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RESEARCH ARTICLE

Effects of Ankaferd Blood Stopper (ABS) and Topical Tripeptide Copper Complex (TCC) on Wound Healing in Rats: An Experimental Study

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Summary

We investigated the effects of Ankaferd Blood Stopper (ABS) and tripeptide copper complex (TCC) on wound healing in rats. A total of 24 outbred, male, Sprague-Dawley rats were randomly divided into (1) ABS, (2) TCC, and (3) control groups. Bilateral experimental wounds were created near the caudal border of the scapula. Each wound in the respective treatment group was treated daily with sponges soaked in ABS solution, topical TCC gel, or saline. On days 0, 7, 14 and 21, unhealed wound area was measured and biopsy samples were taken for histopathological analysis (except day 0). Median time for the first observable granulation tissue was not significantly different in the ABS, TCC, and control groups (5.8, 5.5, and 6.7 days, respectively) (P>0.05). Filling of the open wound with granulation tissue to skin level was significantly slower in the control group than in the ABS and TCC groups (18, 9, and 11 days, respectively). The mean unhealed wound area was significantly smaller and the mean percentage of total wound healing was significantly higher in the ABS- and TCC-treated wounds than in the control wounds on day 7, 14, and 21 (P<0.05); the average time for healing was also significantly shorter in the treatment groups than in the control group (17.4, 16.8 vs. 23.6 days, respectively) (P<0.05). Our results suggest that topical application of ABS and TCC have beneficial effects on wound healing.

Keywords: *Wound healing, Ankaferd Blood Stopper (ABS), Tripeptide copper complex (TCC), Skin, Rat*

Ankaferd Blood Stopper (ABS) ve Topikal Tripeptid Bakır Kompleksinin (TCC) Ratlarda Yara İyileşmesi Üzerine Etkisi: Deneysel Çalışma

Özet

Bu çalışmada, Ankaferd Blood Stopper (ABS) ve tripeptid bakır kompleksinin (TCC) ratlarda yara iyileşmesi üzerine etkilerinin araştırılması amaçlanmıştır. Sprague-Dawley ırkı, dişi, 24 adet rat, rastgele şekilde 1) ABS, 2) TCC, ve 3) Kontrol olmak üzere 3 gruba ayrıldı. Skapulanın kaudal sınırına yakın sahada bilateral deneysel yaralar oluşturuldu. Her bir yara günlük olarak grubuna göre ABS solüsyon emdirilmiş sponjlar, topikal TCC jel ya da serum fizyolojik ile tedavi edildi. İyileşmemiş yara alanı 0, 7, 14 ve 21. günlerde ölçüldü ve histopatolojik analiz için biyopsi örnekleri alındı (0. gün haricinde). İlk gözlenebilir granulasyon dokusu oluşumu ortalama zamanı açısından ABS, TCC ve kontrol grupları arasında anlamlı istatistiki fark yoktu (sırasıyla 5.8, 5.5 ve 6.7 gün) (P>0.05). Açık yara sahasının deri düzeyine kadar granulasyon dokusu ile dolma süresi, kontrol grubunda ABS ve TCC grubundan anlamlı şekilde daha düşüktü (sırasıyla 18, 9 ve 11 gün). ABS ve TCC ile tedavi edilen yaraların 7, 14 ve 21. günlerde yapılan ölçümlerinde ortalama iyileşmemiş yara sahasının kontrol grubuna kıyasla anlamlı şekilde daha küçük ve ortalama total yara iyileşme yüzdesinin kontrol grubuna göre anlamlı şekilde daha büyük olduğu gözlendi (P<0.05); ortalama iyileşme zamanı da uygulama yapılan gruplarda kontrol grubundan anlamlı şekilde daha kısa (sırasıyla 17.4, 16.8 ve 23.6 gün) (P<0.05) olarak bulundu. Elde edilen bulgular ışığında, ABS ve TCC topikal uygulamalarının yara iyileşmesi üzerine yararlı etkileri olduğu sonucuna ulaşılmıştır.

Anahtar sözcükler: *Yara iyileşmesi, Ankaferd Blood Stopper (ABS), Tripeptid bakır kompleksi (TCC), Deri, Rat*

INTRODUCTION

Skin wound healing is the repair process that follows injury to the skin and other soft tissues. Cutaneous wound healing is a complex process involving four major stages: hemostasis, inflammation, proliferation, and maturation/

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remodeling [1,2]. During the treatment period, the goal is to provide an optimal environment for wound contraction and epithelialization through the use of medications and bandages [3].

Ankaferd Blood Stopper® (ABS; Ankaferd Drug Cosmetic Co., Istanbul, Turkey) is a herbal medicine, ingredients of which have been used in Anatolia as a hemostatic agent for centuries for clinical hemorrhages [4,5] when the conventional control of bleeding by ligature and/or conventional hemostatic measures was ineffective [6,7]. Ankaferd is a standardized 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 [4,5,8,9]. ABS is clinically effective in bleeding individuals with normal hemostatic parameters and in patients with deficient primary hemostasis and/or secondary hemostasis [10-13].

Few studies have been published evaluating the effect of ABS, which seems to be an effective hemostatic agent, on wound healing [14,15]. Isler *et al*.[14] investigated the effects of ABS on early bone healing using a rat tibia defect model. The defects treated with ABS showed more intense new bone formation and less necrosis, which may be related to increased speed of healing and decreased inflammation that is associated with anti-oxidant activity of the components of ABS. Akalin *et al*.[15] investigated the efficacy of ABS on healing of dermal wounds in a rat model. The ABS group was superior to the control group in terms of inflammatory scoring, type I/type III collagen ratio, and wound contraction rates. Except these studies, the effect of ABS on wound healing has not been investigated thus far.

Glycyl-L-histidyl-L-lysine (GHK) is a naturally occurring peptide found in several biological fluids, including plasma. A synthetic form of GHK used in the preparation of the tripeptide-copper complex (TCC) acts as a controlled delivery system for copper to the wound site. Glycyl-L-histidyl-L-lysine-Cu^{+2} (GHK-Cu) is a TCC isolated from human plasma by Pickart and Thaler in 1973 [16-18]. The efficacy of TCC in acute wound healing is well established, and it possesses many properties that promote wound healing which include increase in neo-vascularization [19], increase in epithelialization and collagen deposition [3,16,20], acceleration of wound contraction [3,21,22], and improvement of the acute wound environment by increasing proteinases [17].

The aim of this study was to evaluate the clinical and histopathological effects of ABS and TCC applied topically on open wounds created on dorsal skin of rats and to compare the results with saline treated control wounds in an experimental model.

MATERIAL and METHODS

Study Population

The study was performed at the Experimental Animal Breeding and Research Unit of the university. Upon approval of the Animal Ethics Committee (2012-01/04), the procedure was initiated.

A total of 24 outbred, male, Sprague-Dawley rats (mean weight±SD: 230±19 g; mean age: 2 months) were used. The animals were kept in individual cages, in a room with constant temperature (22±4°C) and a 12 h light/12 h dark cycle with free access to food and water. The rats were randomly divided into three groups: (1) ABS group, (2) TCC (Iamin® 5% gel; Procyte Co., Kirkland, WA, USA) group, and (3) control group (given only saline), each group with 8 rats. Prior to anaesthesia and on days 7 and 14 a complete blood cell count was performed on each rat.

Wound Creation

The rats were starved for 24 h preoperatively. On day 0, rats in all groups were anesthetized with inhalation chamber of sevoflurane (4.0-5.0%, vol). After induction, anesthesia was maintained with facial mask by inhalation of sevoflurane (2.5-3.5%, vol). Single dose of enrofloxacin (10 mg/kg, sc) (Baytril®; Bayer, Leverkusen, Germany) was administered immediately preoperatively for prophylaxis. Carprofen (4 mg/kg, sc) (Rimadyl®; Pfizer Inc., Zaventem, Belgium) was injected to all animals once just before the operation for analgesia.

The hair on the dorsum was clipped widely from the scapula to the ilium region and the clipped area was surgically prepared with polyvidone-iodine (Betadine®; Kansuk, Istanbul, Turkey). Each rat was positioned in sternal recumbency and surgically draped.

One full thickness experimental wound (1.5 cm x 1.5 cm) was created on each side, nearly 1 cm from the dorsal midline just caudal to the caudal border of the scapula on each rat. The skin, including panniculus carnosus, was excised with a no. 11 scalpel blade and scissors to create wounds perpendicular to the spine. Hemorrhage was controlled by sterile surgical sponges. After application of the topical treatments, the wound areas were left open.

Treatment Protocol

Each rat in the respective treatment groups was treated once daily, with sponges soaked in ABS solution and topical TCC gel. Enough medication to cover the wound with a thin layer was applied by delivering a segment of the medication diagonally across the square wound. In the control group, sponges soaked in saline solution was administered on wounds.

Evaluation of Wound Healing

Observations during daily wound care: Each wound was evaluated at the time of daily examination for wound healing and presence of any exudate until day 21. On days 14 and 21, any hair that had grown around the wounds was trimmed away. The day that the first granulation tissue was observed and the days that the wound was covered and then completely filled with granulation tissue and epithelialized were recorded.

Planimetry: Planimetry was performed on days 0, 7, 14, and 21 on anesthetized animals (the anesthesia protocol used to create the wounds was repeated) by tracing the perimeter of the square wounds onto a sterile piece of clear acetate film with a special marking pen. The examiner, wearing a 2.5x loupe, traced the wound margin at the border between the normal skin and the wound. The outlined area was defined as 'total wound area'. Thereafter, the examiner traced the margin at the leading edge of the advancing epithelium. The area within the margin of the advancing epithelium was defined as 'unhealed wound area'. Wound tracings were scanned and transferred to a computer, and the area (mm^2) and perimeter were calculated for each wound using the Sigma Scan® software (SPSS Inc., Chicago, IL, USA). The percentage of total wound healing was calculated for wounds on the right side by using a previously described two-step formula [3].

Step 1

Open wound day_n as percentage of original = Open wound area day_n x 100/Original wound area day_0

Step 2

% total wound healing day_n = 100 - Open wound day_n as percentage of original.

The unhealed wound area and the percentage of total wound healing were recorded at each day of measurement and used for statistical analysis.

Histopathological Examination

Four-millimeter punch biopsy instruments were used to take skin specimens from different corners of the left-side wound of each rat on days 7, 14 and 21 immediately after the planimetry was performed. Skin specimens were fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. Five-micrometer sections were stained with hematoxylin and eosin (H&E). Progressive decrease in neutrophil number and progressive increase in angiogenesis were selected for monitoring the healing process histopathologically as reported in previous studies [23,24]. In every skin section, an area just beneath the epidermis or crust formation was randomly selected. Thereafter, four consecutive areas moving towards the deep dermis were selected. The five selected areas were examined under 400x magnification. The number of neutrophils was scored as 0-25=1, 26-50=2, 51-75=3, and > 75=4. The same areas were also examined for the number of vessels and the actual count was noted. All histological sections were blindly evaluated by the same investigator.

Statistical Analysis

The percentage of total wound healing, unhealed wound area, time for the first observable granulation tissue, time for the coverage of the wound with granulation tissue, and filling of the open wound to the skin level with granulation tissue for each wound at each time of measurement as compared to the original wound size were calculated and the mean values were compared among the three groups using repeated measures model for analysis of variance (ANOVA). Where differences existed, the differences were determined by Duncan's multiple-range test. Neutrophil scores and vessel counts were analysed using one-way ANOVA. When differences among the groups were significant, Mann Whitney U test was used. All analyses were performed using SPSS 13.0 (SPSS Inc.). A *P* value lower than 0.05 was considered significant.

RESULTS

Observations During Daily Wound Care

Appearances of the wounds in ABS, TCC, and control groups on day 7, 14, and 21 are shown in *Fig. 1*. The day following surgery, the wounds appeared clean; however, some of the wounds treated with TCC were covered with a bluish film reflecting the blue color of the gel. On the second and third days of treatment, TCC-treated wounds developed a tenacious, purulent appearing exudate. The tissue underlying the exudate, in most cases, was dark red or mottled on day 7 and was considered as granulation tissue. Wounds in ABS and control groups were clean and free of exudate throughout the study.

On day 7, a superficial brown colored scab was observed on the wounds in ABS group. In the control group, granulation tissue formation was less remarkable on day 7.

On day 14, the rapid growth of new tissue at the wound edges was remarkable in ABS and TCC groups. In addition, a small elevation of granulation tissue was present at the center of the wounds in TCC group *(Fig. 1E)*. The wound size in both ABS-treated and TCC-treated animals was reduced.

On day 21, all wounds were covered with granulation tissue and epithelialization in the ABS and TCC groups (mean 16.1 and 19.2 days, respectively ($P<0.05$), whereas wounds of four rats in the control group were not completely epithelialized *(Fig. 1C, 1F, 1I)*.

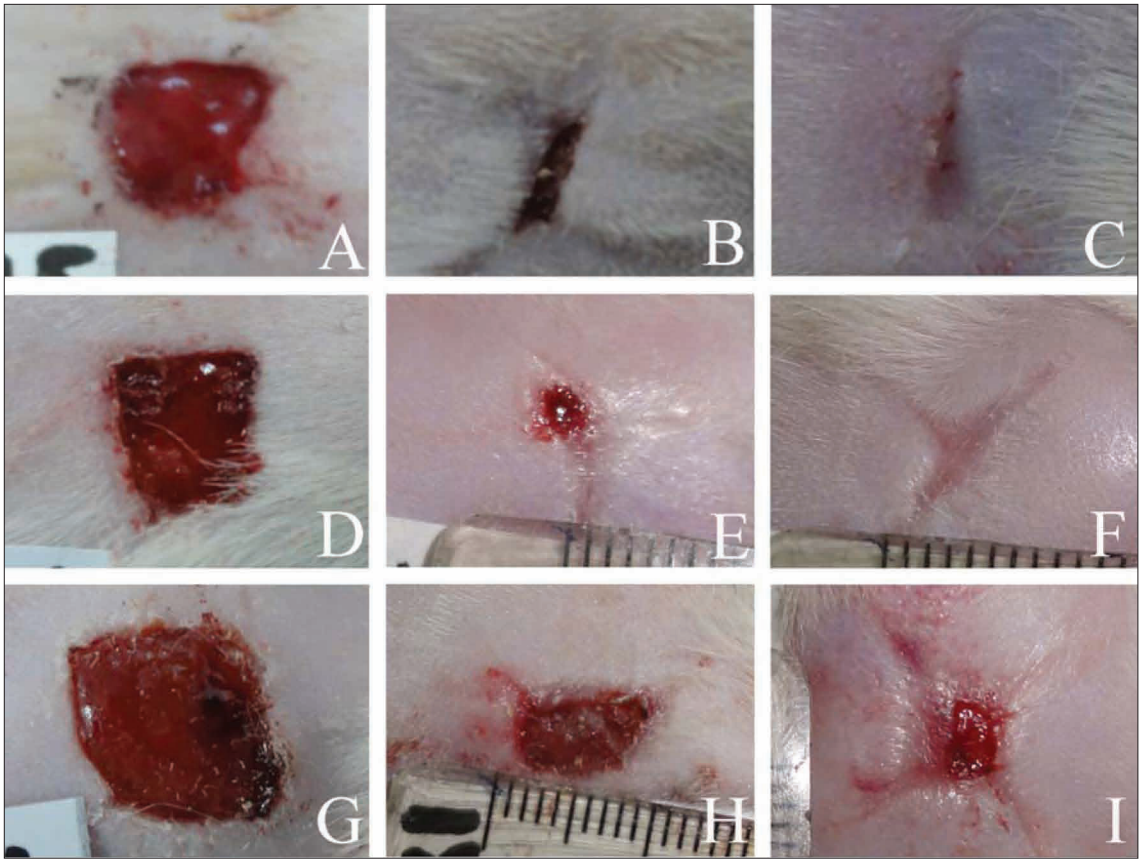

Fig 1. The figure shows the progression of wound healing in the ABS (1A to 1C), TCC (1D to 1 1F) and control (1G to 1I) groups on day 7 *(left column),* day 14 *(middle column)* and day 21 *(right column).* On day 21, all wounds in the ABS and TCC groups were covered with granulation tissue and epithelialization, whereas there were still open wounds in the control group

Şekil 1. ABS (1A-1C), TCC (1D-1F) ve kontrol (1G-1I) gruplarında 7. günde *(sol sütun),* 14. günde *(orta sütun)* ve 21. günde *(sağ sütun)* yara iyileşmesini göstermektedir. Yirmi birinci günde ABS ve TCC gruplarında tüm yaralar kapanırken, kontrol grubunda hala açık yaralar mevcuttu

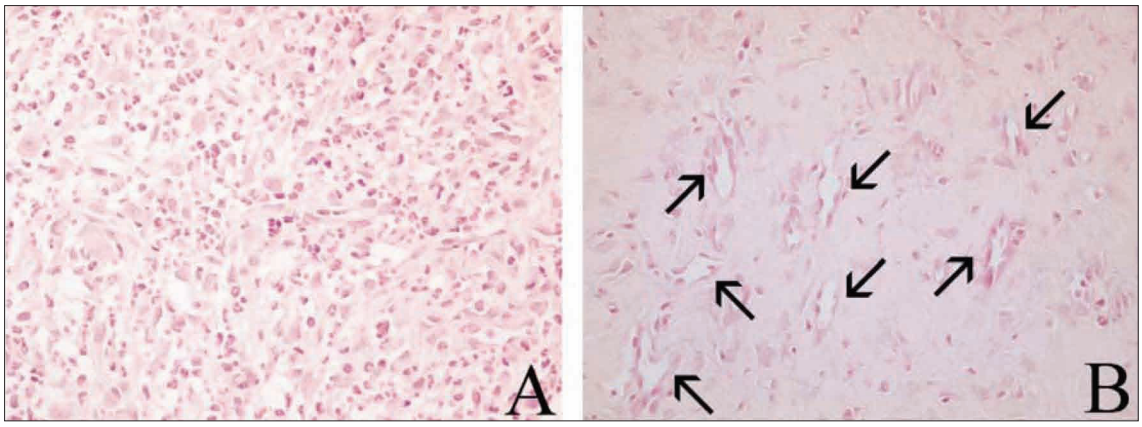

Fig 2. Histopathologically wound healing was characterized by a decrease in the neutrophil count and an increase in the number of vessels. A shows high number of neutrophils on day 7 in the ABS group, whereas B shows the increased number of new capillary vessels *(arrows)* with almost no inflammatory reaction on day 21 in the same group, H&E staining, x400 magnification

Şekil 2. Histopatolojik olarak yara iyileşmesi nötrofil sayısında azalma ve damar sayısında artış ile kendisini gösterdi. A 7. günde ABS grubunda çok sayıda nötrofili gösterirken, B aynı grupta 21. günde artan sayıdaki yeni kapillar damarları *(oklar),* ve hemen hemen hiç yangısal reaksiyonun olmadığını ortaya koymaktadır, H&E boyama, x400 büyütme

The median time for the first observable granulation tissue was not significantly different in the ABS, TCC, and control groups (5.8, 5.5, and 6.7 days, respectively) ($P>0.05$). Filling of the open wound with granulation tissue to skin level was significantly slower in the control group than in the ABS and TCC groups (18, 9, and 11 days, respectively)

Table 1. *Comparison of mean unhealed wound area and percentage of total wound healing on days 0, 7, 14, and 21 among groups of rats treated with the Ankaferd Blood Stopper (ABS), tripeptide copper complex (TCC) and saline treated controls (mean ± standard error of mean)*

Tablo 1. *Ankaferd Blood Stopper (ABS), tripeptid bakır kompleksi (TCC) ve serum fizyolojik (kontrol) uygulanan ratlarda 0, 7, 14 ve 21. günlerde ortalama iyileşmemiş yara alanı ve total yara iyileşmesi yüzdesinin karşılaştırılması (ortalama ± ortalamanın standard sapması)*

Group	Day 0	Day 7		Day 14		Day 21	
	Unhealed Wound Area (mm^2)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)	Unhealed Wound Area ± SE (mm^2)	Total Wound Healing (%)
ABS	225	102.25±34.11*	54.55±15.16*	4.50±3.12*	97.99±1.39*	0*	100*
TCC	225	124.12±47.18*	44.83±20.97*	11.12±9.08*	95.05±4.03*	0*	100*
Control	225	169.37±38.07†	24.72±16.92†	23.50±13.09†	89.55±5.82†	16.80±4.15†	95.33±4.11†

**,†Different superscripts within the same column indicate significant difference among groups (P<0.05)*

Table 2. *Mean neutrophil scores and vessel counts of open wounds in rats treated with Ankaferd Blood Stopper (ABS), tripeptide copper complex (TCC) and saline (control) for 21 days (mean ± standard error of mean)*

Tablo 2. *21 gün boyunca Ankaferd Blood Stopper (ABS), tripeptid bakır kompleksi (TCC) ve serum fizyolojik (kontrol) uygulanan ratlarda ortalama nötrofil skoru ve damar sayıları (ortalama ± ortalamanın standard sapması)*

Treatment Group¶	Neutrophil Score	Vessel Count
ABS	1.75±1.03	5.58±2.75
TCC	1.75±0.74	4.83±2.81
Control	1.91±0.83	5.00±2.32
P	0.559	0.588
Biopsy day††		
Day 7	2.38±0.92*	3.42±1.89*
Day 14	1.88±0.74*	4.25±1.67*
Day 21	1.17±0.38†	7.75±1.94†
P	0.000	0.000
Treatment group and biopsy day		
ABS- Day 7	2.50±1.20	3.75±1.28
TCC- Day 7	2.12±0.83	2.87±2.30
Control- Day 7	2.50±0.76	3.62±2.06
P	0.721	0.316
ABS- Day 14	1.75±0.89	4.63±2.39
TCC- Day 14	1.88±0.64	4.12±1.46
Control- Day 14	2.00±0.76	4.00±1.06
P	0.767	0.749
ABS- Day 21	1.0±0	8.38±1.92
TCC- Day 21	1.25±0.46	7.5±2.33
Control- Day 21	1.25±0.46	7.38±1.60
P	0.317	0.553

**, † Different superscripts within the same column indicate significant difference (P<0.05), ¶ The values are the mean ± standard error of mean (SEM) of days 7, 14, and 21 in the same group, †† The values are the mean ± standard error of mean (SEM) of the three treatment groups on the same day*

(*P*<0.05), but was similar in the ABS and TCC groups. The average time for healing was shorter in the ABS and TCC groups than in the control group (17.4, 16.8 vs. 23.6 days, respectively) (*P*<0.05), but was not different between the ABS and TCC groups. Complete blood cell count values were within normal limits for all samples taken on days 0, 7 and 14 (data not shown).

Planimetry

The mean unhealed wound area was significantly smaller and the mean percentage of total wound healing was significantly higher in the ABS- and TCC-treated wounds than in the control wounds on day 7, 14, and 21 (*P*<0.05, *Table 1*), but were similar between the ABS and TCC groups (*P*>0.05, *Table 1*).

Histopathological Examination

The mean±SEM neutrophil scores and vessel numbers are presented in *Table 2*. Neutrophil count was significantly higher and the vessel count was significantly lower on days 7 and 14 than on day 21 (*P*<0.05). In all groups, a steady increase in vessel numbers and steady decrease in neutrophil scores were observed from day 7 to day 21, but this could not be substantiated statistically (*P*>0.05) *(Fig. 2)*. The vessel count tended to be higher in the ABS group than in the other groups on all measurement days (*P*>0.05).

DISCUSSION

An understanding of the process of wound healing is essential for effective management of wounds. Topical medications should provide a specific desired effect during the appropriate stage of healing [25]. This study shows that the average time for healing was shorter and the mean total wound-healing percentage was significantly higher in the TCC- and ABS-treated wounds than in the control group.

ABS is a folkloric medicinal plant extract product, ingredients of which have been used in Turkish traditional medicine as a hemostatic agent. Its composition have been shown to have some effect on hematological and vascular parameters, and cellular proliferation [8,26-30]. Antiinfective [31,32], wound healing [14,33], antineoplastic [34], and homeostatic properties [5] have also been reported. Ozturk *et al*.[35] have shown beneficial effects of ABS in patients with osteoarthritis.

In their experimental study investigating the role of ABS on dermal wound healing, Akalin *et al*.[15] stated that all parameters that were important for effective wound healing, including polymorphonuclear leukocyte and mononuclear leukocyte infiltrations, vascularisation and fibroblast proliferation, were better in the ABS-treated group when compared with the control group [15]. Therefore, these authors concluded that the positive effects of ABS on wound healing might be attributable to these useful histopathological alterations.

Isler *et al*.[14] showed that histopathologically over sixty percent of the defects treated with ABS were free of inflammation, which is probably related to the anti-inflammatory activity of some components of this hemostatic agent. Although the occurrence of fibrosis was statistically similar in both groups, the ABS-treated group showed lower fibrosis rate than the non-treated control group, which may be attributed to the increased speed of healing in the test group.

On day 7, we observed formation of a brown colored scab over the wound area after application of ABS. This finding has been observed also by other researchers [12,36] and is believed to be due to the formation of an encapsulated protein network causing delayed degradation of erythrocytes and later hemosiderin-loaded histiocyte accumulation [12].

TCC is a naturally occurring peptide-copper complex that has been shown to possess potentially interesting properties in the wound healing process in animal [21,37] and human models [20,38]. Observation of a positive effect by TCC on wound healing in the current study is consistent with other studies [23,24] which reported that lamin, a TCC containing hydrogel, accelerates wound healing and wound contraction, and promotes epithelialization by creating a moist wound environment. The use of a hydrogel helps to produce more rapid healing by creating a moist environment which has superior features over a dry wound, such as prevention of tissue dehydration and cell death, accelerated angiogenesis, increased breakdown of dead tissue and fibrin, and potentiation of the interaction of growth factors with their target cells [39].

Swaim *et al*.[3] observed that TCC-treated wounds tended to form exuberant granulation tissue in most cases. We also observed granulation tissue formation at the center of wounds in the TCC group on day 14, but the degree was not exuberant as reported by these researchers [3]. On the other hand, we did not observe separation of the granulation tissue from the caudal edge, resulting in seroma formation and splinting of wound edges in any of the wounds as reported by Swaim *et al*.[3].

Wound healing begins as soon as the trauma has occurred. For the histopathological investigation of wound healing several parameters, such as re-epithelialization, wound cellularity [23,24,40,41], collagen deposition and blood vessel formation [23,24,42] have been used by different researchers. Decrease in neutrophil score and increase in vessel numbers were used to monitor the healing process in this study. With the progress of wound healing, a steady decrease in neutrophil scores and steady increase in vessel numbers were observed from day 7 to day 21 in all groups, although the difference was not statistically significant.

No abnormal finding was observed in the complete blood counts throughout the study, which suggests that topical applications of ABS and TCC do not result in systemic abnormalities indicated by this test. Chemistry profiles and urinalyses were not performed in this study, precluding any definitive conclusion on the safety of these topical treatments in rats. However, all rats were clinically healthy throughout the study.

In summary, our results suggest that although the median time for the first observable granulation tissue is not significantly different between the treatment groups and control, application of ABS and TCC accelerates filling of the open wound with granulation tissue to skin level and shortens the average time for healing. Histopathologically, decrease of the infiltrating neutrophil leukocytes and increase of neovascularisation are indicative of the healing process.

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