

The Effect of Algan Hemostatic Agent (AHA) on Wound Healing

Halil Aksoy¹ , Azize Sener¹ , Dilek Akakin² , Ali Sen³ , Ozlem Bingol Ozakpinar¹ , Sinemcan Ozcan² , Ahmet Kaan Simsek⁴ , Turgut Sekerler¹ , Sevket Ergun Guzel⁵ , Ahmet Midi⁶ 

¹ Marmara University, Faculty of Pharmacy, Department of Biochemistry, İstanbul, Turkey.

² Marmara University, Faculty of Medicine, Department of Histology-Embryology, İstanbul, Turkey.

³ Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, İstanbul, Turkey.

⁴ Bahcesehir University, Faculty of Medicine, İstanbul, Turkey.

⁵ Kahta State Hospital, Department of Orthopedics, Adiyaman, Turkey.

⁶ Bahcesehir University, Faculty of Medicine, Department of Pathology, İstanbul, Turkey.

Correspondence Author: Halil Aksoy

E-mail: aksoyhalil@yahoo.com

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ABSTRACT

Objective: The Algan Hemostatic Agent (AHA) is a novel herbal originated blood stopper. The aim of this study is to investigate the effect of AHA on wound healing on excisional wound model in rats.

Methods: In this study, 54 adult Wistar albino rats were used. Rats were divided into 3 groups (saline, Madecassol® and AHA). Each group was then divided into 3 subgroups as the 3rd, 7th and 14th days. Two wounds were created in the dorsal thoracic region of the rats. One of the lesions was used for histopathological examinations and the other for hydroxyproline measurement. In order to evaluate the wound healing, wound area were measured during the whole treatment period and animals were sacrificed at the end of the 3rd, 7th and 14th days and tissue samples were taken for the determination of hydroxyproline levels.

Results: AHA treatment did not cause significant difference in hydroxyproline level on days 3, 7, 14. The contraction percentage of wound area was higher in the AHA group on day 7 than that of the control group. However, the difference was not statistically significant ($p>0.05$). On days 3 and 14, no significant difference was detected in the contraction percentage of wound area between the control and the AHA groups. AHA and Madecassol® results of epidermis regeneration on the 14th day, neutrophil infiltration on the 7th day and edema on the 3rd, 7th and 14th days were different in terms of histopathological parameters compared to the control group.

Conclusion: Despite good histological findings, AHA did not significantly accelerate wound healing, but did not adversely affect wound healing as well.

Keywords: Algan hemostatic agent, Madecassol®, wound healing, hydroxyproline

1. INTRODUCTION

Wound healing is a complex physiological process involving soluble mediators, blood cells, and extracellular matrix in damaged tissue. It consists of four programmed phases as hemostasis, inflammation, proliferation, and tissue remodeling (1). The presence of certain factors such as diabetes, obesity, infection, aging, stress, deficiency of steroids such as observed in postmenopausal period, and poor nutrition impair wound healing processes (2). It is important for proper wound healing to be rapid without infection and not leave a scar tissue. If the contraction of the scar tissue formed in the final stage of wound healing is excessive, it may also causes deformities in the internal organs (3). Different local and systemic therapies are being investigated to accelerate wound healing. Some extracts obtained from species such as *Aesculus hippocastanum*, *Cotinus coggygia*

and bioactive molecules have been reported to accelerate the wound healing process by promoting collagen deposition, increasing fibroblasts, preventing infection or suppressing oxidative stress (4-7).

The Algan hemostatic agent (AHA, Algan Group Health Services Import and Export Industry and Trade Ltd., Co., İstanbul, Turkey) is a novel polysaccharide based hemostatic agent and has been patented (Patent application no: a2015 / 00018, application publication no. TR2015 0018 A2). It is in the form of powder, and liquid and produced for bleeding control. It consists of a standardized mixture of 6 different plants (*Achillea millefolium*, *Juglans regia*, *Lycopodium clavatum*, *Rubus caesius*, *Viscum album*, *Vitis vinifera*). Preclinical studies of AHA have been completed and clinical studies are continuing. It has been shown to be

effective in some bleeding models like experimental partial splenectomy (8), renal venous bleeding (9), liver laceration (10) and hepatectomy bleeding (11). According to the hemodynamic test, AHA primarily provides hemostasis by forming a mechanical barrier in the area where it is applied (9). It has been reported that the coagulation mechanism forms by confining and gelling blood elements into the polymeric network (12). AHA has a similar mechanism of action with polymer-based hemostatic products (8,9). Local hemostatic (antihemorrhagic) agents promote hemostasis in postoperative and posttraumatic bleeding. An ideal hemostatic agent should be safe. A hemostatic agent is expected to have a positive effect on wound healing or at least not to have a negative effect. Besides their favourable hemostatic effects, some hemostatic agents are shown to accelerate wound healing (13-15).

In this study, we aimed to investigate time-dependent effects of AHA on wound healing on the excisional wound model created in rats. The effects of AHA on wound healing is important because it is a product that will be used as a local hemostatic agent in internal and external bleedings.

2. MATERIALS AND METHODS

2.1. Animals

In this study, female Wistar albino rats, weighing 250-300 g and 16 weeks old, were used. Rats were obtained from Marmara University Experimental Animal Research and Application Center. The animals were kept in individual wire-bottomed cages, in a room at a constant temperature ($22^{\circ} \pm 2^{\circ}\text{C}$) with 12-h light and dark periods, and fed with standard rat chow. All procedures for the experimental protocols of this study were approved by the Marmara School of Medicine Animal Care and Use Ethics Committee (protocol number: 15.2020.mar). During the experiments, all animals were subjected to the same stress under the same conditions.

2.2. Treatments

Test agent, Algan Hemostatic Agent, was obtained from Algan Group Health Services Import and Export Industry and Trade Ltd., Co., Istanbul, Turkey. The rats were divided into the following three groups of eighteen rats each: Control group, AHA treatment group, Madecassol® (Bayer, as reference ointment) treatment group. Each group was then divided into 3 subgroups (as the 3rd, 7th and 14th days), each consisting of six rats.

2.3. Excisional Wound Model

This model is a convenient method to monitor wound contraction. The study was conducted as described in the literature (6). Under anesthesia, the dorsal region hairs of rats were removed with shaving machine and the wound

area was cleaned with 0.2% chlorhexidine solution. Two circular wounds were excised from the skin with a 6 mm biopsy punch to create wounds in the dorsal thoracic region of each animal. The wounds were left open throughout the study. The progressive changes in wound area of all groups were monitored.

Saline was administered topically once daily to the control group. AHA solution (liquid form) and Madecassol® (Bayer) were also applied topically once daily. These applications were continued until the end of the 3rd, 7th and 14th days. Tissue samples were taken at the end of the experiment. One of the two wound tissues created in each animal was used for the determination of hydroxyproline content and the other for histopathological examinations.

2.4. The contraction Percentage of Wound Area

In order to evaluate the contraction percentage of the wound areas, sizes of wounds were measured on the end of days 3, 7, and 14. The shape of each wound was drawn on transparent paper. Then, wound shapes were transferred on a 1 mm chart paper. The contraction percentage of wound area was calculated by using the following formula (16). The contraction percentage of wound area = (initial wound size – specific day wound size)/initial wound size) × 100.

2.5. Determination of Hydroxyproline Content

Tissue samples were taken at the end of the 3rd, 7th and 14th days. Hydroxyproline content was measured using the method by Reddy and Enwemeka (17). Briefly, tissue samples were mixed with 2 N NaOH and incubated for 20 minutes at 120 °C. The samples were oxidized with chloramine T and Ehrlich reagent was added. Then, the absorbance of the coloured product formed at 65 °C was read at 550 nm. The concentration of hydroxyproline was calculated using the standard curve. The results were presented as mg/g tissue.

2.6. Histological Scoring

For histological examination, at the end of the 3rd, 7th and 14th days wounded skin specimens were collected from the experimental groups and fixed in 10% neutral buffered formalin solution. After fixation, tissue samples were dehydrated in graded ethanol series (70%, 90%, 96% and 100%), cleared in toluene and mounted in paraffin. Sections were cut into 4- μm -thick sections by rotary microtome from paraffin-embedded blocks and stained with hematoxylin and eosin (H&E). Finally, the sections were viewed under a light microscope (Olympus BX51, Tokyo, Japan) for epidermal and dermal regeneration, fibroblast density, angiogenesis, neutrophil infiltration and edema scored semiquantitatively by two blinded histologists according to the scoring system outlined in Table 1.

Table 1. System for Scoring the Histological Features of Wound Tissue Samples.

Score	Epidermal and dermal regeneration	Fibroblast density	Angiogenesis	Neutrophil infiltration	Edema
1	Little epidermal and dermal organization	Mild fibroblast density	Altered angiogenesis (1-2 vessels per site)	Mild	Mild
2	Moderate epidermal and dermal organization	Moderate fibroblast density	Few newly formed capillary vessels (3-6 vessels per site)	Moderate	Moderate
3	Complete remodeling of epidermis and dermis	Increased fibroblast density	Newly formed capillary vessels (7-10 vessels per site)	Severe	Severe
4			Newly formed and well-structured capillary vessels (>10 vessels per site)		

2.7. Statistical Analysis

Data were analyzed using GraphPad Prism 4.0 (La Jolla, CA). Statistical differences between groups were evaluated by one-way ANOVA and Tukey's post-hoc test. Data were expressed as mean \pm standard deviation (SD). A p value <0.05 was considered as statistical significance.

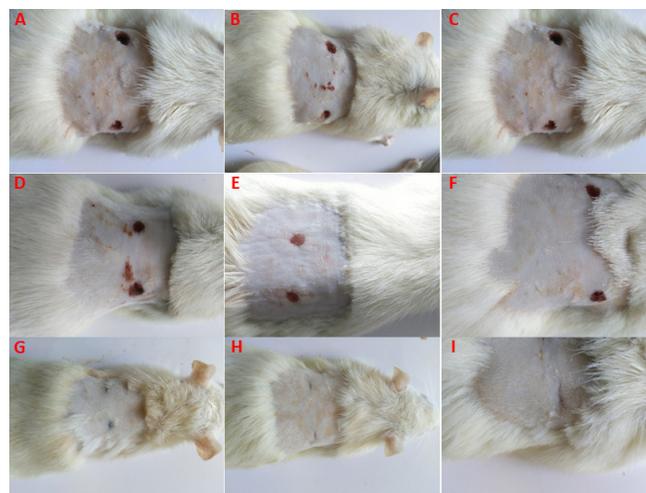
3. RESULTS

The wound closure values and hydroxyproline levels of control, AHA and Madecassol® treated groups were as shown in Table 2. The contraction percentages of wound areas were observed as 43.83%, 74.34%, and 86.57% at the end of the 3rd, 7th and 14th days in the AHA group, respectively. The contraction percentages of wound areas of the AHA group were close to those of the control group on the 3rd and the 14th days, whereas the contraction percentages of wound areas of the AHA group on the 7th day was 16% higher than those of the control group. However, this difference was not statistically significant. In the reference drug Madecassol® group, the contraction percentages of wound areas were higher on the 7th day compared to those of the control group, whereas they were higher on the 14th day compared to those of the control and the AHA groups ($p<0.05$, $p<0.01$ respectively). Figure 1 shows the wound images of rats randomly selected from each group.

Table 2. Wound Tissue Hydroxyproline Levels and Contraction Rates of Wound Areas (%) of the Control and the Treatment Groups.

	Hydroxyproline (mg/g tissue), mean (SD)	Contraction rate of wound area (%), mean (SD)
3rd day		
Control	24.74 \pm 4.88	47.33 \pm 14.45
AHA	24.16 \pm 1.53	43.83 \pm 4.50
Madecassol®	32.14 \pm 5.81*,#	48.67 \pm 3.30
7th day		
Control	26.50 \pm 4.50	64.00 \pm 2.76
AHA	29.65 \pm 2.37	74.34 \pm 9.00
Madecassol®	31.21 \pm 4.85	78.86 \pm 3.24*
14th day		
Control	24.80 \pm 1.21	88.00 \pm 4.78
AHA	22.97 \pm 2.40	86.57 \pm 6.03
Madecassol®	24.89 \pm 1.66	95.00 \pm 1.44*,##

* $p<0.05$, versus control group; * $p<0.05$, ** $p<0.01$ versus AHA group; SD: standard deviation; AHA: Algan Hemostatic Agent

**Figure 1.** The wound images of randomly selected rats from each group. A, B, C: 3rd day; D, E, F: 7th day; G, H, I: 14th day. On day 3, the wound closure was similar in all groups.

Hydroxyproline levels of the AHA group on days 3 and 14 were close to those of the control group. There was no difference between hydroxyproline levels of the control and the AHA groups on day 7. However, hydroxyproline level of the AHA group was found to be higher than that of the control group. Hydroxyproline levels of the Madecassol® group on day 3 were significantly higher than those of the control and the AHA groups ($p<0.05$). No significant difference was observed in the hydroxyproline levels of the Madecassol® group on days 7 and 14 compared to those of the AHA and control groups.

There was no difference between the groups regarding the histopathological parameters evaluated in terms of epidermis regeneration, on the 3rd and 7th days. However, on the 14th day, histopathological parameters of the Madecassol® and the AHA groups were found to be better than those of the

control group. Similarly, there was no difference in fibroblast density between the groups on the 3rd and the 7th days. On day 14, fibroblast density was found to be less in the Madecassol[®] and the AHA groups compared to that of the control group. Angiogenesis was found to be less in the AHA and Madecassol[®] groups on the 3rd and 14th days compared to that of the control group. Neutrophil infiltration was found to be less in the AHA and Madecassol[®] groups on the 7th day than that in the control group. Edema was found to be less in the AHA and the Madecassol[®] groups on the 3rd, 7th and 14th days compared to the control group (Figure 2). The results are shown in Table 3.

Table 3. Comparison of Histological Parameters Between Groups.

	Epidermis regeneration	Fibroblast density	Angiogenesis	Neutrophil infiltration	Edema
3rd day, mean (min.-max.)					
Control (Group 1)	0	0	2.80 (2-3)	2.83 (2-3)	2.66 (2-3)
Madecassol [®] (Group 2)	0.33 (0-1)	0	3.66 (3-4)	2.33 (1-3)	1.33 (0-2)
AHA (Group 3)	0.16 (0-1)	0	3.50 (2-4)	2.50 (1-3)	1.50 (1-2)
P	1&2&3 > 0.05	1&2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05	1&2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05
7th day, mean (min.-max.)					
Control (Group 1)	0.5 (0-1)	2.33 (1-3)	3.16 (2-4)	2.16 (1-3)	2.33 (1-3)
Madecassol [®] (Group 2)	1.16 (1-2)	2.66 (2-3)	3.50 (3-4)	1.33 (0-3)	1.50 (1-2)
AHA (Group 3)	1.00 (1-2)	2.66 (2-3)	3.50 (3-4)	1.83 (1-3)	1.66 (1-2)
P	1&2&3 > 0.05	1&2&3 > 0.05	1&2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05
14th day, mean (min.-max.)					
Control (Group 1)	1.66 (1-2)	2 (1-3)	2.16 (1-2)	0.5 (0-1)	0.83 (0-2)
Madecassol [®] (Group 2)	2.66 (2-3)	1.5 (1-2)	1.66 (1-2)	0	0
AHA (Group 3)	2.33 (2-3)	1.66 (1-2)	1.66 (1-2)	0	0.16 (0-1)
P	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05	1&2&3 > 0.05	*1&2 < 0.05 *1&3 < 0.05 2&3 > 0.05

Min.: minimum; max.: maximum; AHA: Algan Hemostatic Agent

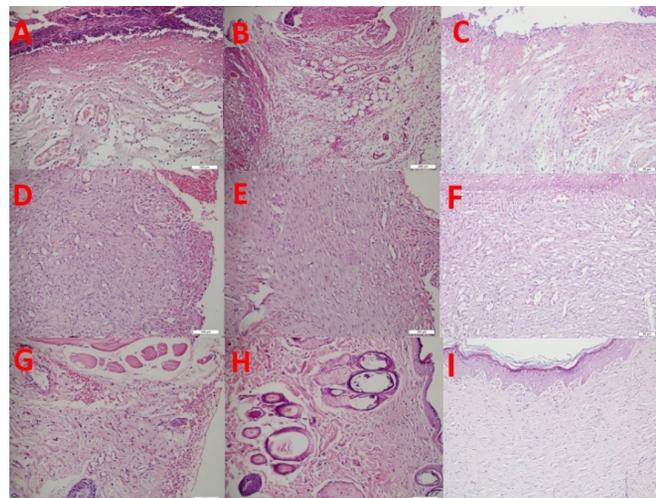


Figure 2. Histopathological view of the wound in the control, AHA and Madecassol[®] groups, respectively. A, B, C: 3rd day; D, E, F: 7th day; G, H, I: 14th days. On the 3rd day, histopathologic examination revealed acute inflammatory cell infiltration with intense bleeding in all groups. Intense edema is noted in the control group (A, B, C). On the 7th day, granulation tissue was rich in cells in the control group and the other two groups had less cellularity. On day 14, the connective tissue rich in healing area was observed in the Madecassol[®] and the AHA groups compared to the control group. In the control group, vascular granulation tissue is still present.

4. DISCUSSION

Many studies have clinically and histologically investigated the healing potential of natural products on incisional and excisional wound models. It has been shown that plants and extracts with pro-collagen synthesis, antioxidant, antiinflammatory and antimicrobial activities have accelerated wound healing (4). AHA is a new local hemostatic agent and a herbal biopolymer. Its effectiveness on experimental bleeding models is being investigated (8-11). However, its effects on wound healing have not been investigated. Therefore, this study was planned to evaluate the potential effect of AHA with biochemical and histological parameters in the excisional wound model.

Based on the ethnobotanical use of plants, making scientific research in this direction is very important in revealing the products or medicines that will be used as primitive raw materials in the treatment (if the active compound is to be isolated). Proof of this effect of these plants, which are used by the public for wounding purposes, is of great importance when evaluated in this respect. As AHA will be used in bleeding areas, it will be directly related to wound healing. Therefore, its effect on wound healing was tested. There are many studies conducted in the literature about the effect of many other haemostatic agents on wound healing. In these studies, it has been shown that many hemostatic products have a positive effect on wound healing (13-15). In a study conducted by Akalin et al. (18) with a herbal hemostatic agent, it was reported that the hemostatic agent was superior to the control group in terms of wound contraction

rates, type I / type III collagen ratio and inflammatory scoring in the dermal wound model.

AHA is an effective product in preclinical studies (8-11). Furthermore, the ability to produce AHA in powder and liquid forms is important in terms of ease of use in clinical practice. In splenectomy hemorrhage model with AHA, it is shown that it does not have intra-abdominal adhesion (8). Biopolymers are naturally occurring biomolecules synthesized by bacteria, plants and animals. Their bioactive properties such as antimicrobial, immune modulator and cell proliferative can create a micro-environment suitable for wound healing process (19).

AHA consists of a standardized mixture of 6 different plants. Several studies were carried out on this standard mixture of wound healing. *Achillea millefolium* has been shown to accelerate wound healing with its effect on collagen production, wound proliferation phase (20) and edema (21). *Juglans regia* has antioxidant, antimicrobial and wound healing activity (22,23), *Lycopodium clavatum* has been shown to have potential effects on inhibition of ROS production and tissue repair associated signaling pathways during wound healing (24,25). *Viscum album* accelerates cell migration (26). *Vitis vinifera* accelerates wound healing by acting on collagenation (27). *Rubus caesius* has high antioxidant potential (28). Wound healing activities of different *Rubus* species such as *Rubus niveus* (29), *Rubus ellipticus* (30), *Rubus fairholmianus* (31), *Rubus sanctus* (32) have also been reported.

In our study, the effect of liquid form of AHA on wound healing was examined histopathologically as well as by measurement of wound areas and hydroxyproline levels. Wound area measurement is a good indicator for monitoring wound healing (7). Collagen is synthesis by the healing tissue and the level of hydroxyproline in the tissue is an indicator of collagen concentration. As the concentration of hydroxyproline increases, wound healing accelerates (28). In our study, no significant difference was observed in hydroxyproline levels in the wound tissue of the AHA groups compared to the control group. Parallel to hydroxyproline levels, AHA did not cause a significant difference in wound closure compared to the control group. The effect of AHA on wound healing in terms of histopathological parameters was close to that of the Madecassol® group. However, positive histopathological findings were not reflected in macroscopic and biochemical findings.

As a result, when applied to wounds, AHA did not adversely affect wound healing and had a low positive effect compared to Madecassol®. AHA may be used safely on excision wounds as a herbal product.

5. CONCLUSION

The present study affirms that AHA does not delay wound healing and has a low positive effect on wound healing in an excision wound model. Further studies are needed to

demonstrate the possible effects of AHA on different wound models such as burn and diabetic wounds.

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