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EXPERIMENTAL STUDY

TNF-α, IL-1β, and oxidative stress during fracture healing with or without ankaferd

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Abstract: *Aims*: Whether ankaferd blood stopper (ABS) has a negative or positive effect on bone union during fracture healing is unknown. The purpose of this study was to evaluate the serum changes of oxidative stress markers, Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-10 (IL-10) during fracture healing process with or without ABS application to bone fracture.
Material and methods: Eight rats were used as a control group (1) that was not subject to fracture. The remaining 48 rats were divided into six groups, 8 rats in each. The femoral shaft fracture was produced by cutting with bone-scissors. One ml of ABS was applied on the fracture region in groups 3 (7th day), 5 (21st day), and 7 (45th day) or saline instead of ABS on the fracture regions in groups 2 (7th day), 4 (21st day), and 6 (45th day). Radiographs and above parameters were examined on post-fracture days 7, 21, and 45.
Results: Malondialdehyde and protein carbonyls were measured in high levels in the groups 2 and 4 with respect to control. Their levels did not change statistically in the experimental groups after ABS application. The values of TNF-α and IL-1β were elevated on 7th post-fracture day according to control, but were lower (by 11.86 % and 44.48 %) in the group 3 treated with ABS comparing to group 2. Radiographic examination indicated a low callus formation on fracture union in the femoral fractures of groups 3 and 5 treated with ABS.
Conclusion: The present findings may suggest that ABS application seems to be ineffective on fracture union in early fracture healing period, except for bleeding control *(Tab. 2, Fig. 3, Ref. 26).* Full Text in PDF *www.elis.sk.*
Key words: ankaferd, fracture healing, TNF-α, IL-1β, oxidative stress.

Healing of fractured bones is a complex physiologic process in which bone repairs itself with the help of tissues such as cortical bone, the periosteum, blood, bone marrow, surrounding soft tissue, and restores its own ability of mechanical loading (1–3). Fracture healing process involves initial haematoma formation followed by inflammation, repair, and finally remodeling. Immediately following the bone fracture, the response starts, hematoma forms and coagulates in between and around the fracture ends, and within the medulla forming a template for callus formation and then the injury initiates an inflammatory response that is necessary for the healing to progress (3). Despite it is indicated that inflammatory cytokines have a negative effect on bone, joints and implanted material when prolonged or chronic expression occurs, highly regulated secretion of pro-inflammatory cytokines following the acute injury is critical for tissue regeneration (4). In general, key inflammatory cytokines like IL-1, IL-6 and TNF-α are secreted by macrophages and other immune cells as monocytes, leukocytes and lymphocytes recruited to the fracture site. Their main effects are: induction of extracellular matrix synthesis, angiogenesis stimulation, chemotactict effect on circulating immune and mesenchymal cells, recruitment of endogenous fibroblasts (2, 5, 6). IL-10 is released by stimulated monocytes and T-cells as part of the auto-regulation of inflammation.

Oxidative stress is a cytotoxic condition that occurs in the tissue when antioxidant mechanisms are overwhelmed by reactive free radicals. Human and experimental studies have reported that there is an increase in the production of free radicals by macrophages, polymorphonuclear leukocytes and osteoclasts during first month after a fracture has been sustained. Oxidative stress clearly prolongs the inflammatory and repair periods of fracture healing (7–10).

ABS is a novel effective haemostatic agent that has been used in management of dental surgery bleedings and hemorrhage in Turkish medicine in the last years (11, 12). ABS is a standardized mixture of herbs 0.10 mg *T. vulgaris*, 0.18 mg *G. glabra*, 0.16 mg *V. vinifera*, 0.14 mg *A. officinarum* and 0.12 mg *U. dioica* in an ampoule of 2 ml.

The present study evaluates the values of TNF-α, IL-1β, IL-10 and oxidative stress during fracture healing process in the absence and presence of ABS in a rat femur fracture model. Another aim of this study is to examine the effectiveness of ABS on bone union in fracture healing.

Material and methods

The present study was approved of the local ethical committee for animal studies (Project No: 2009/43). This study was performed

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in the Experimental Research Center, Ondokuz Mayıs University, Samsun, Turkey. A total of 56 Sprague-Dawley male rats, weighing 250–300 g, were allowed to adapt to laboratory environment for 1 week before the onset of the experiment. The rats were housed in per cage with wood chip bedding and fed on standard laboratory chow and water ad libitum. They were maintained on a 12 hour light: dark cycle with a constant room temperature at 22±1 °C.

Animal treatment

To determine the basal TNF-α, IL-1β, IL-10, protein carbonlys and MDA levels of blood specimens were taken from 8 rats, which had not been subjected to fracture of the femur and these rats were no treated with ABS or 0.9 % NaCl (1.control group). The remaining 48 rats were divided into six groups of 8 animals each representing post-fracture days 7, 21, and 45. Experimental group rats were anaesthetized with 50 mg/kg Ketamin (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. For this aim, an anterior incision was made to the femur and knee joint. 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 medullar canal of the femur, the bone was cut with bone-scissors. One ml of ABS (the standardized ampoules (2 ml) of the ABS [patent number 2007–906002] used in the experiments were supplied free of charge from Ankaferd Drug Inc., Istanbul, Turkey) was applied on the fracture region of groups 3 (7th day), 5 (21st day), and 7 (45th day) or saline instead of ABS treated on the fracture region of groups 2 (7th day), 4 (21st day), and 6 (45th day) before closure of surgical area. Rats were observed for 7, 21, and 45 days after fracture. At the end of experimental periods, all animals of experimental groups were X-rayed laterally on 7th, 21st, and 45th post-fracture day. After blood specimens were obtained by direct intracardiac puncture with an injector for assays, all rats were sacrificed by exsanguinations under anesthesia.

Biochemical assays

In the serum samples, MDA was estimated spectrophotometrically by the tiobarbituric acid-reactive substance (TBARS) method with slight modifications and expressed in terms of malondialdehyde (MDA, a marker of lipid peroxidation) (13). Measurements of protein carbonyls (a marker of protein oxidation) in the serum samples were made by the method of Evans et al. (14) with slight modifications. Carbonyl concentration (nmol mg^{-1} of protein)= nmol ml^{-1} of carbonyl groups/protein concentration in mg ml^{-1}. Circulating TNF-α, IL-1β, and IL-10 cytokines were measured with ELISA kits (Invitrogen™ rat TNF-α elisa kit (cat.no: KRC3011), rat IL-1β immunoassay kit (cat.no:KRC0011) and rat IL-10 Elisa kit (cat.no: KRC0101) in the serum samples.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 for windows (SPSS, Chicago, IL., USA). Data were analyzed by the Shapiro-Wilk test for normal distribution of the quantitative outcomes. They were not distributed normally. Therefore non-parametric statistical analyses were used for all comparisons. Kruskal–Wallis test was used to determine the statistical significance of the differences in the groups. Then Mann–Whitney–U test (with Bonferroni correction) was used for comparisons between the groups. $p<0.05$ was taken as statistically significant.

Results

In the present study, the levels of MDA and protein carbonyls in serum were determined as oxidative stress indices. The mean values of protein carbonyls and MDA were measured high in the experimental groups with respect to the control group (not fractured femur) on 7th, 21st and 45th post-fracture days ($p<0.05$). The values of oxidative stress markers were decreased in the group 6 (45th post-fracture day) according to groups 2 and 4. No statistically significant difference was obtained for any of oxidative stress indices studied between the ABS-treated and untreated groups as shown in Table 1.

The basal TNF-α and IL-1β values which had a mean of 20.24 and 32.67 in the non-fractured control group reached a level of 34.56 and 37.43 on 7th day in the fracture group 2, respectively. These cytokines in sera were decreased in the fracture group 3 after

Tab. 1. The serum levels of MDA and protein carbonyls during fracture healing in rats untreated and treated with ankaferd®.

Groups (n=8)	Parameters	
	MDA (μmol/L)	Protein Carbonyls (ng/mg protein)
1 – Control	[a]0,133±0,018	[b]3,60±0,15
2 – 7th day fracture healing	[c]0,188±0,066	[d]4,14±0,33
3 – Ankaferd® + 7th day fracture healing	0,160±0,070	4,33±0,28
4 – 21st day fracture healing	0,236±0,145	4,30±0,24
5 – Ankaferd® + 21th day fracture healing	0,210±0,070	4,14±0,43
6 – 45th day fracture healing	0,166±0,056	4,02±0,22
7 – Ankaferd® + 45th day fracture healing	0,158±0,070	4,01±0,13

Values are mean ± S.D. [a]$p< 0.05$ versus experimental groups; [b]$p< 0.05$ versus experimental groups. [c,d]$p< 0.05$ versus 21-day group without ABS.

Tab. 2. The serum levels of TNF-α, IL-1β and IL-10 during fracture healing in rats untreated and treated with ankaferd®.

Groups (n=8)	Parameters		
	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-10 (pg/ml)
1 – Control	20,24±5,96	32,67±13,82	54,27±7,05
2 – 7th day fracture healing	34,56±12,12	[a]37,43±13,62	55,06±9,77
3 – Ankaferd® + 7th day fracture healing	30,46±8,60	20,78±9,86	59,01±15,90
4 – 21th day fracture healing	18,31±5,63	19,69±5,88	45,32±12,98
5 – Ankaferd® + 21th day fracture healing	20,08±6,35	18,52±4,20	49,79±13,23
6 – 45th day fracture healing	16,05±11,59	16,13±1,63	48,16±9,93
7 – Ankaferd® + 45th day fracture healing	12,60±3,33	19,86±4,40	45,97±5,03

Values are mean ± S.D. [a]$p< 0.05$ versus 7-day group treated with ABS.

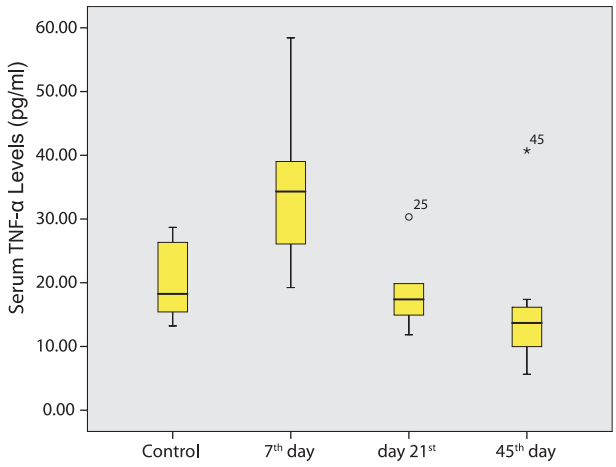

Fig. 1. Serum levels of TNF-α during fracture healing in the groups untreated with ABS.

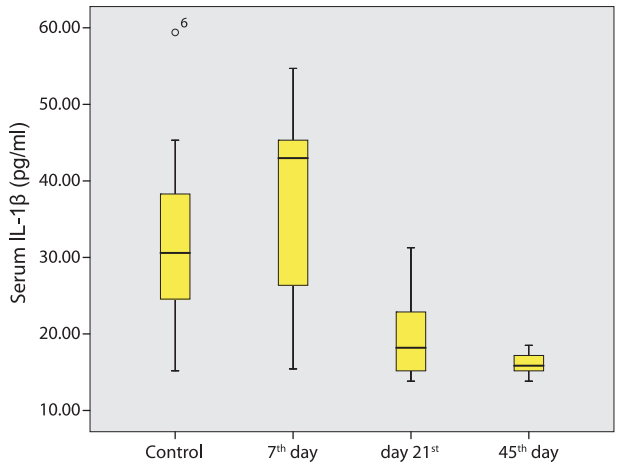

Fig. 2. Serum levels of IL-1β during fracture healing in the groups untreated with ABS.

ABS application. Also, the levels of these cytokines in experimental groups declined from 7th day to 45th day as indicated in Table 2, and Figures 1 and 2. No significant difference was obtained for IL-10 measured between the groups. However, macroscopically a low callus formation on fracture union of the femur fractures in groups 3 and 5 with respect to groups 2 and 4 untreated with ABS was observed that was detected radiographically (Fig. 3).

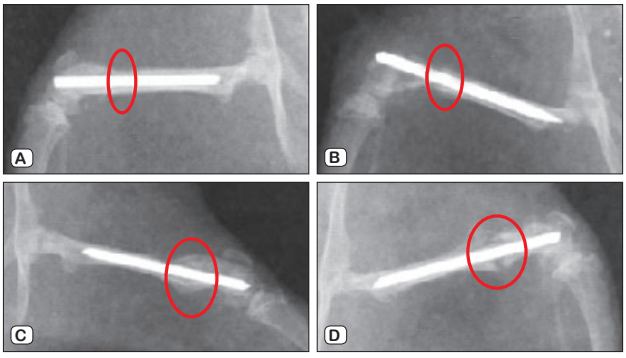

Fig. 3. Radiographic evaluation of fracture healing at 7 and 21 days. A and C) show a normal callus formation at 7th and 21st post-fracture days, B and D) represent a smaller callus formation at 7th and 21st post-fracture days treated with ABS®.

Discussion

Many studies indicate that growth factors, inflammatory cytokines, hormones, antioxidants, nutrients, some drugs and various bio-molecules may have an effect to speed up bone union process in fracture healing (1–5). ABS is a medicinal product used as a haemostatic agent, and it possesses anti-inflammatory actions, anti-thrombin, anti-platelet, anti-microbial activity, antioxidant characteristics, and has no reported side effects (11, 12, 15, 16). As a blood stopper agent, ABS is sometimes used in orthopedic application areas such as pelvis fracture and shoulder prosthesis operations, knee-ankle-foot orthosis, and vertebral column (backbone) surgery (www.ankaferd.com).

The fracture healing process has been divided into consecutive but overlapping stages. Fracture healing processes can be divided in following phase: hematoma formation, inflammation, neovascularization and granulation tissue formation, fibrous tissue formation, fibrocartilage, hyaline cartilage (soft callus), cartilage mineralization, woven bone (hard callus), and finally remodeling (17, 18). Bone fracture is an injury, and thus inflammation is an immediate response to bone injury. A growing body of evidence indicates that the signaling cascade initiated during the week-long acute inflammatory response plays an essential role in healing after fracture (18). TNF-α, and IL-1β are known as the potent inflammatory cytokines that are up-regulated during inflammation, and these play a pivotal role in enhancing fracture healing (19–21). Together with a variety of growth factors and fracture-induced inflammatory mediators they recruit inflammatory cells and osteoblasts, promote angiogenesis, and guide mesenchymal stem cell differentiation, and they affect cell proliferation as well as collagen synthesis (17, 18, 21). In the present study the values of TNF-α and IL-1β were elevated on 7th post-fracture day compared to control. In contrast to this finding, both cytokines levels were reduced in the groups 4 (21st post-fracture day) and 6 (45th post-fracture day). The levels of most inflammatory mediators return to baseline after the week-long acute inflammatory phase (18). However, the levels of TNF-α and IL-1β were lower (by 11.86 % and 44.48 %) in group 3 treated with ABS than in group 2 untreated with ABS on the 7th post-fracture day. Moreover, Isler and et al. have reported that the application of ABS decreased the occurrence of inflammation on the 7th post-operative day in early bone healing period (15). The application of ABS to blood, blood components, and tissues resulted in formation of a protein network acting as an anchor for vital erythrocytes aggregation including activated leukocytes without disturbing individual coagulation factors and platelets (16). In this condition, physiological hematoma formation in the fracture region can be affected negatively by the formation of encapsulated protein network after ABS application. Also, radiographic examinations presented a smaller callus formation in fracture healing process on the 7th and 21st post-fracture

days treated with ABS as shown in Figure 3. As a result of these findings, ABS solution seems to be ineffective in early fracture healing period, except for bleeding control.

IL-10 forcefully suppresses cell-mediated immunity by suppressing macrophage secretion of pro-inflammatory cytokines as well as by suppressing effector functions of T and NK cells (22). Hauser et al. have reported that human fracture hematomas are a rich repository of IL-10 within the first 24 h (23). No prior studies suggested how or where IL-10 is generated in bone trauma. In our study, IL-10 levels in the serum of experimental groups with or without ABS were not significantly different in comparison to all groups.

MDA and protein carbonyls in the serum of experimental group rats were higher than the basal value of oxidative stress markers of the control group. These data suggest that the production of oxygen free radicals during fracture healing is highest particularly on 7th and 21st post-fracture days. Oxidative stress occurs after sustaining a fracture (8, 9). So, administration of antioxidants might help in the acceleration of healing of fractured bones. On the other hand, *G. glabra* and *T. vulgaris* which are two of the ingredients of ABS mixture have been shown to exhibit antioxidative activity (24, 25). In our study, oxidative stress indices did not diminish statistically in the serums of ABS-treated rats with respect to untreated rats. This finding might imply that ABS has no effect on fracture healing as an antioxidant.

Fracture hematoma, inflammation and fracture-induced inflammatory mediators like TNF-α and IL-1β, and callus formation are required for fracture healing. Fracture hematoma involves the basic elements such as inflammatory cell functions, blood, lymph, endosteum, periosteum and surrounding soft tissue (26). The administration of ABS to fracture region resulted in bleeding control, but fracture-hematoma formation interferes with ABS-induced haemostatic protein network or aggregation. Therefore, the application of ABS in the early fracture healing period in the presence of femur fracture led to reduction of TNF-α, IL-1β and callus formation. Oxidative stress markers might not decrease in rats treated with ABS. Early bone healing process may be delayed by ABS application. Further studies are needed to confirm benefits or possible adverse effects of the ABS® application on fracture healing including molecular and pathological data in fracture healing periods.

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