

1-1-2017

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### Recommended Citation

AKTOP, SERTAÇ; ÇEVRELİ, BURCU; GENÇ, DENİZ; KAŞIKÇI, EMEL SERDAROĞLU; ÜSTÜNDAĞ, NİL ÇOMUNOĞLU; ZİBANDEH, NOUSHİN; ÖZCAN, ELİF MERVE; GÖÇMEN, GÖKHAN; GÖKER, MEHMET KAMİL; UZBAY, İSMAİL TAYFUN; and AKKOÇ, TUNÇ (2017) "Effects of Ankaferd BloodStopper on dermal healing in diabetic rats," *Turkish Journal of Medical Sciences*: Vol. 47: No. 2, Article 45. <https://doi.org/10.3906/sag-1604-145>

Available at: <https://journals.tubitak.gov.tr/medical/vol47/iss2/45>

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## Effects of Ankaferd BloodStopper on dermal healing in diabetic rats

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Received: 29.04.2016 • Accepted/Published Online: 11.09.2016 • Final Version: 18.04.2017

**Background/aim:** Diabetes mellitus inhibits wound-induced angiogenesis, impairs the wound healing process, and leads to the development of chronic wounds. Ankaferd BloodStopper (ABS) is a new and promising local haemostatic agent. Although the mechanism of ABS-mediated haemostasis is well established, little is known about the associated histological and biochemical tissue reactions. The aim of this study was to evaluate the effects of this new-generation local haemostatic agent on short-term soft-tissue healing in streptozotocin (STZ)-treated rats.

**Materials and methods:** The 24 Wistar albino rats used in this study were divided into STZ-treated (STZ, n = 12) and nontreated groups (control, n = 12). Four days prior to surgery, rats in the STZ group were subcutaneously administered 60 mg/kg STZ intraperitoneally, while rats in the control group were administered 1 mL saline/kg. An incision was made in the dorsal dermal tissue of all rats, and either ABS or no haemostatic agent (NHAA) was applied to the wound before suturing. All of the rats were euthanised on postoperative day 4. Blood and skin samples were evaluated biochemically and histologically.

**Results:** The results showed that STZ treatment impaired soft-tissue healing, assessed by measuring glutathione and lipid peroxidation levels. Moreover, while good histological results were obtained in the control group treated with ABS, there were fewer benefits in the STZ-treated group.

**Conclusion:** ABS's benefits in the control group seemed to lose their effectiveness under STZ medication.

**Key words:** Streptozotocin, Ankaferd BloodStopper, glutathione, lipid peroxidation, vascular endothelial growth factor, transforming growth factor-beta

### 1. Introduction

Diabetes mellitus (DM) is a chronic, life-long metabolic disease occurring worldwide. Affected individuals require continuous follow-up and suffer numerous disease-related complications. The high levels of blood glucose that characterise DM contribute to an increase in free radical production, enhanced oxidative stress, and changes in antioxidant capacity (1-3).

Streptozotocin (STZ) is the chemical agent most frequently used to induce experimental diabetes in animal models. By binding to glucose receptors in the plasma membrane, STZ blocks glucose-induced insulin secretion (2,3) and alters blood insulin and glucose concentrations.

Among the many effects of free radical production is lipid peroxidation (LPO). Oxidation of membrane polyunsaturated fatty acids disrupts cell structure and

function and induces cytotoxic, hepatotoxic, mutagenic, and genotoxic effects related to the release of aldehydes (4,5).

The tripeptide glutathione (GSH) is found in the cells of all organisms. In humans, GSH concentrations are highest in the liver; in hepatocytes, GSH accounts for 90% of nonprotein sulfhydryl groups. As an important reducing agent and antioxidant, GSH maintains the cellular oxidation-reduction balance and protects cells against the toxic effects of oxidants, whether of endogenous or exogenous origin (5-7).

Ankaferd BloodStopper (ABS) (Ankaferd Health Products Ltd., İstanbul, Turkey) is a medicinal extract from the plants *Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica* (7,8). ABS is not only an effective haemostatic agent but also

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exhibits tissue antioxidant properties (5,9). In Turkey, ABS is approved for the management of postsurgical dental bleeding and external haemorrhage (5). ABS forms an encapsulated protein providing focal points for erythrocyte aggregation, without affecting other components of the coagulant system.

Studies on wound healing often involve assessments of collagen, a protein crucial to restoring the integrity of the skin (10,11), and angiogenesis, in which vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF- $\beta$ ) play prominent roles (12,13). VEGF controls a variety of endothelial cell functions involved in angiogenesis and protects these cells from apoptosis (14). Fibroblast growth factor-2 (FGF-2) and TGF- $\beta$  induce VEGF expression in vascular endothelial cells (15). In addition, TGF- $\beta$  is an important regulator of tissue morphogenesis and a potent inhibitor of proliferation for most cell types (13).

Several studies have shown that ABS promotes tissue healing (5,9,16–21). Therefore, in this study, we addressed the effectiveness of ABS in diabetes, in which wound healing is severely impaired. Specifically, we evaluated the effects of ABS on short-term dermal soft-tissue healing in rats with STZ-induced diabetes.

## 2. Materials and methods

### 2.1. Animals and treatment

Twenty-four male Wistar albino rats weighing 280–450 g were divided into STZ-treated and control groups ( $n = 12$  each). The animals were obtained from the Department of Experimental Research Unit, Üsküdar University (İstanbul, Turkey), where the surgery and postoperative care were performed. All experimental protocols were approved by the Animal Care and Use Ethical Committee of Marmara University (no: 40.2013.mar)

The 12 control rats were injected subcutaneously with 1 mL saline/kg intraperitoneally and the 12 rats in the STZ group were injected subcutaneously with a single dose of STZ (60 mg/kg, freshly dissolved in 1 mL of saline) intraperitoneally 4 days before surgery. In the latter group, the animals were considered to be diabetic, based on blood glucose levels  $\geq 250$  mg/dL. All 24 rats underwent surgery and on postoperative day 4 were euthanised by the injection of a high dose of anaesthetic.

The animals were anaesthetised with a combination of 90 mg/kg ketamine (Ketalar, Pfizer İlaçları Ltd. Şti, İstanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer HealthCare, Leverkusen, Germany). Surgery was performed under aseptic conditions.

The dorsal skin was shaved, and an incision 2 cm long and perpendicular to the head-to-tail direction was made. In 6 of the 12 animals in each group, the wounds were sutured without application of haemostatic agent and left

to heal naturally. The incisions of the other 6 animals were treated with 0.25 ml ABS, applied before suturing.

The dorsal skin was excised completely from the euthanised animals, and the wounded tissue area was prepared for histological evaluation at the Department of Medical Pathology, Cerrahpaşa Faculty of Medicine, İstanbul University (İstanbul, Turkey). Blood samples taken from the euthanised animals were used in biochemical evaluations, performed at the Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Üsküdar University (İstanbul, Turkey).

### 2.2. Biochemical tests

Blood GSH concentrations were determined according to the method of Beutler, using metaphosphoric acid for protein precipitation and 5'5'-dithiobis-2-nitro-benzoic acid for colour development (22). Blood LPO levels were assayed by measuring serum malondialdehyde levels, determined as thiobarbituric acid reactive substances according to the method described by Yagi (23).

### 2.3. Histological evaluation

Surgical specimens were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin blocks. The 4- $\mu$ m-thick sections cut from the blocks were stained with haematoxylin and eosin. In addition, 4- to 6- $\mu$ m-thick sections were cut, floated on positively charged microscope slides, and labelled with an indirect avidin-biotin-peroxidase complex for automated immunohistochemistry analysis (Ventana Medical Systems, Tucson, AZ, USA). Tissue sections were deparaffinised, rehydrated in decreasing concentrations of alcohol, and washed with distilled water. Antigen retrieval was achieved by incubating the slides in 10 mM sodium citrate at 36 °C for 30 min. The slides were then incubated for 1 h with TGF- $\beta$  rabbit polyclonal antibody (1:500, Abcam, Cambridge, UK), VEGF rabbit polyclonal antibody (1  $\mu$ g/mL, Abcam), and collagen 4 rabbit polyclonal antibody (1/1000, Abcam) and then counterstained with haematoxylin. Positive controls for TGF- $\beta$ , VEGF, and collagen consisted of human placental tissue, haemangioma tissue, and human epidermal keratinocyte lysate, respectively. The negative control consisted of PBS instead of the primary antibody.

#### 2.3.1. Histological scoring

Immunostaining was scored by a pathologist blinded to the clinical data and was evaluated using a double-headed BHS Olympus microscope. The intensity of staining was graded as 0, negative; 1+, weak; 2+, moderate; and 3+, strong. The extent of immunostaining was graded as 0 (0%), negative, 1+ (0%–25%), 2+ (26%–50%), and 3+ (51%–100%).

**2.4. Statistical analysis**

All statistical analyses were performed using the Statistical Package for the Social Sciences, version 22.0 (SPSS, Chicago, IL, USA). The results were evaluated using the Shapiro–Wilk test for data with a normal distribution and are expressed as means ± standard deviation. Quantitative data that fulfilled the parametric criteria were analysed using Student’s t test; nonparametric data were analysed using the Mann–Whitney U test. A P value <0.05 was considered to indicate statistical significance.

**3. Results**

**3.1. Biochemical results**

**3.1.1. Comparison of GSH and LPO levels in blood samples from the control and STZ groups**

There were no significant differences in the blood samples from ABS versus NHAA rats in either the control or STZ group. The GSH values were significantly higher

in the control group than in the STZ group for both the NHAA- and the ABS-treated rats (P = 0.0001; P = 0.003, respectively). LPO values were significantly higher in the NHAA blood samples of the STZ group than those of the control group (P = 0.0001). The LPO values in the ABS-treated animals were slightly, but not significantly, higher in the STZ group than in the control group (Table 1).

**3.2. Histological results**

**3.2.1. Comparison of histological scores in tissues of the control and STZ groups**

In the control group, the scores for both the intensity and extent of collagen 4 staining were significantly higher in the ABS-treated than in the NHAA-treated tissues of the control group and the ABS-treated tissues of the STZ group (P = 0.021 and P = 0.021, respectively). The VEGF intensity scores within the control group were significantly higher in ABS-treated than in NHAA-treated tissues (P = 0.034) (Table 2).

**Table 1.** GSH and LPO levels in blood samples on postoperative day 4.<sup>a</sup>

		Control group (n = 12)			STZ group (n = 12)			P
		Mean	±	SD	Mean	±	SD	
GSH (mg/g p)	ABS (n = 6)	31.35	±	4.62	18.58	±	3.1	0.0001*
	NHAA (n = 6)	42.03	±	10.14	20.52	±	3.01	0.003*
P			0.51			0.298		
LPO (nmol MDA/mg p)	ABS (n = 6)	5.95	±	1.7	7.8	±	1.23	0.06
	NHAA (n = 6)	5.88	±	0.67	8.52	±	0.82	0.0001*
P			0.922			0.264		

Abbreviations: SD: standard deviation; ABS: Ankaferd BloodStopper; NHAA: no haemostatic agent administered, p: protein, GSH: glutathione, LPO: lipid peroxidation

<sup>a</sup>Values are means ± SD.

\*P < 0.05 according to Student’s t test

**Table 2.** Histologic scores of tissue samples on postoperative day 4.

	Collagen 4	Collagen 4	VEGF	VEGF	TGF-β	TGF-β
	extent	intensity	extent	intensity	extent	intensity
Control - NHAA	-	-	++++	++++	+	+
Control - ABS	++*	++*	++++	+++++*	++	+
STZ - NHAA	-	-	++++	+++++	++	++
STZ - ABS	-	-	++++	+++++	++	+

Abbreviations: ABS: Ankaferd BloodStopper; NHAA: no haemostatic agent administered; VEGF: vascular endothelial growth factor; TGF-β: transforming growth factor-beta

\*Statistically significant according to Student’s t test or the Mann–Whitney U test

#### 4. Discussion

Many studies have investigated the healing potential of particular agents in animal models of diabetes, examining clinical effects or histological outcomes (24–27). ABS has been used as a local haemostatic agent in surgery, but it was also shown to promote healing (5,9). Its effects on soft-tissue healing have been investigated (17,19–21), but not in a STZ model of diabetes. Therefore, the aim of the present study was to histologically assess the effects of ABS on collagen 4, blood GSH levels, and LPO to evaluate its potential effects in early-stage soft-tissue healing on STZ-treated rats.

The STZ group was injected with a single dose of STZ (60 mg/kg, freshly dissolved in 1 mL of saline solution) intraperitoneally for 4 days before surgery. Many other successful STZ administration protocols are described in the literature (2,28–30).

Collagen, which is beneficial for endoepidermal growth and therefore healing, is a major functional extracellular matrix protein in the dermal layer of the skin (20). Collagen 4, VEGF, and TGF- $\beta$  are commonly used markers of healing potential (24,26,27,30–32) and were evaluated in this study as well. Wound healing comprises four primary stages that occur in a partly sequential, partly overlapping process. During the proliferation (third) phase, fibroblasts migrate from the surrounding connective tissue, proliferate, and begin to synthesise a matrix of ground substance, fibronectin, and extracellular proteins such as collagen, elastin, and integrins. In addition, macrophages release numerous growth factors that promote angiogenesis, including basic FGF and VEGF (33).

Huri et al. applied chitosan and ABS as haemostatic agents to the excision area in 40 Wistar rats with partial nephrectomy and found no significant differences in their effects on haemostasis. They suggested that ABS was just as effective as other haemostatics, none of which led to fibrosis, adhesion, or calcification, and all achieved good histopathological results (34).

Satar et al. compared ABS and oxidised cellulose in experimentally injured rats. The bleeding time was shorter in the ABS-treated group, as determined in liver sections. The favourable histopathological changes included fewer signs of inflammation than in the oxidised-cellulose-treated group on days 7 and 14 (17).

In a study based on a rat skin defect model, Akalin et al. reported higher collagen deposition scores and lower inflammatory scores in the ABS group. The fibroblast proliferation scores were higher in the ABS group on day 14 (35). The authors suggested that ABS can be safely used for the repair of full-thickness wounds. Yüce et al. also recommended ABS to promote wound healing (19).

Our study demonstrated that, in the control, nondiabetic animals, collagen 4 extent and intensity were significantly higher in ABS tissues than in NHAA tissues ( $P = 0.021$ ). VEGF scores were also significantly higher in ABS tissues ( $P = 0.034$ ). These histologically based observations suggest that ABS supports wound healing. Moreover, the extent and intensity of collagen staining were significantly higher in the ABS tissues of the control group than in those of the STZ group ( $P = 0.021$ ), indicating that ABS did not compensate for the effects of STZ treatment on wound healing.

Isler et al. examined the effects of ABS on bone healing during the first 7 days and noted that ABS decreased inflammation and necrosis and increased new bone formation (16). Bulut et al. evaluated ABS and routine antibiotic prophylaxis (AP) on the early healing of bone defects in diabetic rats but were unable to detect significant differences in new bone formation between the AP- and ABS-treated animals (18).

ROS are produced in metabolic and physiological processes, but in healthy individuals their harmful oxidative effects are prevented (35) via enzymatic and nonenzymatic antioxidative mechanisms. A shift in the oxidative and antioxidative balance towards the former, as occurs in many disorders including DM, results in oxidative stress (22,36).

An increase in the concentration of the end products of LPO is the most prominent evidence of free radical involvement in human disease (37). However, rather than accelerating the bulk peroxidation of cell membrane lipids (22), oxidative stress is likely to cause cell damage, which in turn leads to a secondary increase in LPO (38). Thus, LPO is often a late event that accompanies rather than causes cell death (39). In our study, LPO levels were significantly increased in the NHAA blood samples but not in the ABS blood samples of the STZ group ( $P = 0.0001$ ,  $P = 0.06$ , respectively). Oxidative stress has been associated with insulin resistance (29) and thus may develop in response to STZ treatment, resulting in poor soft-tissue healing (25,29,40–42). The absence of a significant difference in the blood samples of the ABS-treated rats in the control versus STZ groups suggests that ABS reduces the oxidative stress that occurs in DM.

Koluman et al. revealed the presence of several antioxidant molecules (including tocotrienols, vitamin E, tryptophan, estriol, galangin, apigenin, oenin, 3,4-divanillyltetrahydrofuran, TBHQ, thymol, BHA, BHT, lycopene, glycyrrhetic acid, and tomatine), which may have clinical implications in the pharmacobiological actions of ABS. They concluded that the antioxidant content of ABS should be investigated in future studies (43).

Conversely, GSH is part of an integrated antioxidant system that protects cells and tissues from oxidative damage (44). In DM, a decrease in tissue GSH could be due to a decrease in the synthesis of GSH or to an increase in its degradation by oxidative stress (40). We found that STZ treatment caused a significant reduction in blood GSH values, both in ABS-treated and NHAA-treated rats ( $P = 0.0001$ ,  $P = 0.03$ , respectively).

Aktop et al. found that warfarin treatment in rats inhibited antioxidant capacity, similar to the treatment effects of STZ treatment (5). Similar to our own results, in the control group of their study, there were no statistically significant differences in GSH and LPO levels in ABS-treated versus NHAA-treated tissues ( $P = 0.051$ ,  $P = 0.922$ ). The same authors evaluated catalase and superoxide dismutase activities in warfarin-treated rats and found positive effects of ABS on soft-tissue healing (9). In our

study ABS administration improved soft-tissue healing, consistent with the histologically determined increase in collagen 4 levels, but there were no significant benefits with respect to blood GSH and LPO levels.

Aydin et al. applied ABS to the healing tendons of rats but found no beneficial effects, as determined histologically (21). Evren et al. reported significantly increased fibrosis and necrosis in the auricular cartilages of New Zealand rabbits treated with ABS (45).

In conclusion, STZ treatment may impair soft-tissue healing in rats by altering the antioxidant–oxidative stress balance. ABS had histologically confirmed benefits on wound healing in control rats but not in STZ-treated rats. Nonetheless, ABS seems to reduce the oxidative stress associated with diabetic metabolism, based on GSH and LPO levels that were similar to the control levels.

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