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ORIGINAL ARTICLE

The Effect of Co-administration of *Aloe vera* Gel and *Cinnamon zeynalicum* Hydroethanolic Extract on Wound Healing Process in Diabetic Mice

Javad Arbab[1], Mohammad Reza Farahpour[2*], Amir Hossein Roohollahi-Masoumi[1]

[1] *Graduated Veterinary Medicine, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran.*

[2] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran.*

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Hydroxyproline;
Diabetes.

Abstract

Objective- This study was conducted to evaluate the effect of co-administration of *Aloe vera* gel and *Cinnamon zeynalicum* bark hydroethanolic extract on process of wound healing in diabetic mice model.

Design- Experimental Study.

Animals- Seventy-two male BALB/c mice.

Procedures- A single full-thickness excisional wound was created on back of each mouse with 7-mm punch biopsy. Animals were divided into four groups including control, 5% *Aloe vera* gel (*A. vera)*, 5% cinnamon extract (*C. zeynalicum*) and combination of 5% *Aloe vera* gel + 5% cinnamon extract (*C. zeynalicum* + *A. vera*). The rate of wound closure, histological assessment, hydroxyproline content and biochemical evaluation for total antioxidant capacity (TAC), superoxide dismutase (SOD) and malondialdehyde (MDA) were done at 3, 7 and 14 day after wound creation.

Results- The rate of wound closure, number of fibroblast, collagen deposition, epithelium thickness and tissue hydroxyproline, TAC and SOD content were significantly enhanced in treated animals in comparison to control group ($p<0.05$). Moreover, tissue edema, immune cells infiltration and MDA content were significantly decreased in treated animals versus control group ($p<0.05$).

Conclusion and Clinical Relevance- Topical co-administration of *Aloe vera* gel and *Cinnamon zeynalicum* bark hydroethanolic extract have synergistic interaction effect and improved wound healing in diabetic rat and could be recommended as a new topical herbal drug production for treating of the diabetic wound.

* Correspondence to: Mohammad Reza Farahpour, Department of Clinical Sciences, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran.
E-mail: mrf78s@gmail.com

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1. Introduction

Diabetes mellitus has been reported as one of health problem which influences the major population of people in all over world. Diabetic population is predicted to be increased by 439 million patients in 2030.[1] It is reported that 15% of patients with diabetes suffers faulted wound healing.[2] Wound healing is known as one of complex process which involves major list of growth factors and cellular events.[3] It is shown that hyperglycemia in diabetic conditions causes to produce the neuropathy, vascular dysfunction and other complications which finally fault wound healing process.[4] Inflammatory phase is first phase in wound healing which is followed by a proliferation of fibroblasts and endothelial cells, production and reorganization of the extracellular matrix.[5] Initial phase of wound healing is faulted due to impaired in reactive oxygen species (ROS) resulting from faulted antioxidant enzymes.[6-8] Chronic diabetic wounds are accompanied with increased the levels of malondialdehyde content and proteases together with faulted expression of growth factors.[9-10]

Medicinal herbs are administrated to treat the various disorders such as wound healing. Studies have shown the medicinal characteristics of some herbs on animal by different wound models.[11-12] *Aloe vera* (AV) is a medicinal plant that belongs to the Liliaceae Family and are traditionally used in wound healing.[13] AV is known to have some medicinal properties such as anti-inflammatory,[13] antibacterial and antioxidant properties.[14] AV is known to have beneficial effects on wound healing by decreasing the inflammation phase significantly and supplying mature granulation tissue.[15] Topical administration of AV stimulated activity and proliferation of fibroblast and accelerated collagen synthesis.[16]

Cinnamomum zeynalicum (cinnamon), a common spice from the Lauraceae family, is administrated to treat the diabetes and it is known to have insulin secretory property,[17] insulin sensitizing property,[18] antidiabetic and antioxidant.[19] Cinnamon is known to have significant levels of polyphenols that may enhance glucose uptake in animals.[20] Studies have shown that cinnamon alcoholic and aqueous extracts accelerating the wound healing by their antioxidant properties.[21-22]

Aloe vera gel and cinnamon bark extract are known to have wound healing effects and the both have antioxidant properties which help to accelerate the wound healing. This study was conducted to evaluate the effect of co-administration of *Aloe vera* gel and cinnamon hydroethanolic extract on wound healing process in diabetic mice.

2. Materials and Methods

Preparation of cinnamon extract and Aleo vera gel

The *Cinnamon zeynalicum* bark powder was prepared from local market and identified by an expert botanist in the Department of Botany Sciences, Agriculture and Natural Resources Research Center, Hamadan, Iran (Herbarium No. 958).

The powdered bark (150 g) was suspended in 600 ml of hydroethanolic solution, at room temperature for 96 h. The mixture was filtered by fine muslin cloth and filter paper (Whatman No 1), then dried in oven at 40°C and finally kept at -20°C for subsequent experiments.[12,22,23] The *Aloe vera* was purchased from Barij Essence Company (Kashan-Iran) which contains benzoic acid, p-toluic acid, p-coumaric acid, psalicylic acid and protocatechuic acid.

Animals and induction of diabetes mellitus

All the used procedures were approved by Standard Committee, Islamic Azad University (No. 1104). Seventy-two 12-week-old BALB/c mice were purchased and kept in individual cages in a temperature and humidity-controlled room (22 ± 1 °C and 50± 1% humidity with a 12-hr light/dark cycle) with allowed access to distilled water and food, except for fasting periods.

BALB/c mice were intraperitoneally administrated with single dose of streptozotocin (50 mg/kg body weight) dissolved in citrate buffer (0.1M, pH 4.5). The mice with fasting blood glucose over 300 mg/dl were determined as diabetic mice, 72 h after administration.[12, 24]

Preparation the therapeutic ointments

In order to prepare the therapeutic ointments which, contain 5% *Cinnamon zeynalicum* (*C. zeynalicum*), 5% *Aloe vera* (*A. vera*) and combined from *C. zeynalicum* + *A. vera*, 5 g cinnamon extract, 5 g *Aloe vera* gel and 5 g cinnamon extract + 5 g Aloe vera gel were included into commercial ointment (white petrolatum), respectively. [12]

Induction of wound

After induction of diabetes, animals were intraperitoneally anesthetized by using ketamine hydrochloride and xylazine hydrochloride (ketamine 60 and xylazine 20 mg/kg), cocktail. One wound (5 mm diameter circular full thickness wounds/animal) was surgically incised on the

dorsal surfaces of each mouse.[12] Sixty male BALB/c mice were divided into 4 groups and treated with basal formulation (Control) and 5% *A. vera*, 5% *C. zeynalicum* and 5% *A. vera*+ 5% *C. zeynalicum*. The ointments were topically applied once/day for 14 consecutive days. Six animals from each group were anesthetized on the 3rd, 7th and 14th day after wounding and wound tissue collected for histological, hydroxyproline and biochemical analysis. After tissue collection, all animals euthanized by compressed CO_2.

The wound healing ratio was computed as recommended by our previous studies.[22,23,25,26] Rate of wound healing was calculated as follows:

% wound size =wound area on day X / wound area on day zero × 100

Histological analysis

Animals were euthanized on the 3rd, 7th and 14th day after wounding, and full thickness tissue samples excised with 1 to 2 mm from the surrounding normal skin were fixed in neutral-buffered 10% formalin, then processed, embedded with paraffin wax, sectioned at 5µm in thickness and stained with Masson's trichrome.

Briefly, Cellular infiltration including inflammatory cell infiltration and fibroblasts/fibrocytes proliferation, and epithelial thickness were quantitatively evaluated per one mm^2 of the tissue for each section under ×400 magnification.

Edema and collagen production and density were also evaluated qualitatively and graded as negative (–), mild (+), mild to moderate (++), moderate (+++), and intensive (++++) calculated manually.[25,26]

Hydroxyproline assessment

In order to evaluate the presence of collagen, hydroxyproline content was evaluated by a standard biochemical assay. Hydroxyproline content of individual wounds was evaluated in 2 animals per group. Summary, the frozen tissue was hydrolyzed in 2 ml of 6 NHCl overnight at 110°C. One ml of a 0.05M chloramine T solution was added into 2 ml of the neutralized/diluted solution and incubated for 20 min at room temperature. One ml perchloric acid (3.515 M) was added it and incubated for 5 min at room temperature. One ml of 20% p-dimethylaminobenzaldehyde was then added and the mixture was incubated for 20 min at room temperature and then cooled. The hydroxyproline level (µg/mg) was evaluated in 557 nm.

Biochemical analysis

The wound granulation tissue weighing 300-400 mg each wound sample were homogenized in ice-cold KCL (150 mM) and the mixture was then centrifuged at 3000× *g* for 10 min. The supernatant was used to evaluate the superoxide dismutase (SOD), total antioxidant capacity (TAC) and malondialdehyde content (MDA).

Assessment of the TAC was carried out based on the reduction power of ferric antioxidant assay. The MDA content (based on nmol per mg protein) of the collected granulation tissue was used to determine the lipid peroxidation rate. SOD activity was measured by commercial kits.

Statistical analyses

All the data were analyzed by PASW version 18.0. Two-way ANOVA was used to analyze the results. Dunnett's test for pair-wise comparisons was used to evaluate the effect of time and treatments. Differences were considered significant if $p<0.05$.

3. Results

Wound area

Figure 1A shows effects of experimental treatments on wound area in diabetic rats. Topical administration of *A. vera* and *C. zeynalicum*, singly and combined form, reduced wound area in days 3, 7 and 14 in comparison to control group ($p<0.05$). Topical administration of *A. vera* showed better response in comparison to *C. zeynalicum* and a combination of the both showed better response in comparison to *A. vera*.

Hydroxyproline content

Results indicate that topical administration of ointments could increase hydroxyproline content in diabetic mice and the best responses were observed in combined group (Figure 1B).

Histological evaluation

Results showed that mice treated with *C. zeynalicum* 5% and *A. vera* 5%, especially in co-administrated group, in the third and seventh days after the wound creation, showed lower edema score (Table 1) and immune cells

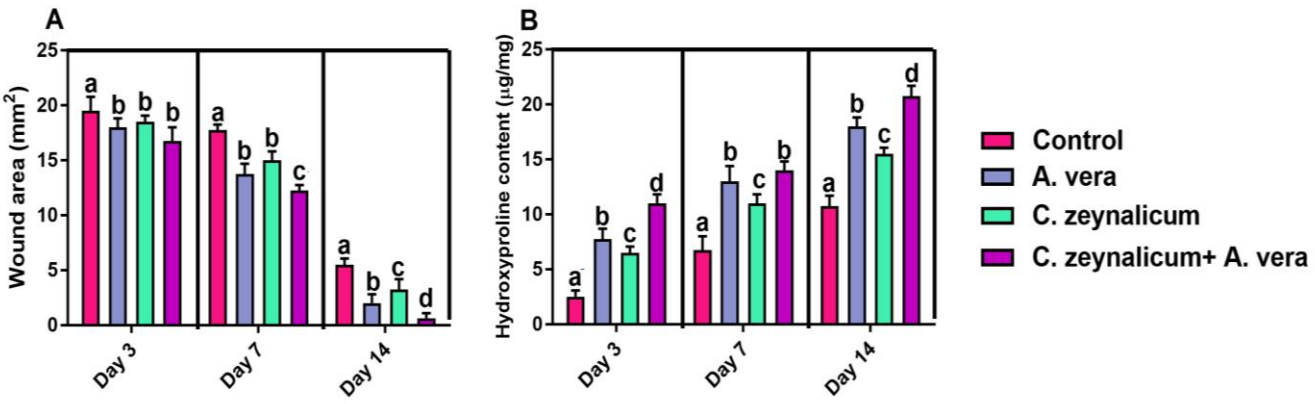

Figure 1. (A) Effects of experimental treatments on wound area (mm^2). (B) Effects of experimental treatments on hydroxyproline content. The data are presented as mean ± SD (n = 6 in each group). Superscripts (a-d) show significant differences ($p<0.05$) in same day.

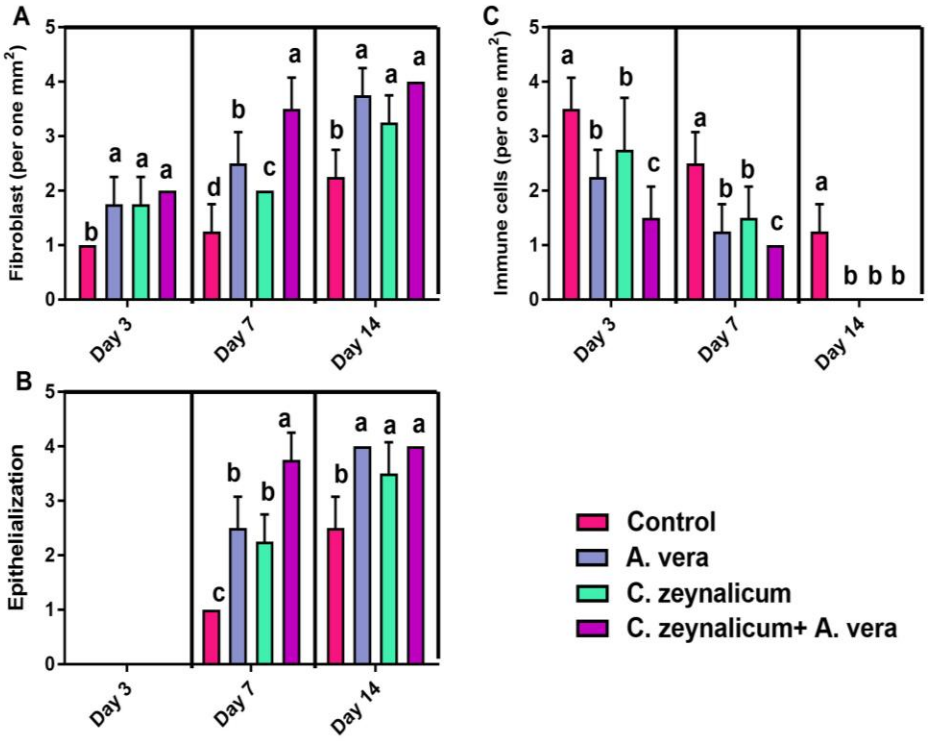

Figure 2. Effects of experimental treatments on edema (A), fibroblast (B), epithelization (C), immune cell infiltration. The data are presented as mean ± SD (n = 6 in each group). Superscripts (a-d) show significant differences ($p<0.05$) in same day.

infiltration (Figure 2C) compared to control animals. More light microscopic analyses showed that fibroblasts proliferation (Figure 2A) and re-epithelialization (Figure 2B and 3) enhanced in all treated groups, especially in combined group. Moreover, more analyses indicated that collagen deposition and collagen bundles formation sore remarkably enhanced in all treated animals, especially in animals treated with combination of *C. zeynalicum* + *A. vera*, compared to control animals (Table 1 and Figure 3).

Biochemical analysis

Results indicates that administration of different ointments prepared from *C. zeynalicum* and *A. vera*, up-regulated the antioxidant power and could enhanced tissue TAC and SOD levels in treated animals ($p<0.05$). More analyses for lipid peroxidation showed that *C. zeynalicum* and *A. vera*, especially in combined group, significantly reduced tissue MDA content (Figure 4).

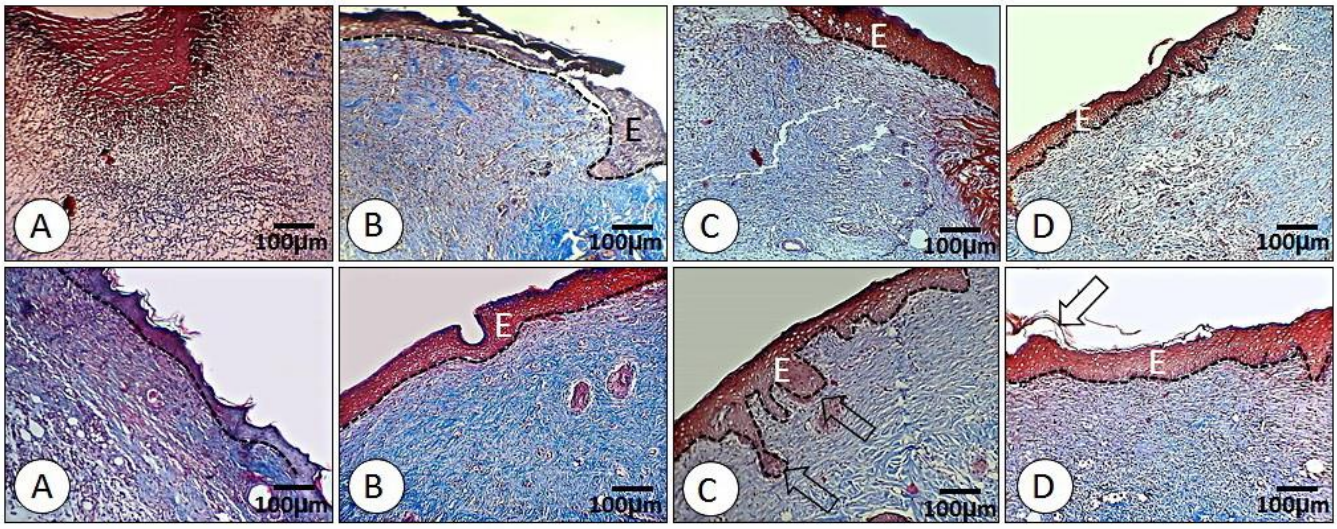

Figure 3. Cross section from wound area; (A) control, (B) 5% *C. zeynalicum*-treated, (C) 5% *A. vera*-treated and (D) 5% *C. zeynalicum* + *A. vera*-treated groups. Note well-formed collagen deposition in cross sections from treated animals on day 7 after wound induction (first row), which is significantly increased on day 14 after injury (second row). Moreover, see well-re-epithelialization in *C. zeynalicum* + *A. vera*-treated animals. The re-epithelialization initiated on day 8 after wound induction in *C. zeynalicum* + *A. vera*-treated animals. However, the cross sections from control and control group are not representing epithelialization. Indications well-show organized dermis and complete epithelialization with well-formed papillae in *C. zeynalicum* + *A. vera*-treated animals in comparison to control group. Masson-trichrome staining, 100×.

Table 1. Effects of the topical co-administration of *C. zeynalicum* and *A. vera* on edema and collagen score in different groups. *C. zeynalicum* and *A. vera.* Note: The Masson trichrome staining was scored into negative (–), mild (+), mild to moderate (++), moderate (+++), and intensive (++++).

Groups	Edema	Collagen
Day 3		
Control	++++	-
A. vera 5%	+++	+
C. zeynalicum 5%	+++	+
A. vera + *C. zeynalicum* 5%	++	++
Day 7		
Control	+++	+
A. vera 5%	++	++
C. zeynalicum 5%	++	++
A. vera + *C. zeynalicum* 5%	+	++++
Day 14		
Control	++	+++
A. vera 5%	+	+++
C. zeynalicum 5%	+	+++
A. vera + *C. zeynalicum* 5%	-	++++

4. Discussion

Wound healing is known to have several interdependent phases including inflammatory, proliferation and extracellular matrix formation phases. Faulted inflammatory phase causes defects in fibroblast migration, collagen synthesis and wound contraction.[27-28] The inflammatory stage is a main step in diabetic wound healing which can promote reactive oxygen species (ROS) resulting from chronic non-healing diabetic ulcers.[29] Delayed inflammatory phase faults proliferative phase. Immune cells such as neutrophils, macrophages and lymphocytes are infiltrated into injury site during inflammatory phase.[30] However; our findings showed that topical administration of *C. zeynalicum* and *A. vera* especially in combination form decreased inflammatory phase and immune cell infiltration. It is shown that cinnamaldehyde, 2-hydroxycinnamaldehyde and quercetin [31] have anti-inflammatory effects. In addition, water extract of cinnamon inhibits monocyte-to-macrophage differentiation.[32] It is accepted that phenolic compounds have antioxidant properties and protective effects against inflammation due to their antioxidant properties.[3,23] High production of reactive oxygen species is known to have adverse effects on cellular proliferation.[34] It can be stated that *C. zeynalicum* and *A. vera* decrease inflammatory phase by their antioxidant properties and promotes proliferative phase. The best response in combined group can be explained by more powerful antioxidant properties. Our findings for antioxidant properties confirm our claim.

Results also showed that ointments prepared from extract and gel especially in combined from fasten proliferative phase by increasing collagen deposition and

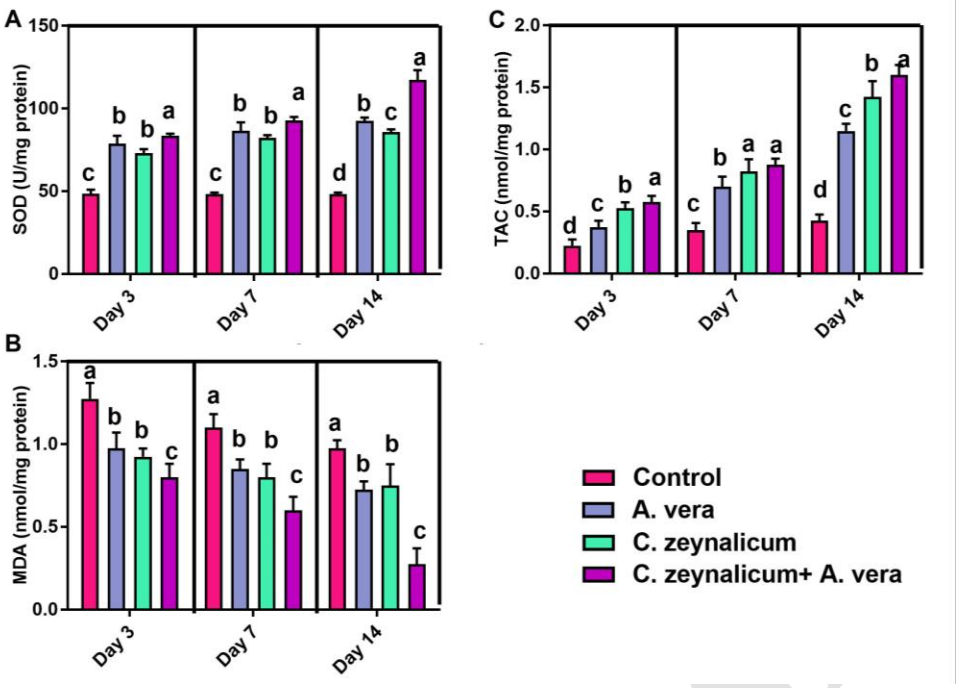

Figure 4. Effects of experimental treatments on TAC (nmol/mg protein), MDA (nmol/mg protein) and SOD (U/mg protein). The data are presented as mean ± SD (n=6 in each group). Superscripts (a-d) show significant differences ($p<0.05$) in same day.

reepithelization. It is well known that rapid cellular proliferation and differentiation is essential for shortening the healing time.[23,35] Medicinal plants accelerate wound healing and proliferative phase by their antioxidant properties.[36] Main activity of fibroblast is production of collagen in skin.[37] It is shown that simultaneous administration of *C. zeynalicum* and *A. vera* increase fibroblast and fibrocytes proliferation and collagen biosynthesis by their antioxidant properties. Reduced the tissue MDA level in the treated groups implicates on antioxidant status in the prepared ointments. Increased ROS can be related with the infiltration rate of neutrophils in the high-glucose environment of diabetic wounds.[37,38] It is accepted faulted antioxidant defense system under diabetic conditions and suggested to use the plant derivate (extracts, essential oils and active compounds) for improving the antioxidant capacity.[39] Increased levels of SOD confirms antioxidant properties in the treated groups. Increased hydroxyproline is accompanied with increased collagen deposition. The exact mechanism is unknown for increased hydroxyproline, however, it can be attributed to prevent the oxidation of proteins by medicinal plants.

In conclusion, a combination of *C. zeynalicum* and *A. vera* could decrease inflammatory phase and increase proliferative phase by their antioxidant properties. A combination of the both improved wound healing which can be attributed to their antioxidant capacity.

Acknowledgment

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Conflicts of interest

None

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نشریه جراحی دامپزشکی ایران
سال۲۰۱۸ ، جلد ۱۳ (شماره ۲)، شماره پیاپی ۲۹

چکیده

بررسی اثر موضعی ترکیب عصاره هیدرو الکلی دارچین و ژل آلوئه ورا بر روند ترمیم زخم پوستی در موش آزمایشگاهی دیابتی

جواد ارباب[۱]، محمدرضا فرهپور[۲]، امیرحسین روح‌اللهی معصومی[۱]

[۱] داتش‌آموخته دامپزشکی، دانشکده دامپزشکی، واحد ارومیه، دانشگاه آزاد اسلامی، ارومیه، ایران.

[۲] گروه علوم درمانگاهی، دانشکده دامپزشکی، واحد ارومیه، دانشگاه آزاد اسلامی، ارومیه، ایران.

هدف– این مطالعه به‌منظور بررسی اثر موضعی ترکیب عصاره هیدرو الکلی دارچین و ژل آلوئه ورا بر روند ترمیم زخم پوستی در موش آزمایشگاهی دیابتی انجام شد.

طرح مطالعه– مطالعه تجربی.

حیوانات– شصت موش آزمایشگاهی نژاد بالب/سی

روش کار- یک زخم تمام ضخامت برشی توسط پانچ بیوپسی ۷ میلی‌متری بر پوست ناحیه پشت هر موش ایجاد شد. همه موش‌ها به چهار گروه کنترل، ژل آلوئه ورا ۵٪ (w/w)، عصاره دارچین ۵٪ (w/w) و ترکیب ژل آلوئه ورا ۵٪ با عصاره هیدرو الکلی دارچین ۵٪ (w/w) تقسیم شدند. سرعت بسته شدن زخم تا ۱۴ روز بعد از القای زخم اندازه‌گیری شد. به لحاظ آسیب‌شناختی بافتی، روند ترمیم زخم ازنظر ظرفیت آنتی‌اکسیدانی، سطح مالون دی آلدئید، سوپر اکسید دیسموتاز و میزان هیدروکسی پرولین بین گروه‌ها مورد مقایسه قرار گرفت.

نتایج- میزان انقباض زخم، تعداد فیبروبلاست، رسوب کلاژن، ضخامت بافت پوششی، ظرفیت آنتی‌اکسیدانی، سوپر اکسید دیسموتاز و میزان هیدروکسی پرولین در گروه‌های درمانی نسبت به گروه کنترل افزایش معناداری ($p<۰/۰۵$) را نشان داد. میزان ادم، ارتشاح سلول‌های ایمنی و سطح مالون دی آلدئید در هر سه گروه درمانی نسبت به گروه کنترل کاهش یافت ($p<۰/۰۵$).

نتیجه‌گیری و کاربرد بالینی- تجویز هم‌زمان پمادهای تهیه‌شده از آلوئه ورا و عصاره دارچین اثرات سینرژیسمی را نشان داده و التیام زخم را در موش‌ها بهبود دادند. پیشنهاد می‌شود ترکیبی از آلوئه ورا و عصاره دارچین به‌عنوان یک داروی جدید گیاهی برای درمان زخم دیابتی استفاده شود.

واژه‌های کلیدی- ژل آلوئه ورا، دارچین، التیام زخم، دیابت، موش.