on endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and cell mediators. Many studies have demonstrated its safety and efficacy as a topical hemostatic agent. Recently, use of ABS has been approved in the management of dermal, external traumatic/postsurgical and dental hemorrhage in Turkey. Its basic mechanism of action is currently unknown, although recent reports have indicated that ABS promotes formation of an encapsulated protein network acting as an anchor for erythrocyte aggregation without significantly interfering with individual coagulation factors. Since bone formation depends on the cooperation of several factors including some molecular pivots, vascularization, inflammation, and specific cell types by poorly understood complex mechanisms, ABS may play a possible role on bone repair and represent a new agent supporting bone regeneration. Moreover, cranium is one of the main workplaces of neurosurgeons and calvarial bone has a low regenerative capacity. Thus, we aimed to assess the potential effects, if any of ABS, on bone development in experimentally created calvarial defects in rats.

MATERIAL AND METHODS

After obtaining ethical consent from the Ethics Committee, 14 male Wistar-Albino rats weighing 25-30 g (14-day-old) were included in the study. Animal study was performed in Experimental Surgery, Research, and Animal Laboratory of Karacemis University School of Medicine. All rats were kept at 22-25°C with an appropriate humidity and were supplied with sufficient fluid and food; 12 hours daylight and darkness cycle was maintained before and after surgery.

The subjects were divided into two groups. In the control group (G1; n: 7) only craniectomy was performed. In the drug group (G2; n: 7) a total dose of 0.5 ml ABS (Trend Technology and Drugs, No: 2007-0-114485; Istanbul, Turkey) was topically applied over the dura mater with a syringe, after craniectomy.

Two weeks later, rats were anesthetized by intraperitoneal injection of ketamine hydrochloride 40 mg/kg (Ketalar®, Parke Davis, Berlin, Germany) and xylazine chloride 5 mg/kg (Rompun®, Bayer AG, Leverkusen, Germany). Their heads were in a free position. Under aseptic conditions, coronal sutures were exposed by midline scalp incisions and right frontal craniectomies with an average area of 12 mm² (4 mm x 3 mm) were performed by a ronguer. Care was taken not to disturb the dura mater or superior sagittal sinus (Figure 1). In G2 group, 0.5 ml ABS was topically applied into the surgical area and a ring-shaped plastic mould was used to prevent the drug slipping out until it was resorbed. Scalp incisions were then sutured with 4/0 vicryl. Animals were housed in a light- and temperature-controlled environment and given food and water ad libitum. On the fourth week, defect sites were re-explored and measured with a caliper compass (0-150 mm; Vernier). Scalp incisions were re-sutured, as done before.

For biochemical analysis, blood samples were collected into sodium citrate-containing tubes (concentration of sodium citrate was 3.2%; ratio of sodium citrate and blood was 1: 9) to evaluate coagulation parameters. The blood sample was centrifuged at 1500 ×g for 15 minutes to collect plasma. Prothrombin time (PT)/international normalized ratio (INR) and activated partial thromboplastin time (aPTT) were measured using Trinity Biotech (Wicklow, Ireland) test kits and Amax 200 (Lemgo, Germany) automatic coagulation analyzer.

Three-dimensional images were concurrently obtained by helical CT scanning (CT Securo, Philips, Amsterdam, Netherlands).

Rats were sacrificed using a lethal dose of ketamine hydrochloride at four weeks by the time they were re-operated for the second measurements. Their skulls were harvested for histologic examination. Tissue samples were fixed in 10% buffered formalin solution for 10 days. All samples were decalcified in 5% formic acid for seven days and embedded in paraffin. 5-μm-thick coronal sections through the center of circular defects were stained with hematoxylin and eosin, and evaluated under light microscope.
Kolmogorov-Smirnov test was used to assess the normality of distribution for defect diameter, and PT, INR and aPTT values. Independent samples t-test was used to determine the difference between defect sizes, PT, INR and aPTT in G1 and in G2, on fourth week. Although data were evenly distributed, both parametric independent samples t-test and non-parametric Mann-Whitney U test were used for data analysis due to small sample size. Because results from both tests were similar, we only reported the results of independent samples t-test. A P value < 0.05 was considered statistically significant. SPSS (ver. 11.5) program was used for all calculations.

RESULTS

All animals survived after surgical procedures and no signs of infection were observed during the healing period or at the time of sacrifice.

At the end of the fourth week, mean defect area in G1 (Mean ± SD; 7.20 mm² ± 2.62) was significantly larger than those in G2 (1.83 mm² ± 1.65) (p< 0.001). Mean PT values in G1 and G2 were 24.72 seconds (SD ± 2.14) and 23.84 seconds (SD ± 1.88), respectively. Mean INR values in G1 and G2 were 1.68 seconds (SD ± 0.13) and 1.60 seconds (SD ± 0.12), respectively. Mean aPTT values in G1 and G2 were 17.04 seconds (SD ± 2.59) and 15.97 seconds (SD ± 2.42), respectively. Mean PT, INR and aPTT values showed no statistical difference between G1 and G2 (p= 0.541; p= 0.938; p= 0.541).

In 3D CT analysis, defects in G2 with ABS exhibited more prominent osteogenesis with substantial calvarial defect closure when compared to control defects in G1 (Figure 2a, 2b).

Histologically, on the fourth week, control defects were primarily filled with loose fibrous connective tissue in G1 group. Minimal new bone formation confined to defect margins was also noted (Figure 3a). Defects in G2 group with relatively smaller defect size revealed dense fibrous connective tissue and prominent fibrous-osteoblastic activity (Figure 3b). No necrosis, tumorigenesis or infection was observed in either group.

DISCUSSION

ABS is a unique folkloric medicinal plant extract composed of a standardized mixture of the plants Thymus vulgaris (thyme), Glycyrrhiza glabra (liquorice), Vitis vinifera (grape vine), Alpinia officinarum (blue ginger), and Urtica dioica (nettle) and has long been used in Turkish traditional medicine as a hemostatic agent. All those plants are individually suggested to have some effects on the endothelium and blood cells via cellular proliferation and
Neurosurgery Gül et al

angiogenesis as well as vascular dynamics and mediators. Cranium is the province of the neurosurgeon, who must respect and understand its unique biological nature no less than that of the brain itself. However, it is well known that calvarial bone has the lowest regenerative capacity when compared to any bone in the body. On the other hand, craniotomy for various cerebral lesions requires safe passage to and from cephalic areas with eventual restoration of conformity of the head. Whatever the cause, surgical defects larger than 2 to 3 cm over the cerebral convexity and defects of any size in the glabrous frontal areas are universally repaired. Additionally, bone wax is widely used for bone-derived hemorrhage, however it inhibits osteogenesis.

Clinically, it has been observed that children retain the ability to reossify calvarial defects until approximately two years of age. Since there is no capacity for healing in even small defects in older children and adults, insufficient calvarial healing occurs with fibrous non-union and patients without sufficient reconstruction are prone to cerebral injuries. Therefore, various techniques have been developed to provide adequate defect closure although some problems still exist. Research mainly including experimental calvarial defect models are also currently conducted to find out materials with potential, if any, osteopromotive effect in bone repair or regeneration. Among many procedures developed for promoting bone formation bioimplants containing bone morphogenic proteins (BMPs) such as demineralized bone matrix are the most well-known bone graft substitute having osteoinductive potential and they are used in surgical practice for calvarial defects. Much has been learnt about the underlying mechanism of BMPs in bone repair; many studies have demonstrated that they stimulate the differentiation of primitive mesenchymal cells into bone forming cells via various molecular interactions including cytokines. Other experimental studies including chitosan, calcium phosphate, and simvastatin and calcium sulfate combination also demonstrated bone promoting effect with significant defect closure. Although calcium phosphates such as hydroxyapatite, tricalcium phosphate, and biphasic calcium phosphate as well as bioglass have been widely used as bone-graft materials for repairing bone defects, they are inadequate for large bony defects due to their slow degradation rate. Still, some other studies that used other agents with a bone-forming potential revealed no such association.

ABS has been widely used in Turkish folkloric medicine as a hemostatic agent although its precise mechanism of action is unclear. ABS is officially licensed by the Turkish Ministry of Health and commercially available in compress, spray and liquid forms. Glycyrrhiza glabra has anti-inflammatory, anti-thrombin, anti-platelet, anti-oxidant, anti-atherosclerotic, and anti-tumor activities. It inhibits angiogenesis, decreases vascular endothelial growth factor production, and cytokine-induced neovascularization. Thymus vulgaris has anti-oxidant action, such as prevention of lipid peroxidati-

FIGURE 3: Representative photomicrographs of (A) control defects with loose fibrous connective tissue between defect margins (arrows) with minimal new bone formation confined to the defect margins and (B) defects with ABS administration showing smaller defect size filled with dense fibrous connective tissue (arrows) and prominent fibrous-osteoblastic activity (inset).
on. Vitis vinifera exerts anti-tumor and anti-atherosclerotic effects. Alpinia officinarum inhibits nitric oxide production by lipopolysaccharide-activated mouse peritoneal macrophages. Urtica dioica causes vasodilation via inducing nitric oxide production by endothelium. Therefore, the hemostatic effects of ABS seem largely not to related with well-studied properties of its individual ingredients. On the other hand, in their unique in vitro study, Goker et al. found that adding ABS to normal plasma resulted in formation of an encapsulated protein network and that this network formation depended on interactions between ABS and blood proteins including mainly fibrinogen. They also proposed that this network might provide focal points for erythrocytes aggregation without affecting individual clotting factors and with decreased plasma fibrinogen activity concomitant with prolonged thrombin time. Beneficial effects of topical administration of ABS on hemostatic parameters were also supported by some in vivo studies suggesting that modulation of platelet activation by ABS might contribute to the hemostatic process. Although not investigated or confirmed with a number of studies, no adverse effects or toxicity has been reported to date. Therefore, ABS may be a novel effective hemostatic agent with a therapeutic potential for the management of hemorrhage in medical practice. On the other hand, it is clear that the mechanism(s) underlying the hemostatic control by ABS require further investigation and other possible mechanisms that might contribute to its less known consequences including toxic and adverse effects should also be regarded.

Considering our study, we attempted to find out the effect of ABS on bone formation either as an osteoinductive or an osteoinhibitor. Bone healing is a complex event typically characterized by four overlapping stages: the initial inflammatory response, soft callus formation, hard callus formation, initial bony union and bone remodeling. Endochondral ossification adjacent to the fracture site forming bony callus span a period up to 28 days. Remodeling of this woven bone proceeds for several weeks. Thus, we examined the defect sites four weeks after craniectomy. We observed that ABS did not have any effects on PT, INR and aPTT values, a finding that may be attributed to topical administration of ABS. On the other hand, we found that surface area of the bone defects decreased significantly after ABS administration, suggesting that ABS promoted bone formation. Osteogenic activity was also confirmed by 3D CT analysis and histology. Bone formation depends on the cooperation of several factors including specific cell types such as mesenchymal stem cells and osteoclasts, hydroxyapatite, extracellular matrix molecules, soluble molecules such as cytokines and growth factors such as bone morphogenetic proteins, hormones, vitamins, and various mechanical stimuli. Several different molecular mechanisms are also known to be crucial for bone formation although the interactions between them are not well known. Several approaches are of particular interest in improving bone repair, including inducing bone regeneration by molecular targeting, inhibition of resorption and the use of mesenchymal stem cells as therapeutic cells. Briefly, after injury of any type, hypoxia and inflammation induce vascularization. In vessels growing within injured tissue, perivascular mesenchymal stem cells populate the wound site under hypoxic conditions to differentiate along a cartilaginous or an osteogenic lineage in response to growth factors and cytokines released by platelets, inflammatory cells, and neighboring cells. Thus, bone promoting effect of ABS in the current study seems much complicated since ABS is an agent with anti-inflammatory, anti-thrombin, and anti-platelet effects, and it inhibits angiogenesis and neovascularization which are required for bone formation. It appears particularly interesting when we consider that local hemorrhage seems favorable for healing process in the classical bone repair as the blood clot forms a scaffold for the osteoblasts and fibroblasts involved in bone repair, and on the other hand ABS facilitates osteogenesis while preventing hemorrhage. The interactions between ABS and several molecules, molecular mechanisms and associated progenitor cells that contribute bone formation are probably more complicated, as in the case of hemostasis. For example,
it may be suggested that protein network developed between ABS and blood proteins may induce other possible molecular pathways or provide a background for mesenchymal stem cells to proliferate or intervene in other unknown mechanisms crucial for bone formation.

In conclusion, our findings support that ABS has a potential to enhance bone formation although the underlying mechanisms still remain unexplained. Besides its hemostatic properties, ABS may be a novel agent to improves bone formation, but more research is needed to explain its exact mode of action as well as its possible adverse or beneficial therapeutic effects.

Acknowledgement

We would like to thank Hasan Ocaş, MD and Bilgehan Acikgöz, MD for their contribution.

REFERENCES


