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Ankaferd Blood Stopper: Does it have a role in fracture healing?

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Aim: To examine the effect of Ankaferd Blood Stopper (ABS) on bone healing using a rat femur fracture model.

Materials and methods: Eight rats were used as a control group (group 1), which was not subjected to fractures. The remaining 48 rats were divided into 6 groups of 8 each. The femoral shaft fractures were produced by cutting with bone scissors. One milliliter of ABS was applied to the fracture region of groups 3 (day 7), 5 (day 21), and 7 (day 45), or rats treated with saline instead of ABS on the fracture region in groups 2 (day 7), 4 (day 21), and 6 (day 45). After the treatments, blood was taken for analyses and fracture healing was evaluated radiologically according to the modified Lane and Sandhu scoring system on postfracture days 7, 21, and 45.

Results: Fracture-hematoma formation interfered with ABS-induced hemostatic protein network or aggregation. Radiological healing scores observed at an average of 50% in group 3 were low compared to group 2 the first week, but fracture healing seemed to be normal at weeks 3 and 6. No statistical difference was observed for bone morphogenetic protein-2 and fibroblast growth factor-2 (FGF-2), alkaline phosphatase, osteocalcin, pyridinoline, and deoxypyridinoline studied among the groups. FGF-2 level in serum decreased by an average of 37.3% and 32.6% in groups 3 and 7, which were treated with ABS, in comparison with groups 2 and 6, respectively.

Conclusion: The application of ABS to femur fractures has no extra positive effect on bone union in fracture healing periods, except for bleeding control.

Key words: Ankaferd, fracture healing, BMP-2, FGF-2, bone formation, resorption markers

1. Introduction

The control of the injured bone or fracture and surrounding tissue bleeding during orthopedic surgery can sometimes be challenging and time-consuming for surgeons. Hemostatic agents such as bone wax, Surgicel, Ostene, fibrin glue, and Ankaferd Blood Stopper (ABS) are frequently used in medicinal and dental surgery to control bleeding at diverse body sites (1,2). From these, ABS, an herbal extract used as a hemostatic agent, is a medicinal remedy for clinical management of surgery bleedings as well as immediate cessation of bleeding of external injuries based on upcoming clinical trials (3–5; www.ankaferd.com).

On the other hand, the fracture healing process includes initial hematoma formation, which is followed by inflammation, repair, and finally remodeling. The biological processes that take place during each of these stages are tightly controlled by signaling molecules, which may be classified into proinflammatory cytokines, transforming growth factor-beta (TGF-β) members and other growth differentiation factors, and the angiogenic factors (6–8). Bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, have been implicated in a variety of functions including the formation of both cartilage and bone in skeletal embryogenesis and to be osteoinductive in vitro and in vivo (9). From these BMPs, BMP-2 is produced by mesenchymal cells, osteoblasts, and chondrocytes during fracture healing. BMP-2 is absolutely required for normal fracture healing (9,10). FGF-2 is recognized as a potent mitogen for a variety of mesenchymal cells. In bone fractures, FGF-2 is produced by mesenchymal cells, osteoblasts, osteocytes, chondrocytes, periosteal cells, and monocytes and macrophages. It is an important modulator of cartilage and bone cell function. Both BMP-2 and FGF-2 take part in processes from inflammation to the remodeling stage in fracture healing (11–13).

Markers of bone formation (e.g., alkaline phosphatase [ALP], osteocalcin [OC]) and resorption (e.g., pyridinoline [PYD], deoxypyridinoline [DPYD]) reflect the overall osteoblastic and osteoclastic activity in the skeleton (14,15). As biomarkers of bone turnover, they clinically provide useful evidence of the normal and pathologic

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processes reflecting bone metabolism or cell activity in the bone tissues (16).

The present study evaluates the changes in serum growth factors (e.g., BMP-2, FGF-2) and markers relating to bone formation and resorption during fracture healing in the absence and presence of ABS in a rat femur fracture model. In addition, we also examine the effectiveness of ABS on bone union after ABS application to the fracture region.

2. Materials and methods

The study had the approval of the local ethics committee for animal studies. This study was performed in the Experimental Research Center of Ondokuz Mayıs University, Samsun, Turkey. A total of 56 Sprague Dawley male rats, weighing 250–300 g, were allowed to adapt to the laboratory environment for 1 week before the onset of the experiment. They were permitted to take water and a standard laboratory diet. The rats were kept in a room at a constant temperature of 22 ± 1 °C in individual cages.

2.1. Animal treatment

To determine the basal BMP-2, FGF-2, ALP, OC, PYD, and DPYD levels of blood, specimens were taken from 8 rats that had not been subjected to fracture of the femur, and these rats were not treated with ABS or 0.9% NaCl (group 1, control group). The remaining 48 rats were divided into 6 groups of 8 animals each representing days 7, 21, and 45 after fracture. Experimental group rats were anesthetized with 50 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, Turkey). When the appropriate depth of anesthesia had been achieved, fractures were produced with a minimal open method in the experimental groups. To do this, an anterior incision was made to the femur and knee joints. Exposing the femur condyles, a 1-mm Kirschner wire was employed to make an opening of the medullar canal placed into the femur. After Kirschner wire placement to the medullar canal of the femur, the bone was cut with bone scissors. Standardized ABS ampoules of 2 mL (patent number 2007-906002) were supplied free of charge from Ankaferd Drug Inc., İstanbul, Turkey. One milliliter of ABS was applied to the fracture region of groups 3 (day 7), 5 (day 21), and 7 (day 45) before closure of the surgical area, while saline instead of ABS was applied to the fracture region of groups 2 (day 7), 4 (day 21), and 6 (day 45) before closure of the surgical area. Rats were followed for 7, 21, and 45 days after fracture. At the end of experimental periods, radiographs of the femur were taken under anesthesia at weeks 1, 3, and 6 after fracture operation. The Lane and Sandhu (17) method was used for radiological scoring system on fracture union with or without ABS application. All radiographs were evaluated by an orthopedic surgeon using the radiologic criteria with respect to the modified Lane and Sandhu method. After blood specimens were obtained by direct intracardiac puncture with an injector for assays, all rats were sacrificed by exsanguinations under anesthesia.

2.2. Biochemical assays

Circulating BMP-2, FGF-2, ALP, OC, PYD, and DPYD levels were measured with ELISA kits (USCN rat BMP-2 [cat. no: E0013Ra], USCN rat FGF-2 [cat. no: E0551Ra], USCN rat ALP [cat. no: E1091Ra], USCN rat OC [cat. no: E0471Ra], CSB rat PYD [cat. no: E11870r], and CSB rat DPYD [cat. no: E08400r]) in the serum samples.

2.3. Statistical analysis

Data were analyzed by the Shapiro-Wilk test for normal distribution of the quantitative outcomes. They were not normally distributed. Therefore, nonparametric statistical analyses were used for all comparisons. The Kruskal–Wallis test was used to determine the statistical significance of the differences in the groups. The Mann–Whitney U test (with Bonferroni correction) was then used for comparisons between the groups. $P < 0.05$ was taken as statistically significant.

3. Results

The Figure represents the control of the fracture and surrounding tissue bleeding after ABS application to femur fracture. In addition to this, fracture-hematoma formation seems to have declined. The findings of radiologic fracture healing scores observed at an average of 50% in group 3 treated with ABS were low with respect to group 2 at the end of first week. However, statistical analysis showed that there was no significant difference between median radiological healing scores of the groups on postfracture days 7, 21, and 45 (Table 1).

Changes in BMP-2, FGF-2, markers of bone formation (ALP, OC), and resorption (PYD, DPYD) levels following femur fractures in all groups are shown in Tables 2 and 3.

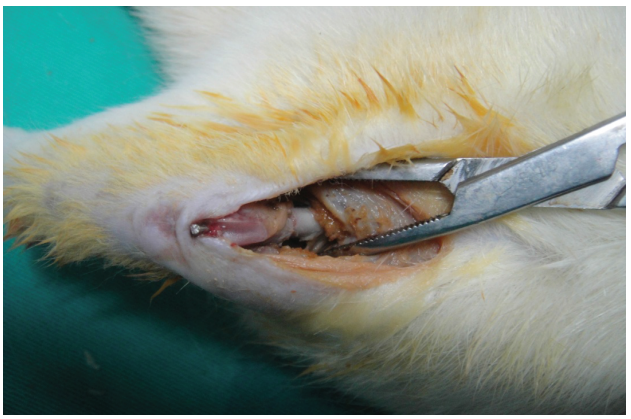

Figure. A photograph after the application of ABS on rat femur fracture (←) area.

BMP-2 levels in serum were slightly increased in groups 6 and 7 at week 6 after fracture. The serum values of FGF-2 were raised during fracture healing without ABS application at weeks 1, 3, and 6 with respect to the control (baseline). Its level was decreased by a median of 37.3% and 32.6% in groups 3 and 7 treated with ABS compared to groups 2 and 6, respectively. Serum ALP levels were slightly increased in fracture healing periods after fracture. OC levels fluctuated during fracture healing in the serum of all experimental animals. No statistical difference was observed for PYD and DPYD studied among the groups. However, DPYD was measured high at a median of 34.3% in serum of rats treated with ABS at week 6 according to group 6.

4. Discussion

ABS, which is a standardized hemostatic agent, comprises a mixture of plants: *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*. Besides its hemostatic activity, it possesses antiinflammatory actions, antimicrobial activity, antithrombin, antiplatelet, wound healing, and antioxidant characteristics, and antineoplastic properties in vitro, and it does not exhibit any side effects (1,3,5,18,19).

Application of ABS in tampon forms, sprays, and solutions has the therapeutic potential to be used for the management of external postsurgical bleedings and hemorrhages in medical and dental clinics in Turkey (18). In recent years, ABS has been used experimentally in medical orthopedic areas such as pelvis fracture and shoulder prosthesis operations, knee-ankle-foot orthosis, vertebral column (backbone) surgery, the control of sternal bleeding, the management of hemarthrosis, femur fracture, and tibia defect models (1,5,18–20; www.ankaferd.com). In relation to this issue, İşler et al. (19) indicated that the application of ABS to tibia defects decreased the occurrence of inflammation and necrosis in the early bone healing period. Recently, Amanvermez et al. (20) reported that the values of proinflammatory cytokines (e.g., TNF-α, IL-1β) were elevated in the postfracture first week according to the control (baseline), but circulating levels of these cytokines were found to be low after Ankaferd application to the fracture region. In addition, radiological examination indicated a low callus formation on fracture union in the femur fractures treated with ABS in the early fracture healing period. Normally, bone fracture is an injury, and thus inflammation is an immediate response to bone injury. In fracture-hematoma, following injury, disruption of blood vessels leads to activation of the coagulation cascade and formation of a hematoma, which encloses the fracture area. Hematoma is the source of signaling molecules that have the capacity to initiate the cascades of cellular events, which are critical to fracture healing. Removal of the hematoma significantly attenuates fracture repair as well. A growing body of evidence indicates that the signaling cascades initiated during the week-long acute inflammatory response plays an essential role in healing after fracture (21,22). Thus, fracture-hematoma and inflammation are required for fracture healing.

The control of bleeding after ABS application to the fracture region is shown in the Figure. Fracture-hematoma formation, as seen in the Figure, appeared to decline after ABS application; hematoma formation most likely interferes with the ABS-induced hemostatic protein network or aggregation. In other words, radiologic fracture healing scores were low, observed at an average of 50% in group 3 treated with ABS with respect to group 2 at the first week (Table 1). As a result of these findings, removal of the fracture-hematoma or lesser hematoma formation and inflammation suppressed by ABS application on the first days may have resulted in delayed union in fracture healing.

There was no significant difference in healing scores in groups treated and untreated with ABS at weeks 3 and 6 in the view of fracture union, according to radiologic findings. It is noteworthy that ABS application has no adverse effect on total bone fracture healing. Therefore, ABS can be used

Table 1. Statistical findings of the study groups radiologically.

Groups	Control (n = 8)	ABS (n = 8)	P
Day 7 fracture healing Median (min–max)	2 (1–3)	1 (1–3)	>0.05
Day 21 fracture healing Median (min–max)	2 (1–3)	2 (1–3)	>0.05
Day 45 fracture healing Median (min–max)	3 (3–4)	3 (0–4)	>0.05

The Lane and Sandhu radiological system was used, which scores fracture healing as (0): nonunion, (1): callus formation, (2): initiation of bone union, (3): beginning of disappearance of fracture line, (4): complete bone union.

Table 2. The serum values of BMP-2 and FGF-2 during fracture healing with or without ABS in all groups.

Groups (n = 8)	Parameters	
	BMP-2 (ng/mL)	FGF-2 (pg/mL)
1- Control (baseline)	0.74 (0.70–0.80)	11.31 (7.33–27.97)
2- Day 7 fracture healing	0.75 (0.72–0.86)	21.62 (7.33–68.09)
3- ABS + day 7 fracture healing	0.73 (0.70–0.90)	13.55 (2.04–29.79)
4- Day 21 fracture healing	0.73 (0.70–0.81)	13.99 (5.56–27.06)
5- ABS + day 21 fracture healing	0.72 (0.69–0.86)	15.34 (9.99–78.68)
6- Day 45 fracture healing	0.81 (0.76–0.91)	23.43 (12.66–57.60)
7- ABS + day 45 fracture healing	0.80 (0.73–0.90)	15.78 (8.22–39.89)

Data are expressed as median (min–max). BMP-2: bone morphogenetic protein-2, FGF-2: fibroblast growth factor-2.

Table 3. The serum levels of ALP, OC, PYD, and DPYD during fracture healing with or without ABS in all groups.

Groups (n = 8)	Parameters			
	ALP (U/L	OC (ng/mL)	PYD (ng/mL)	DPYD (pmol/mL)
1-Control (baseline)	9.95 (8.33–12.35)	6.26 (3.94–9.73)	1.56 (1.18–4.42)	2.50 (1.76–3.95)
2- Day 7 fracture healing	11.42 (9.77–14.61)	6.70 (4.43–8.66)	1.56 (1.22–2.99)	2.46 (1.94–5.98)
3- ABS + day 7 fracture healing	11.79 (11.24–14.2)	5.43 (3.94–8.79)	1.67 (1.12–2.07)	2.27 (1.07–3.34)
4- Day 21 fracture healing	10.50 (8.69–11.98)	5.87 (4.56–6.96)	3.10 (1.34–5.01)	3.55 (1.99–5.73)
5- ABS + day 21 fracture healing	11.05 (9.77–11.90)	6.13 (4.06–7.09)	1.66 (1.34–3.12)	3.22 (1.27–7.18)
6- Day 45 fracture healing	10.13 (9.41–15.77)	4.55 (4.31–7.48)	2.25 (1.33–3.48)	2.89 (2.09–5.45)
7- ABS + day 45 fracture healing	11.97 (9.41–14.61)	5.62 (3.94–6.96)	2.97 (1.49–4.54)	4.40 (3.79–5.42)

Data are expressed as median (min–max). ALP: alkaline phosphatase, OC: osteocalcin, PYD: pyridinoline, DPYD: deoxypyridinoline.

safely in a number of hemostatic bleeding disorders and to control intraoperative and postoperative bleedings, and to obtain surgical comfort in dental and orthopedic clinics.

During bone healing, fibroblast growth factors (FGF-1, FGF-2) promote growth and differentiation of a variety of cells like fibroblasts, myocytes, osteoblasts, and chondrocytes (8). FGF-1 and FGF-2 are identified during the early stages of fracture healing. They play an important role in angiogenesis and mesenchymal cell mitogenesis. FGF-1 is important for chondrocyte proliferation and maturation, while FGF-2 is expressed by osteoblasts and is usually more potent than FGF-1 (8,12,13). In this study, serum FGF-2 levels measured at weeks 1, 3, and 6 during fracture healing were high with respect to control (baseline). However, FGF-2 levels decreased by an average of 37.3% and 32.6% in groups 3 and 7 treated with ABS, compared to groups 2 and 6, respectively. According to this finding, it may be suggested that FGF-2 expression may negatively affect fracture healing after the administration of ABS. BMPs induce a sequential cascade of events for chondro-osteogenesis, including chemotaxis, mesenchymal and osteoprogenitor cell proliferation and differentiation, angiogenesis, and controlled synthesis of extracellular matrix (13). For instance, BMP-2/4 causes precursor cells to become chondroblasts during fracture healing and express the proteins needed for production of woven bone (23). In this study, serum BMP-2 levels were slightly raised at week 6 according to the other groups.

In order to determine the markers of bone formation and resorption in fracture healing periods with or without Ankaferd, we also examined the serum levels of ALP, OC, PYD, and DPYD, respectively. All of these serum markers increased or decreased after different skeletal bone fractures and fluctuated during fracture healing in agreement with the literature data (14,24–26). For example, OC elevated at 24 weeks after fracture of the tibial shaft and rose at 1 week after distal radial fracture. ALP increased at 4 weeks after fracture of the tibial shaft and remained elevated at 1

year (26). PYD-DPYD peaks 1 to 8 weeks after proximal femoral fracture, and PYD peaks 1 to 4 weeks after fracture of the tibial shaft. PYD is a less specific marker of bone resorption than DPYD since it is abundant in all connective tissues; however, DPYD is distributed generally in bone and dentin, and 90% of the bone matrix consists of type I collagen (14,26). In the present study, no significant changes were observed for the markers of bone formation and resorption analyzed between the groups.

In conclusion, the application of ABS to the fracture region resulted in bleeding control. However, fracture-hematoma formation interferes with ABS-induced hemostatic protein network or aggregation. Additionally, the application of ABS to the fracture area in the presence of femur fracture led to reduced radiologic fracture healing scores at the first week of fracture healing, except for weeks 3 and 6. FGF-2 level in serum decreased by a median of 37.3% and 32.6% in groups 3 and 7 treated with ABS as compared to groups 2 and 6, respectively. It may be suggested that, based on the present findings, ABS application to femur fracture has no extra positive effect on fracture healing process. However, further studies with a larger number of animals are necessary to confirm benefits or possible adverse effects of the ABS application on fracture healing, including growth factor expressions and pathological data of fracture area in fracture healing periods.

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