Beneficial effects of Ankaferd Blood Stopper on dermal wound healing: an experimental study

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Key words
Ankaferd Blood Stopper; Topical haemostatic agents; Wound healing

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doi: 10.1111/j.1742-481X.2012.01063.x

Abstract
Ankaferd Blood Stopper® (ABS) is a folkloric medicinal plant extract used as a haemostatic agent in traditional Turkish medicine. The aim of this study was to investigate the efficacy of ABS on the healing of dermal wounds in a rat model. Twenty Wistar albino rats were divided into two groups. Standard full-thickness skin defects were created on the back of the rats. In the control group (group 1), dressings moisturised with saline were changed daily. In the study group (group 2), the wounds were cleaned daily with saline, Ankaferd solution was applied, then the wounds were covered with moisturised dressings. The contraction percentage of wound areas were calculated on the 3rd, 7th, 10th and 14th days using a planimetric programme. On day 14, the wound areas were excised for histopathological examination, inflammatory scoring and evaluation of collagen deposition. The study group was superior to the control group in terms of inflammatory scoring, type I/type III collagen ratio and wound contraction rates. ABS® may be used effectively and safely on full-thickness wounds as a natural product.

Introduction
The process of wound healing is a progressive and dynamic event with predictable stages that occur with varying intensities (1). Most skin wounds can heal naturally. The quality of skin wound healing can be improved by the application of different materials. There is a wide range of materials currently in use, some of which are natural products. Many of these preparations as a necessity were derived from natural sources, including a diverse array of plants, animal products and minerals (2). Ankaferd Blood Stopper® (ABS) is a unique folkloric medicinal plant extract, which has historically been used in Turkish traditional medicine as a haemostatic agent (3). The aim of this study was to investigate the effectiveness of ABS on dermal wound healing in a rat model.

Materials and methods
Experimental model
Twenty healthy adult male Wistar albino rats weighing 200–250 g were used. All the rats were obtained from our

Key Messages
• in ABS group, the wound closure was almost complete on day 14
• in the control group, the granulation tissue had been slowly moved from the wound base to the wound surface
• the wound contraction progressed more slowly in the control group
• the wound contraction rate is significantly higher in the ABS group than the control group both during and at the end of the experiment
• there was a significant difference between the control and ABS groups according to the mean inflammatory scores (P < 0·05). The scores were better in the study group when compared with the control group
• the mean collagen scores and type I/type III collagen ratios were different between the groups (P < 0·05). The collagen scores and type I/type III collagen ratios were higher in the ABS group than in the control group
• Ankaferd Blood Stopper® may be used effectively and safely on full-thickness wounds as a natural product
animal research centre. The rats were housed in stainless steel cages in an animal room maintained at 22°C with 12-hour dark–light periods. All were fed with the same amount of a laboratory pellet diet and water ad libitum and fasted for 12 hours before the procedures. The procedures in this experimental study were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and approved by the Animal Ethics Committee of Ankara Research and Training Hospital.

**Surgical technique**

Two groups were randomly constituted of 10 rats each. The rats were anaesthetised with intramuscular injections of 50 mg/kg ketamine hydrochloride (Ketalar, Parke-Davis-Eczacıbası, Istanbul, Turkey). The operation sites were shaved and disinfected with povidone-iodine. A 2 × 1 cm rectangular-shaped incision was made on the back of the rats centred on the midline, then a standard full-thickness skin defect, including panniculus carnosus, was created on this site. In the control group (group 1), no intervention was made on the dermal wounds. The wounds were cleaned daily with saline and covered with moisturised dressings. In the study group (group 2), the wounds were cleaned daily with saline, Ankaferd solution was applied, then the wounds were covered with moisturised dressings. All wounds were followed for 14 days and no complications developed during this period. Open wounds were drawn on graph acetate paper with a marker pen on the 3rd, 7th, 10th and 14th days and photographed with a digital camera. All rats were euthanised with high-dose ketamine hydrochloride on postoperative 14th day.

**Histopathological analysis**

The wound area was excised en bloc together with the scar tissue. All specimens were fixed in 10% phosphate-buffered formaldehyde solution for 24 hours at room temperature. Histopathological assays were performed in a blind manner by a pathologist. Specimens were washed in tap water and dehydrated through graded alcohol series. After passing through the routine histological series, tissues were embedded in paraffin blocks. Sections of 5 µm were cut, deparaffinised and rehydrated. Sections were counterstained with haematoxylin and eosin (H&E), and Masson’s trichrome and reticulin stain (method of silvering). The intensities of polymorphonuclear leucocytes and mononuclear leucocytes and the extent of fibroblast proliferation and vascular proliferation were evaluated by inflammatory scoring to determine the general characteristics of scar tissue in the sections stained with H&E (Figure 1). The qualitative assessment of total collagen deposition was performed using the Masson’s trichrome stain. The collagen fibres were identified as blue colour stained with Masson’s trichrome (Figure 2). In the reticulin stain, the fibres observed in the form of thin black fibres were determined as type III and yellow fibres as type 1 collagen (Figure 3).

The number of polymorphonuclear leucocyte and mononuclear leucocyte and the degrees of vascular proliferation and fibroblast proliferation were measured on a numerical scale from 0 to 3 to determine inflammatory scores.

The structural density of collagen was scored on a numerical scale from 0 to 5. (0) indicates the lack of collagen, (1) indicates the presence of collagen in the form of a single fibre, (2) indicates the presence of collagen in the form of few fibres, (3) indicates more intense but loose collagen, (4) indicates that collagen overlays a microscopic field, but
have gaps between them and (5) indicates that collagen overlays a microscopic field and have a very dense structure.

The examination of wound contraction rate
Open wounds were drawn on graph acetate paper with a marker pen on the 3rd, 7th, 10th and 14th days and photographed with a digital camera. The surface area of the wounds was measured with a planimetric programme on computer by scanning the acetate sheets. The percentage of contraction was calculated by the following formula:

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\text{Percentage of contraction (xth day)} = 100 - \left[\frac{\text{Total wound area on xth day}}{\text{Total wound area on day 0}}\right] \times 100.
\]

Total wound area on xth day was the contraction percentage of the wound area obtained by the planimetric programme on the 3rd, 7th, 10th and 14th days. The original wound area on day 0 was assumed to be 100% for all wounds.

Statistical analysis
Multiple comparisons between the groups were performed with one-way analysis of variance and post hoc tests. Differences between the groups were analysed with the Mann–Whitney U-test. Statistical analysis was performed with the SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Values of \( P < 0.05 \) were considered to be significant.

Results
In the study group, the wound closure was almost complete on day 14. In the control group, the granulation tissue had been slowly moved from the wound base to the wound surface.

The wound contraction progressed more slowly in the control group. Wound contraction rates were evaluated in the form of mean and standard deviation. The wound contraction rate was 0.913 ± 0.01% in the study group and 0.836 ± 0.02% in the control group. The results of the statistical analysis determined the wound contraction rate to be significantly higher in the study group than the control group both during and at the end of the experiment (\( P < 0.05 \)).

According to the mean inflammatory scores, a significant difference was found between the control and study groups (\( P < 0.05 \)). The scores were better in the study group when compared with the control group.

When the mean collagen scores and type I/type III collagen ratios were compared statistically, a significant difference was found between the groups (\( P < 0.05 \)). The collagen scores and type I/type III collagen ratios were higher in the study group than in the control group.

Discussion
Damage or loss of skin integrity caused by a skin or cutaneous wound may impair skin functions to various degrees ranging from significant disability to even death (4). Skin wounds can arise from mechanical trauma, surgical procedures, reduced blood circulation, burns or ageing (4).

The process of wound healing is a progressive and dynamic event with predictable stages that occur with varying intensities. Wound healing comprises four primary stages that occur in a sequential cascade of overlapping processes: haemostasis, inflammation, proliferation and remodelling (1). Haemostasis, which is initiated by a traumatic event, occurs as a response to tissue injury and is initially a vascular response. During this acute phase, platelets release growth factors, haemostatic factors and inflammatory mediators. It is these factors that are responsible for driving the initial wound-healing cascade (1).

In the inflammatory phase, the polymorphic neutrophils migrate to the site of local injury, phagocyte bacteria and assist in the removal of devitalised tissue (1). During the proliferation phase, monocytes differentiate into macrophages, which are then responsible for amplifying, coordinating and sustaining the wound-healing response. Fibroblasts migrate from the surrounding connective tissue, proliferate and begin to synthesise a framework of ground substances, fibronectin and extracellular matrices such as collagen, elastin and integrins. In the remodelling phase, which is last, macrophages and fibroblasts increase their release of collagenase, resulting in the breakdown of excess collagen (1).

Most skin wounds can heal naturally. The quality of skin wound healing can be improved by the application of different materials. Today, there is a wide range of natural and synthetic materials which are used for this purpose. Many of the natural materials are still derived from a diverse array of plants (2).

ABS is a traditional herbal medicine that has been used in Anatolia as a haemostatic agent for centuries. The use of this product was approved by the Ministry of Health, Turkey, on 26 October 2007 (5). ABS is a standardised mixture of the plants, Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum and Urtica dioica, each of which has some effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and/or cell mediators (6).

Each ingredient of this mixture has specific characteristics. G. glabra inhibits angiogenesis, decreases vascular endothelial growth factor production and cytokine-induced neovascularisation. G. glabra also has anti-inflammatory, antithrombin, antiplatelet, antioxidant, antiatherosclerotic and antitumour activities. T. vulgaris has been shown to inhibit varying levels of antioxidant activity, which may help to prevent in vivo oxidative damage, such as lipid peroxidation, associated with atherosclerosis. Inoculation experiments on detached leaves of V. vinifera exhibited enhanced resistance towards pathogens. V. vinefera also has antiatherosclerotic and antitumour effects. A. officinarum has been shown to inhibit nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. U. dioica can produce hypotensive responses through a vasorelaxation effect mediated by the release of endothelial nitric oxide and the opening of potassium channels, and through a negative inotropic action (7).

Goker et al. (3) showed that the ABS-induced network formation is related to the functions of blood proteins and red blood cells. ABS-induced formation of the protein with vital erythroid aggregation covers the entire physiological haemostatic process (3,8). Mainly, there are distinct important components of the ABS-induced haemostatic network. Vital erythroid aggregation takes place with the spectrin and
ankrin receptors on the surface of red blood cells. Those proteins and the required adenosine triphosphate bioenergy are included in the protein library of ABS. Ankaferd Blood Stopper also upregulates globin transcription factor (GATA)/Friend of GATA (FOG) transcription system affecting erythroid functions. Urotensin II is also an essential component of ABS and represents the link between injured vascular endothelium, adhesive proteins and active erythroid cells (8).

The basic mechanism of action for ABS appears to be the formation of an encapsulated protein network that provides focal points for erythrocyte aggregation. However, in plasma which contains fibrinogen, ABS also interacts with fibrinogen as well as the other blood proteins. Plasma fibrinogen activity and antigen levels decrease following the addition of ABS in parallel to the prolonged thrombin time. Total protein, albumin and globulin levels decrease after the addition of ABS (9). As individual clotting factors (coagulation factors 2, 5, 7, 8, 9, 10, 11 and 13) were not affected by the network formation, the antihaemorrhagic process was possibly driven by protein agglutination. Blood cells (erythrocytes and platelets) also aggregated and participated in the network formation, with the erythrocytes forming a mass (3). Therefore, ABS could be effectively used both in individuals with normal haemostatic parameters and in patients with deficient primary and/or secondary haemostasis (8).

The general action mechanism of agents in the local haemostatics group is as follows: creating a plug by introducing a foreign object (gelatin sponge, oxidase cellulose and oxidase regenerated cellulose) or activating thrombocytes upon contact and ensuring the release of mediators that trigger the natural haemostatic process (thrombin and microfibril collagen). Some agents have additional mechanisms of action, such as the adhesive effect of fibrin preparations and the vasoconstrictor effect of adrenalin. ABS is unlike other local haemostatic agents because its mechanism of action is based on forming a polymerised protein network which becomes a focal point for sedimentation of erythrocyte aggregates (9).

Besides its haemostatic activity, ABS may also inhibit the growth of bacteria (8). The antimicrobial activity of ABS was tested against 102 clinical isolates in a previous study, which reported that ABS was active against all these isolates, with zones of inhibition within the 10- to 8-mm diameter range (10). Antibacterial activities of ABS against gram-positive and gram-negative food and human pathogens have also been reported (8).

Neither local nor systemic adverse effects and/or toxicity have been observed in association with experimental and anecdotal topical application of ABS. Acute mucosal toxicity, haematotoxicity, hepatotoxicity, nephrotoxicity and biochemical toxicity have not been observed during the short-term follow-up of animals (8).

ABS represents an alternative treatment modality for many kinds of bleeding that are resistant to conventional methods. The ability of ABS to induce formation of a protein network not only makes it an effective haemostatic agent, but also confers anti-infective, antineoplastic and healing modulator properties to the extract (11).

Isler et al. (7) investigated the effects of ABS on early bone healing in a rat model. The conclusion was reached that defects treated with ABS showed more intense new bone formation and less occurrence of necrosis, which may be related to the increased speed of healing and decreased inflammation which is associated with the antioxidant activity of the components of the ABS.

Akbal et al. (12) designed an experimental study to show the effectiveness of ABS in enhancing mucosal healing and suppressing stricture formation caused by caustic oesophageal injuries. The results of that experimental study showed that oral ABS application prevents inflammation, scar formation, weight loss and mortality in oesophageal caustic injuries.

Kılıç et al. (13) investigated the efficacy of ABS in the prevention of air leakage in the lungs and bleeding in an animal experiment. The results showed that ABS terminated air leakage in the lung parenchyma significantly and effectively. No significant difference was seen compared with the standard surgery group, although there was significant cessation of bleeding.

Ozaslan et al. (14) applied ABS to the severe radiation colitis of a 71-year-old woman who had undergone pelvic radiotherapy because of cancer of the cervix and was admitted with rectal bleeding. They sprayed ABS solution with a sclerotherapy needle onto the lesion. They reported that ABS may also offer an exciting option in the therapy of radiation colitis, because of the ease of application, speed of action, non-toxicity and low cost.

ABS was also used for different clinical situations such as the management of oral cavity bleedings and persistent nasal bleedings, for controlling upper and lower gastrointestinal bleedings and fundal variceal bleedings, in control of intraoperative and postoperative bleedings during adenoectomy, tonsillectomy, partial nephrectomy, total mesorectal excision and radical prostatectomy (15–23).

For these above-mentioned reasons, this study was planned to investigate the effects of ABS on wound healing in a rat model.

In the current study, the mean histological scores of both polymorphonuclear and mononuclear leucocytes were higher in the study group than in the control group. The polymorphic neutrophils migrate to the site of local injury. These inflammatory cells phagocytose bacteria and assist in the removal of devitalised tissue (1). Throughout this process, especially during the proliferation phase, monocytes differentiate into macrophages, which then release numerous growth factors responsible for angiogenesis and granulation tissue formation (1).

Dermal fibroblasts have numerous functions, not only in synthesising and depositing extracellular matrix components, but also in proliferation and migration in response to chemotactic, mitogenic and modulatory cytokines (24). In the current study, the fibroblast proliferation scores were also higher in study group than in the control group.

During the proliferation phase, basic fibroblast growth factor and vascular endothelial growth factor are responsible for endothelial cell proliferation and the synthesis of new vessels (1). In the current study, vascularisation scores were higher in the study group than in the control group.

Collagen, which is beneficial for endopidermal growth to promote healing, is a major functional extracellular matrix
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protein of the dermal layer of skin. Type I collagen is the predominant collagen of the dermis and forms collagen fibres which maintain dermal configuration (25,26). In the current study, the collagen scores and type I/type III collagen ratios were higher in the study group than in the control group.

All these parameters that were important for effective wound healing, including polymorphonuclear leucocyte, mononuclear leucocyte, vascularisation and fibroblast proliferation, were better in the study group when compared with the control group. Therefore, it was concluded that the positive effects of ABS on wound healing might be attributable to these useful histopathological alterations.

ABS may be used effectively and safely on full-thickness wounds as a natural product. Additional studies are required to evaluate the clinical benefits and any possible adverse effects of the application of ABS on wound healing.

Author contribution

Cagri Akalin and Aziz Mutlu Barlas performed the experiment; Serdar Kuru performed the experiment and wrote the article; Kemal Kismet helped in experimental design and edition of article; Bugra Kaptanoglu and Aydin Demir helped in evaluation and statistical analyses of the results, Hesna Muzeyyen Astarci and Huseyin Üstün in pathological evaluation, and Ertugrul Ertas in experimental design and edition of article.

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