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The effects of ankaferd blood stopper on DNA damage and enzymes with paranchymal damaged rabbits

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Abstract
Ankaferd blood stopper (ABS) is a medical product that is used in several injuries, dental operations, prevention of minor or major bleeding after spontaneous or surgerical operations and have anti-microbial, anti-inflamatory, anti-thrombin, anti-platelet, anti-atherosclerotic, anti-oxidants effects. The present study is aimed to evaluate the effects of ABS on 8-hydroxy-2ˈ-deoxyguanosine (8-OHdG), superoxide dismutase (SOD), myeloperoxidase (MPO) levels over pleural adhesions in rabbits with pulmonary parenchymal damage.16 New Zelland species rabbits were divided in two groups such as control (n=7) and study group (n=7). One rabbit in each group died during anesthesia. In both groups, we performed wedge resections in equal size to the left lower lobes of all rabbits. No interventions were made on control group, whereas 5 puff's (1 cc) ABS was performed to the resection area at study group. Tube thoracostomy that performed both groups were terminated postoperatively at 6th hour after drainage and air leakages follow up. Rabbits were sacrificed with anesthetics at postoperative 8th day. Lung tissues were collected for analyzing of 8-OHdG, SOD, MPO. The 8-OHdG levels were respectively 2.01±0.39 ng/ml in control group and, 0.38±0.12 ng/ml in study. The differences between study and control group were statistically important group ($p<0.001$). SOD and MPO levels did not show any statistically importance in the groups. As a conclusion, we can say that oxidative DNA damage prevented by ABS.

Keywords: Ankaferd, paranchyme damage, 8-hydroxy-2ˈ-deoxyguanosin, superoxide dismutase, myeloperoxidase

Introduction

Ankaferd blood stopper (ABS) approved by Turkey and Bosnia-Herzegovina for using as a topical haemostatic agent in several injuries, dental operations, prevention of minor or major bleeding after spontaneous or surgerical operations [1]. This herbal includes, urtica diocia, vitis vinifera, glycrrhiza glabra, alpinia officinarum and thymus vulgaris. Interestingly, this combination of all five plants in ABS appears to provide a unique composition promoting tissue oxygenation as well as initializing a physiological haemostatic process [2]. The safety of topical use of ABS has been demonstrated in numerous in vitro and in vivo animal models, as well as in a clinical Phase I trial in humans. Besides its haemostatic activity, ABS also has in vitro anti-infectious and anti-neoplastic effects [1,2].

The ABS-induced network formation depended upon interactions between ABS and blood proteins, mainly fibrinogen possibly via agglutination of these molecules. Therefore, the anti-haemorrhagic process was driven. The basic mechanism of ABS appears to be the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation [2]. Vigo et al [3], Akkoç et al [4] and Tsai et al [5] showed the antimicrobial activity of Thymus vulgaris which exhibited inhibitory activity

against E-coli, S.aureus, P.acnes, C.albicans, P.ovale, gram-pozitive and negative bacteria. In addition to, thymus vulgaris inhibits the production of interferon-ɣ via suppression of inducible nitric oxide synthase mRNA expression in murine macrophage cells. Zheng et al [6] determined the highest oxygen radical absorbance capacity (ORAC) values in Tymus Vulgaris. Besides, this property of the Ankaferd medicinal plant extract pointed out its antimicrobial potential as a drug and foods additive [4].

Chemical reactions, including oxidation and reduction of molecules, occur in every cell. These reactions can lead to the productions of free radicals. The presence of free radicals and non-radical reactive molecules are dangerous for living organism due to have an ability to damage cell organelles. Nitrogen monoxide (NO), superoxide anions and related reactive oxygen (ROS) and nitrogen (RNS) species also play important modulating roles in signal transduction pathways. NO and ROS act as signal transducing molecules, modulating vascular tone, monitoring oxygen pressure and production of erythropoietin [7].

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DNA, lipid and proteins are the cellular target of oxidative damage induced ROS. The defense system includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase that decrease ROS concentrations [7,8]. Myeloperoxidase (MPO; donor, hydrogen peroxide oxidoreductase), an enzyme that reacts with the hydrogen peroxide, can oxidize a large variety of oxidants. The products of MPO-Hydrogen peroxide-chloride system are powerful oxidants that can have profound biological effects [9,10] and can contribute to the pathogenesis of diseases. It has been suggested that pulmonary injury, renal glomerular damage, and the initiation of atherosclerotic lesions may be caused by the MPO system [9]. Also, Kotyza et al [11] confirmed that induced pleural inflammation was followed by a significant increase in MPO levels.

It has been shown that the lung is one of the most exposed organs to the oxidative stress, involving animal experimental models. The particular effects of oxidative stress on lungs were investigated in order to quantify the intensity and the extent of the pulmonary damage, featuring the antioxidant enzymatic protective role [12]. Lung accurately reflects the intensity of induced oxidative stress. The most frequent damages of oxidative stress on pulmonary tissue are inflammation, fibrosis and the disruption of vascular walls. It is well known 8-OHdG and 8-hydroxyadenine are certain biomarkers and characteristics for DNA damage in vitro [7, 8, 13-15]. In those adducts, 8-hydroxy-2ˈ-deoxyguanosine (8-OHdG) is the most studied oxidatively modified DNA base product [7,16-18] due to its sensitivity and mutagenic potential [16]. The integrity of genomic DNA is under constant threat due to the effect of environmental factors [19-20]. Besides, biomarkers of oxidative DNA damage are a great of interest and can potentially be used for the early detection of disease, monitoring the progression of disease and determining the efficacy of therapy [18].

Nowadays, the studies determined its anti-microbial, anti-inflamatory, anti-thrombin, anti-platelet, anti-atherosclerotic, anti-oxidants effects. There are a few studies on ABS effects on pleural adhesions with pulmonary parenchymal damage and no studies about ABS effects on antioxidant enzymes such as MPO and SOD and also oxidative DNA damage biomarker. Seen from this aspect, in this study, we aimed to evaluate the effects of ABS, used as a hemostatic agent herbal extract, on superoxide dismutase, myeloperoxidase and 8-hydrox-2-deoxyguanosine levels over pleural adhesions in rabbits with pulmonary parenchymal damage. Additionally, we aimed to clarify whether ABS has an effect on oxidative DNA damage.

Materials and Methods

The study was carried out in Selcuk University Experimental Medicine Research and Application Center and approved by Selcuk University Experimental Animal Ethic Committee (no:2010/077). The biochemical analysis was done at Selcuk University Department of Medical Biochemistry and Medical Biology Laboratories.

Experimental Procedures

In this study, 16 New Zelland species male and female rabbits with mean weight of 2500 g were used. All rabbits were kept under the same experimental conditions. The rabbits were divided into two groups as “Study group” and “Untreated group”. One rabbit in each group died during anesthesia. As an anesthesia % 2-5 ketamin HCl (35 mg/kg) and Ksilazin HCl (5 mg/kg) intramuscular are used without any applied intubation. We performed wedge resections in equal size to the left lower lobes of all rabbits. 5 puff’s (1 cc) ABS (Ankaferd Blood Stopper®; İmmun Gıda İlaç Kozmetik San.ve Tic. Ltd. Ştd., İstanbul, 2007/31) was performed to the resection area at study group. Hereby this group treated with ABS. No interventions were made on untreated group hereby was conducted as control group. Tube thoracostomy that performed both groups were terminated postoperatively at 6^{th} hour after drainage and air leakages follow up. Rabbits were sacrificed with anesthetics at postoperative 8^{th} day.

Sample Collection and Biochemical Analysis

Tissue samples of lungs collected for analyzing of biochemical parameters. The wet weight of the tissue samples were measured, and then divided into two pieces for analysis of 8-hydrox-2-deoxyguanosine and the second piece for analyzing SOD and MPO. For 8-hydrox-2-deoxyguanosine (8-OHdG) analyzing, genomic DNA from lung tissues obtained from 14 rabbits were extracted using Sambrook [21] standard procedures. Then, Bioxytech 8-OHdG-EIA kit (catalog no: 21026) used to determine the levels of 8-hydroxy-2ˈ-deoxyguanosine. Levels were measured as ng/ml.

The second piece of the tissues transferred into tubes and homogenized according to kit procedure. Firstly, pH= 7.4 PBS is used to remove red blood cells and clots form the tissue. The tissues homogenized with 5 ml cold 20 mM HEPES buffer (pH=7.2), containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose per gram tissue using a Misonix’s Microscam ultrasonic cell disruptor. Then, centrifuged at 1,500 x g for 5 minutes at $+4^0$C. Supernatant of the tissues were collected to analyze SOD. SOD was determined by using trade test kits (Cayman’s assay kit, katalog no: 706002). To analyze MPO, the tissues was rinsed 1xPBS and stored overnight at -20^0C. Homogenized with using Misonix’s Microscam ultrasonic cell disruptor and centrifuged for 5 minutes at 5000 x g at $+4^0$C. Supernatants collected and were analyzed with trade test kits (lot no:10280, Aeskulisa assay kit, Germany). SOD and MPO levels were expressed as U/ml. Analysis of parameters were measured by Biotek ELx50 and Biotek ELx800 trade mark Elisa method.

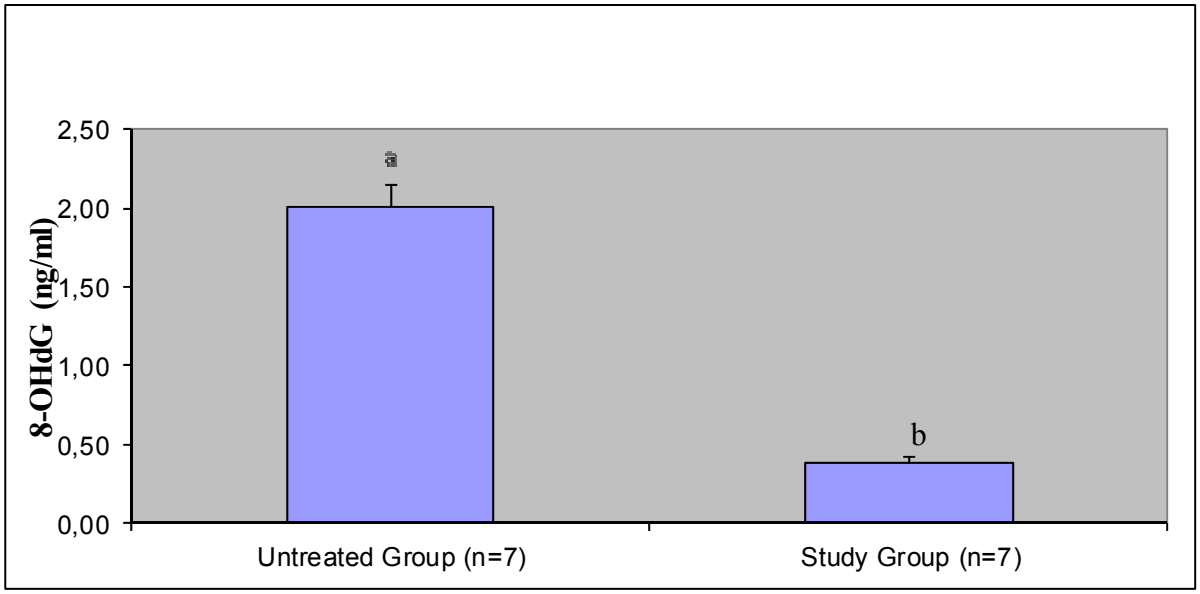
Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Version 18.0, Chicago, IL, USA). The results were expressed as mean ± SD. The comparisons of means between the groups were assessed using Anova test. Correlation analysis was performed using Pearson correlation tests. Statistical significance was set at $p<0.05$ and $p<0.001$.

Results

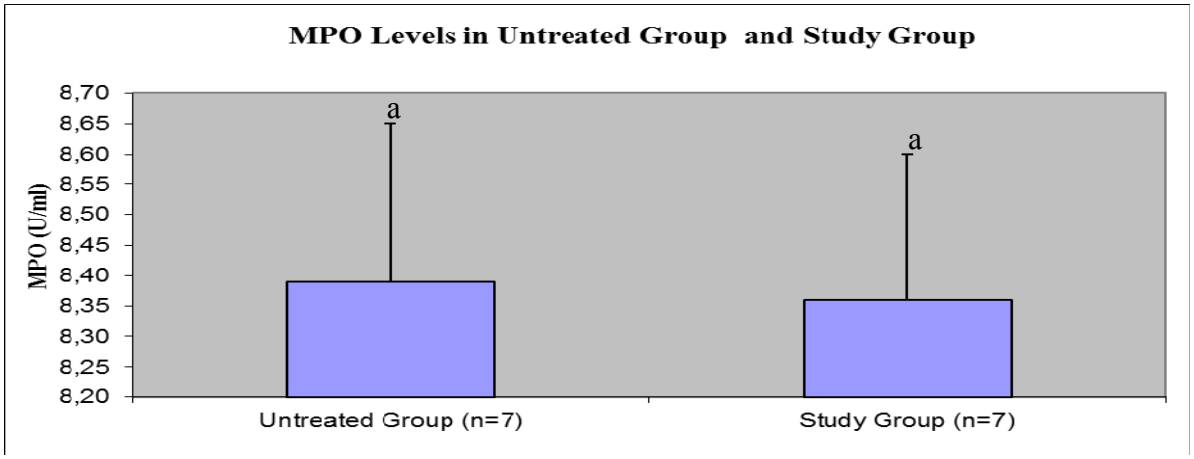
As it shown in figure 1; results obtained of 8-OHdG levels (x±SD) in lung tissue of the group which ABS performed in pulmonary parenchymal damage rabbits were 0.38±0.12 ng/ml and significantly lower than the levels of untreated group (2.01±0.39 ng/ml) ($p<0.001$). Comparing to MPO levels (x±SD), we found highest levels in untreated group (8.39±0.7 U/ml), but the differences between the study (8.36±0.65 U/ml) and untreated groups were not statistically important ($p>0.001$) (figure 2). Furthermore, in figure 3, SOD levels (x±SD) showed highest levels in study group (7.26±0.31 U/ml) which performed ABS in pulmonary parenchymal damage rabbits comparing to untreated group's levels (6.95±0.55 U/ml) ($p>0.001$).

The pearson correlations analysis showed that the correlations between 8-OHdG and MPO levels were statistically important ($r=0.884$, $p<0.05$) (Table 2), whereas there were no relations between these parameters in study group (Table 3).



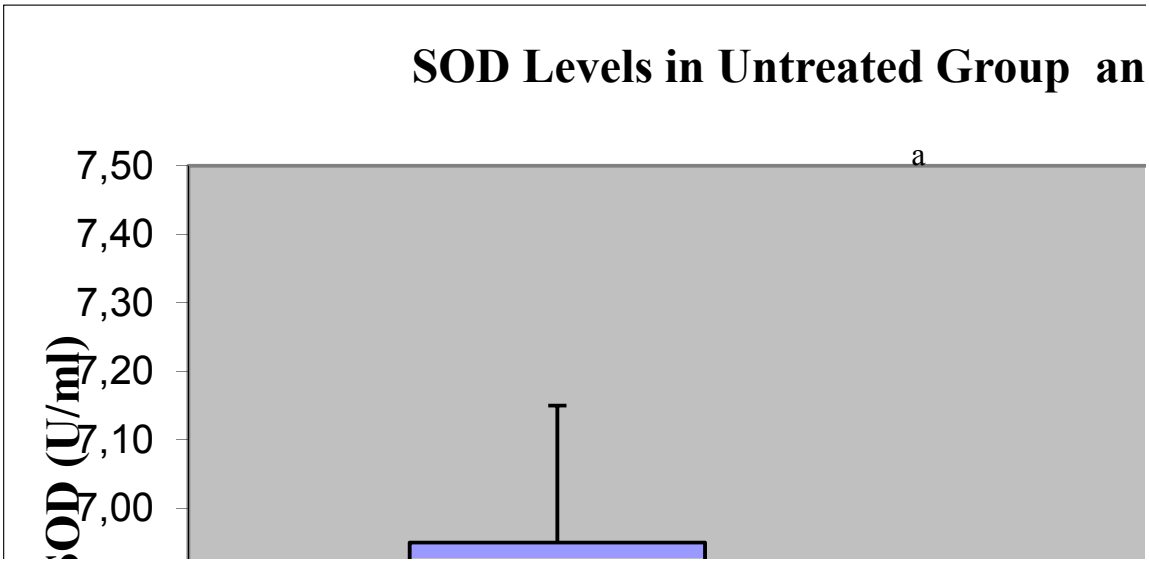
a,b different letter in same column are significant as statistics ($p<0.001$).

Figure 1. Levels of 8-OHdG (ng/ml) in pulmonary paranchyma damage rabbits



a,b different letter in same column are significant as statistics ($p<0.001$).

Figure 2. Levels of MPO (U/ml) in pulmonary paranchyma damage rabbits



a,b different letter in same column are significant as statistics (p<0.001).

Figure 3. Levels of SOD (U/ml) in pulmonary paranchyma damage rabbits

Table 2. Pearson correlations coefficients of 8-OHdG, MPO and SOD levels in untreated group (*p<0.05)

Untreated Group	Pearson correlation and p value	MPO	SOD
8-OHdG	**r**	0.884*	-0.388
	p	0.047	0.519
MPO	**r**		-0.733
	p		0.159

Table 3. Pearson correlations coefficients of 8-OHdG, MPO and SOD levels in study group p<0.05

Study Group	Pearson correlation and p value	MPO	SOD
8-OHdG	**r**	0.338	-0.201
	p	0.513	0.702
MPO	**r**		0.647
	p		0.165

Discussion

It is known that, air leakage and hemorrhage are important causes of morbidity and mortality in operations and traumas of the lung. Surgical suturing or stapler application is a standard method for prevention of air leakage and bleeding following lung resection [22,23]. On the other way, recent years, Turkish researchers studied on herbal which is used on control of bleeding and air leakage in several surgeries. Well-rounded experimantal study is determined that ABS is an efficient as Fibrin Glue, and more advantageous, with respect to side effect potential and cost. The researchers reported that ABS can be used safely in splenectomy [24]. In addition to, Gul Satar et al [25] stated that application of ABS shortened the average time for healing, which was supported in their histopathological examination, as a decrease of the infiltrating neutrophil leukocytes and increase of neovascularisation.

This herbal trade marked as Ankaferd Blood Stopper, use for stopping hemorrhage. It is a topical safety and efficacy agent which has been proven in dermal, external traumatic, postoperative and dental bleedings [2,4,24,26]. Determination of no existence of any allergic reactions and of no color changing in application area along with its antimicrobial activity, pointed out that ABS can be used confidently [4, 26]. The studies showed that usage of topical ABS, on anticoagulant treated rats and patients with developing innate or acquired causes of bleeding diathesis, significantly decreased the time and amount of bleeding [27,28]. Besides, ABS reduced postoperative bleeding and prolonged air leakages without any toxic effects and had an effective role at pleurodesis, increasing the pleural fibrosis level in rabbits with parenchyma damage [29].

Nowadays, the researchers focused on the effects of ABS in experimental studies. These results clarify functional applications of this herbal, but still needs further studies to know its effects on organism. A few studies determined the antioxidant, antimicrobial effects of this herbal. An overall evaluation of the related reports showed that the numbers of studies are limited. It is not clear whether there is an oxidative DNA damage in pulmonary parenchyma

damage. Seen from this aspect, the present study aimed to determine the effects of ABS on antioxidant-oxidant system enzymes such as MPO and SOD and 8-OHdG.

As it is well known hydroxyl radicals attack to bases and leads to mutations. The most affected bases are guanine and cytosine in oxidative stress. Guanine has the lowest ionization potential comparing to other component of DNA, therefore it is attacked by free radicals powerfully. 8-hydroxy-2'-deoxyguanosine, is a modified bases, is formed as the result of attacks to its C8 by hydroxyl radicals. It is sensitive marker of oxidative DNA damage and one of the 23 oxidative bases adducts [13]. Seen from the results, our study showed that in study group the levels of 8-OHdG are lower than untreated group. After the pulmonary resection, using of ABS as a blood stopper showed us initially resection may be under oxidative stress, subsequently ABS reduced oxidative DNA damage. Our evaluations are similar comparing with the study of Williams et al [30]. The researchers studied on patients undergoing elective thoracic surgery and assessed that oxidative damage and lung injury occur following pulmonary resection, changes occur in markers of oxidative protein damage with associated effects on gas exchange, processes which appeared to be related [30]. In our previous study, we supported that using ABS did not show any air leakage [28], therefore this findings may be attributed to the levels of 8-OHdG may not only affected ROS production also affected by gas exchange, oxygen consumption. All the factors which lead to increased ROS production is involved in formation of oxidative DNA damage, and also there is a linear rate between tissue oxygen consumption and basal level of 8-OHdG [13].

Antioxidant and oxidant enzymes neutralize the exposition of ROS. Against to the oxidative DNA damage the second defense way is the mechanism of DNA repairing. Damaged biomolecules are repaired. In spite of the mechanism of DNA repairing, oxidatively modified DNA exits and this adduces that ROS cannot be totally prevented in human tissues [13]. These knowledge can support our findings that SOD levels in study group (7,26 ±0,31 U/ml) are higher than untreated group (6,95±0,55 U/ml). Finding shows ABS's antioxidant's activities are not important, although using ABS increases antioxidant enzyme levels. To point out the effects of ABS on antioxidant system, it is need to increase the number of the subjects in experimental study. Therefore, a negative correlation between 8-OHdG and SOD in study group (r= -0.201, p=0.702) and untreated group (r=-0.388, p=0.519) is not statistically important (figure 2 and 3). That findings show that ABS decreases the oxidation of DNA without important changing in levels of SOD.

Nonetheless, Temneanu et al [12] imply that the correlation between the types of stress, the antioxidant involved enzymes and the lung responsiveness is very important to estimate in a correct manner and they suggest SOD can monitor lung reactivity and animal experimental models suggest the possibility of initiating new-targeted antioxidant therapies.

The cytotoxic effects of free radicals are the initiation of peroxidation of polyunsaturated fatty acids in membrane or plasma lipoproteins, the direct inhibition of mitochondrial respiratory chain enzymes, the activation of membrane sodium channels and other oxidative modifications of proteins [31, 32].

Willy et al [33] demonstrated an increased respiratory burst in granulocytes and reperfusion-associated DNA effects could not prove a causal relationship between these two phenomena. Nevertheless they suggest that DNA effects are due to postischemic oxidative stress. Besides, the researchers implied a close relationship exist between the DNA damaging activity of endogenous reactive oxygen species and their effects on the microcirculation. Seen from this respect, our findings are not same as Willy et al [33]. We could not find a close relationship between SOD and 8-OHdG.

In evaluation of MPO levels, ABS decreases (8,36±0,65 U/ml) MPO but, we did not find an important changing between the two groups. Williams et al [27] found higher baseline plasma MPO levels for the patients undergo elective thoracic surgery (38.3±6.4 ng/ml, n=18) than those in normal controls (28.3± 6.2 ng/ml, n=10). Therefore, our findings are not in agreement with Williams et al [29]. There is no correlation between MPO and 8-OHdG (r=0.318, p=0.513) in study group whereas there is close relation (r=0.884, p=0.047) in control group. We can just educe that using ABS did not show any changing in MPO levels.

As a result, we could not compare our findings with the other studies because of limited research and differences in methods. But it is clear that Ankaferd Blood Stopper reduced the level of 8-OHdG that may mean oxidative DNA damage prevented by ABS.

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Conflict of Interest: Authors have no conflict of interests.

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