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Original Article

A Histopathologic Study of Effects of Olive Oil Plus Lime Water on Third-Degree Burn in Mouse Model

Siamak Kazemi-Darabadi*[1], Ghasem Akbari[2], Seyed-Hossein Jarolmasjed[1], Amir-Ali Shahbazfar[3]

Abstract

Objective- The objective of this study was to compare effects of olive oil and lime water combination with silver sulfadiazine in third-degree burn healing.
Design- Randomized experimental study.
Animals- Sixty-three adult male Bulb/C mice weighing 25±5 g.
Procedures- The mice were anesthetized with an interaperitoneal injection of ketamine 10% and xylazine 2% combination and the third-degree burn wound was created in the area of 1×1 cm at the dorsum of the animals using an innovated electrical device. There were three groups of 21 as follow: Group I – Negative control; which received the topical normal saline solution, Group II – Positive control; with the daily topical application of silver sulfadiazine ointment, and Group III – Treatment; which received topical olive oil plus lime water, daily. Each group was subdivided into three subgroups and topical treatments or saline were applied to each subgroup for 7, 14, and 21 days, respectively. No other dressing was used. The mice of each subgroup were sacrificed on days 7, 14, and 21 and hematoxylin-eosin (H&E) stained slides were prepared. Histopathologic evaluations included epidermal thickness, secondary infection, and percentage of collagen, ground substance, fibroblast and blood vessels.
Results- Group II showed significantly less secondary infection, and secondary infection in group III was significantly reduced compared to group I (P<0.05). The epidermal thickness of group III had a significant difference with group II at 2nd week. Both group II and III showed more collagen synthesis at 2nd week compared to group I. This was also true about ground substance. Group III had more angiogenesis at 2nd week compared to others, but ultimately this difference was diminished (P<0.05).
Conclusions and Clinical Relevance- Despite lime water had some cytotoxic effects, combining with olive oil reduced these undesired effects. Thus, the combination might be beneficial in third-degree burn wounds in mice compared to routinely-used silver sulfadiazine therapy.
Keywords- Lime water, Olive oil, Silver sulfadiazine, Third-degree burn, Mouse
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Introduction

Burns have a high prevalence in modern life.[1] In the United States, 486,000 patients are referred to medical centers because of burns every year. Among these, up to 40,000 persons are hospitalized and 3500 of them die.[2] These patients are usually managed by a staged surgical wound closure and topical wound therapy and dressing before wound closure.[3] Several works have been carried out to develop the more advanced dressing that accelerates the healing process and diminishes the bacterial load in wounds. Recently, traditional medicine, especially the herbal products deployed for centuries, are under scientific investigation for their feature in the wound healing.[4]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[2] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[3] Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
Address all correspondence to Siamak Kazemi-Darabadi (DVM, DVSc)
E-mail: s.kazemi@tabrizu.ac.ir

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Lime water has been traditionally used for burn wound treatment in the northwest of Iran for centuries. Antibacterial effects of lime water have been shown in some researches.[5] In an *in vitro* study, the researchers found that lime cream has an antibacterial activity but also cytotoxic on the fibroblasts. They found out that adding olive oil to lime cream reduces its cytotoxic impact and concluded that mixture of olive oil and lime cream is not cytotoxic and has antimicrobial activity. Olive oil has been called liquid gold because it contains a high percentage of single fatty acid. It is a good source of antioxidants with a high calorific value and a source of essential fatty acids and fat-soluble vitamins tank such as A, D, E, K.[6] The positive effect of this compound on burn wound healing is also proven.[7]

Silver sulfadiazine is a common medicine for dressing burn wounds. This drug stimulates epidermal regeneration and has a role in granulation tissue formation and myofibroblasts proliferation. In addition, it has broad spectrum antibiotic effect on both gram negative and gram positive bacteria.[8] However, some adverse effects such as reduction in platelet and neutrophil number, fibroblast reduction in wound area, delayed healing, suppression of reepithelialization and bacterial resistance may be encountered.[9]

Despite the advances in burn wound management, there is a long way regarding high mortality rate of burn patients. Indeed, proper management of the burn wounds and the resulting scar tissue still remain a problem though various methods of surgical burn wound closure are employed.[3] In the present study, we investigated effects of olive oil and lime water mixture on third-degree skin burn in the mouse model. The objective was to compare this traditional method of wound healing with silver sulfadiazine which is routinely used for the treatment of burn patients. The results of this study could document the value of Iranian traditional medicine in burn patients.

Materials and Methods

Animals

Sixty-three adult male Bulb/C mice weighing 25±5 g were used in this study. The mice were kept in 22° C environment with a 12 h light/darkness cycles. They had free access to tap water and commercial mouse pellet. The period of one week was considered to acclimatize prior to investigation. The study was approved by the Ethics Committee for Animal Investigations of the University of Tabriz (code D/43/7354) in accordance with the Declaration of Helsinki and all aggressive interventions involving the animals were done only after proper sedation and anesthesia.

Burn Model

We employed an innovated device for burn wound induction which is approved and patented by Intellectual Property Center, State Organization for Registration of Deeds and Properties, Iran (Figure 1). The device has an exchangeable iron plate with different sizes that is heated by electric current. The current determines temperature of the plate. We used 1 cm^2 iron plate in this study. The amount of heat and time needed for intended degree of burn wound were determined by a pilot study and histopathologic evaluation of burned skin for every animal species. They were 95 ºC and 10 seconds for mouse skin. The mice were anesthetized with interaperitoneal injection of ketamine 10% (70 mg/kg) and xylazine 2% (8 mg/kg) combination and the dorsum of the animals were clipped. The third-degree burn wound was created at the clipped area under the aforementioned device. In order to prevent shock, 1 ml of normal saline solution was injected interaperitoneally into each mouse after burn induction.

Study design and Treatments

The randomized experimental study design was employed in this research. The mice were numbered and randomized into three groups of 21, using Microsoft Office Excel random number table (Microsoft Office 2016, Microsoft Corporation, Redmond, WA). Three experimental groups were as follows:

Group I – Negative control; which did not receive any topical treatment except for normal saline solution,
Group II – Positive control; with daily topical application of 1% silver sulfadiazine topical cream (Iran Najo Pharmaceutical Co., Tehran, Iran) on the burnt area, and
Group III – Treatment; which received topical olive oil plus lime water, daily. For the preparation of this mixture, 100 g of completely dry lime was dissolved in 200 ml of distilled water and after lucidity, the supernatant was passed through a filter. Afterwards, the equal volume of olive oil (Olitalia, Forli, Italy) was added and mixed with the lime water.

The total period of the study was in three weeks. All treatments were applied once a day. The mice of each group were divided into three subgroups of seven mice. The first subgroup received treatments for 7 days, the second subgroup received treatments for 14 days, and finally, the treatments were applied to the third subgroup for 21 days. No other dressing was used to cover the wounds. In all three experimental groups, the mice of each subgroup were sacrificed by anesthetic overdose

Figure 1: The device for skin burn induction is setup for 95° C and 10 seconds and the heated exchangeable iron plate is applying over mouse skin.

(ketamine 10% [300 mg/kg] and xylazine 2% [35 mg/kg] combination) followed by cervical dislocation on days 7, 14, and 21, respectively, as mentioned above and skin samples were fixed in 10% solution of formalin.

Histopathologic Evaluations

Skin samples were prepared routinely with tissue processor machine (Leica TP1020, Germany) and sections with 5 µm thickness were made using a microtome device. The slides were stained with hematoxylin-eosin (H&E). Histopathologic evaluations include the epidermal thickness (µm), secondary infection, and percentage of collagen, ground substance, fibroblast, and blood vessels. The thicknesses of the epidermal areas were measured in ten different places along the wound surface under the light microscope with an eyepiece lens with 400× magnification and the average of these areas were taken. The number of formed blood vessels and fibroblasts were counted by special morphometric lens. The vessels were counted by detecting their endothelial layer. Ten different areas in the sections were counted and mean values were calculated.[10]

Statistical Analysis

Statistical analyses were done using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). All results are presented as the mean ± standard deviation (SD). Parametric data were analyzed by one-way analysis of variance (ANOVA) with comparisons between groups by Tukey test. Kruskal-Wallis test was employed to analyze non-parametric values, and comparisons between groups were performed by Mann-Whitney U-tests. The results were considered significantly different when $p<0.05$.

Results

The histopathologic results can be found in Figure 2. Although the epidermal thickness of group III had a significant difference with group II at 2nd week, this difference was not significant at the end of the study. Both groups II and III induced more collagen synthesis at 2nd week compared to group I, however, there was not any difference after 3 weeks ($P<0.05$). This was also true about ground substance. We did not observe any significant difference in fibroblast deposition among the groups. Group III had more angiogenesis at 2nd week compared to others, but ultimately this difference was diminished. Finally, as expected, group II showed significantly less secondary infection in all the times, and secondary infection in group III was significantly reduced compared to group I at 2nd and 3rd weeks, although still higher than group II. A series of micrographs of experimental groups at the end of the study is shown in Figure 3. The collagen fibers are irregular and extended to different directions, some inflammation was visible, and the epidermis was thin in group I. The collagen fibers were more matured and regular, the number of fibroblasts was less than the control group, there was no inflammation, and the epidermis was thicker in group II. Finally, in the group III, the collagen fibers were partly regular but regularity was less than group II, the number of fibroblasts was between two other groups, the epidermis was completed and had a normal thickness, and the border of healthy tissue and healed area of burnt skin was visible. Figure 4 shows gross appearance of the burn wounds in different experimental groups.

Discussion

Antibacterial and wound healing acceleration properties of lime water and limited studies about its benefits in second-degree burns,[5] led us to investigate effects of lime water plus olive oil in third-degree burn wound on mouse skin. The findings of this study showed that this combination could increase epidermal thickness, reduce wound infection and ground substance of wound bed, and induce angiogenesis in this model of burn wounds. Burn wounds destroy skin structure leading to functional damages such as loss of thermoregulation, sensation, immunologic responses, integrity against bacterial

invading, and skin hemostasis.[11] Classification of the burn wounds is based on the lost tissues.[12] In the third-degree burns, epidermis, dermis, and sometimes hypodermis, muscles, and even bones undergo necrosis.[13]

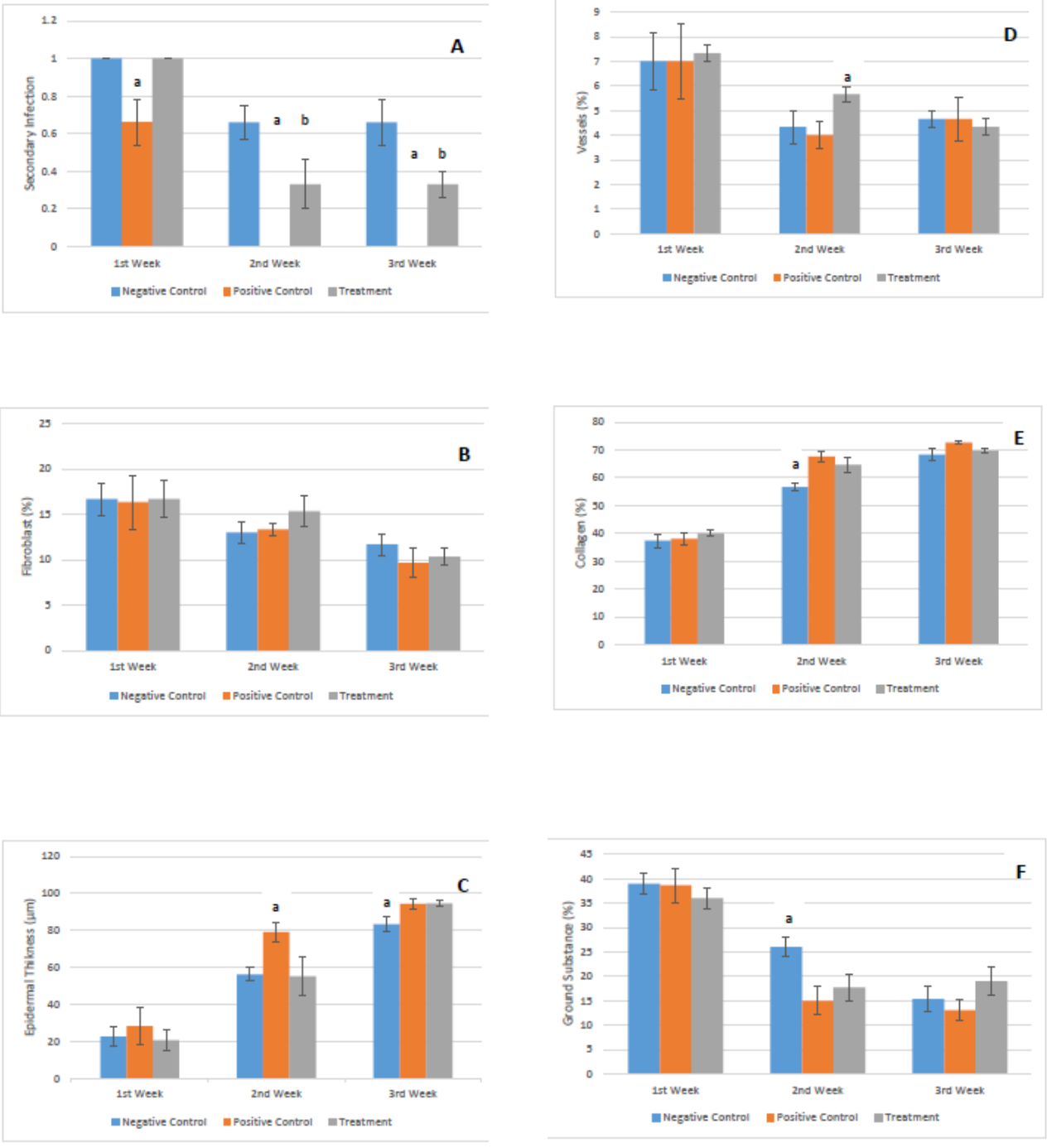

Figure 2: Histopathologic parameters in different groups (mean ± SD). Secondary infection (A), Percentage of fibroblasts (B), The epidermal thickness in µm (C), Percentage of angiogenesis (D), Percentage of collagen (E), Percentage of ground substance (F). Different letters in each column indicate significant difference (P<0.05).

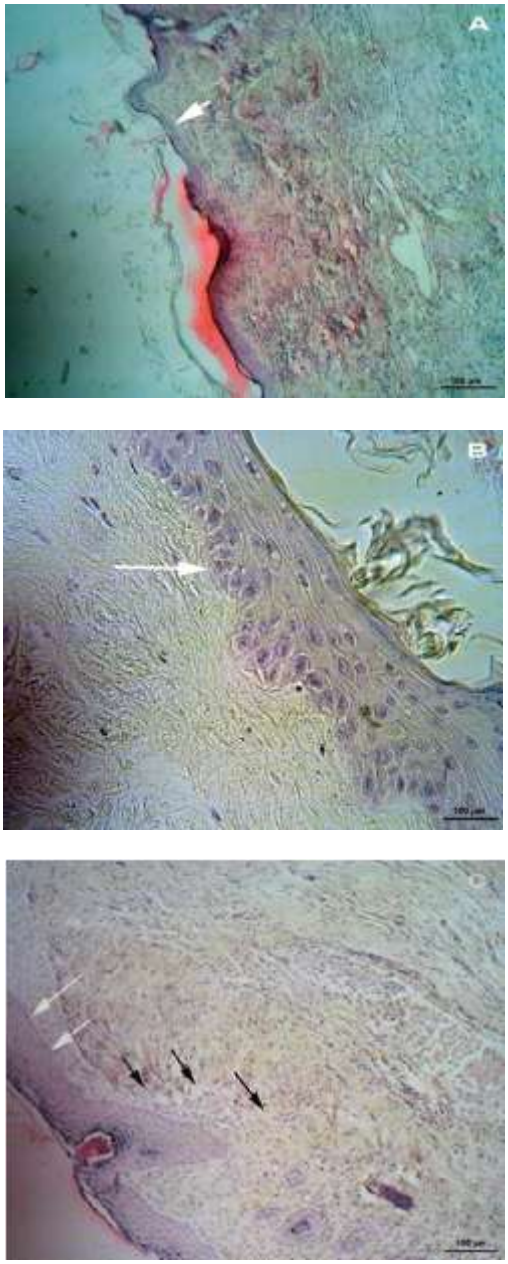

Figure 3. Representative micrographs of the skin of experimental groups after 3 weeks of burning. H&E staining.
Group I: The collagen fibers are irregular and extended to different directions; some inflammation is visible; and the epidermis is thin (white arrow) (A). Group II: The collagen fibers are more matured and regular than group I; the number of fibroblasts is less than the control group; there is no inflammation; and the epidermis is thicker (white arrow) (B). Group III: The collagen fibers are partly regular, however, regularity is less than group II; the number of fibroblasts is between two other groups; the epidermis is completed and has a normal thickness (white arrow); and the border of healthy tissue and healed area of burnt skin is visible (black arrows) (C).

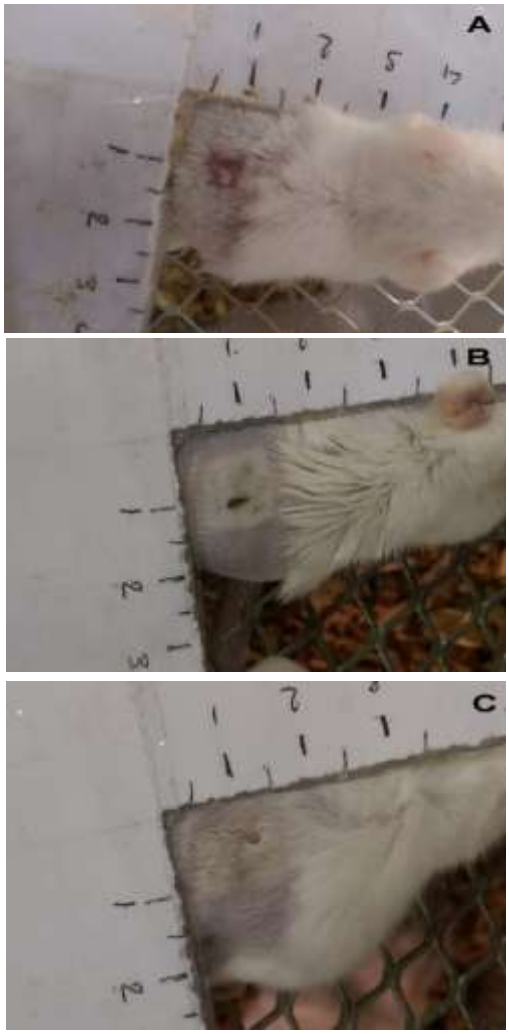

Figure 4: Skin wounds in groups I (A), II (B), and III (C) mice 21 days after third-degree burning.

We ensured about intended burn degree development by the pilot study and specimens confirmed full thickness skin burn. In addition, all wounds represented whitish appearance after three days of the experiment.
Morbidity is greater in pediatric and geriatric burn patients. The immune system is not fully developed in the young patients, and old patients may have a debilitated immune system.[14] None of our mice died during the study because we used adult animals. Infection is the main cause of death in burn patients.[15] Actually, 70% of complications in burn patients are related to infection.[16] The extent and depth of the burn are closely related with the presence of infection. Patients with third-degree burns have approximately twice the risk of infection. Deep burns generate more devitalized tissue and thicker avascular eschar, which is an ideal place for bacteria colonization and reproduction.[14] As devitalized tissue increases, more

stress hormones and proinflammatory mediators are released, increasing inflammatory response and secondary cell damage leading to organ dysfunction.[17] Our results showed infection in all groups after a week of burn, though, silver sulfadiazine had partly reduced infection rate in this period. The amount of infection in antibiotic and lime water groups after two and three weeks were significantly lesser than the control group. Sulfadiazine is the drug of choice in the infectious period in burn wound.[18] The free silver can react with both sulfhydryl groups of bacterial enzymes and DNA, and sulfadiazine can stop the synthesis of DNA by interrupting the production of folate.[19] Antibacterial effect of lime cream on 6 bacteria and 2 yeast strains have been discovered. Although, the mechanism of action is unknown.[6] In addition, olive oil contains a high percentage of fatty acids which have antimicrobial properties, also olive oil can isolate the burn area from the exterior environment, making it potentially an antimicrobial agent.[20]

Silver sulfadiazine has been shown to be cytotoxic *in vitro* toward fibroblasts and consequently to retard wound healing *in vivo*.[21] This explains why fibroblasts were reduced in group II after 3 weeks. Lime water also has cytotoxic effects, but olive oil can prevent this unwanted effect.[6] Previously, it has been reported that olive oil phenolics, even at high concentrations, do not exert cytotoxic effects on mucosal cells.[22] Our results also demonstrated a slight reduction in fibroblasts in group III, but still higher than group II. The last step of the proliferative phase in the healing process is epithelialization, which involves migration, proliferation, and differentiation of epithelial cells from the wound edges to resurface the wound.[23] Epidermal integrity is an important parameter for prevention of wound infection and smooth wound healing.[24] The epidermis was significantly thicker in groups II and III compared to group I after two and three weeks, respectively. Group III had produced more granulation tissue in a two-week period. As a result, keratinocytes had a base to proliferate on this surface, resulting in complete re-epithelialization. Although most reports suggest retardation of epithelialization by silver sulfadiazine, some other investigations have stressed on the acceleration of epithelial layer formation by this compound in earlier phases of healing[25] which supports our results. Angiogenesis, or the process of forming new blood vessels, is critical to wound healing; because it allows the delivery of vital cells and nutrients to the wound.[26] Vascular remodeling is characterized by a reorganization of blood vessel geometry in response to physiological alterations in blood flow. Long-term rise in blood flow will increase lumen diameter and thus accommodate increased blood supply. A physiological role for endothelial cell apoptosis to contribute to vessel regression is suggested.[27] This explains why angiogenesis in group III was significantly greater than two other groups at the end of the second week, but this parameter was reduced a week later. On the other hand, after completion of the healing process, there is no need for excess blood supply in healed area, consequently, proliferation of endothelial cell is diminished and the vessels undergo apoptosis.

It is known that collagen increases the process of healing.[24] Our result demonstrated that collagen synthesis was accelerated in both groups II and III after two weeks, and the regularity of collagen fibers was higher than the control group at the end of the study. Collagen deposits from fibroblast cells and gives the dermis its structural integrity.[28] Although we did not determine the quality of collagen fibers, which may be a limitation of our study, considering silver sulfadiazine inhibits the regeneration of the fibroblasts, collagen deposition in the dermis may result in fragile and stiff membrane.[29] Since the olive oil in combination with lime water did not show cytotoxic effects on fibroblasts, we assumed that the quality of collagen fibers, i.e. tensile strength, in group III may be better than group II, despite micrographs demonstrated less regularity of the fibers in the treatment group.

The synthesis of extracellular matrix to fill in the damaged tissue is another part of tissue repair. The matrix is primarily made up of fibrinogen and fibronectin.[30] Thereafter, proteoglycans are synthesized by cells to make up the ground substance of the extracellular matrix.[31] Despite our findings showed reduced ground substance after two weeks, this matrix had no significant difference ultimately.

In conclusion, despite lime water has some cytotoxic effects, combining with olive oil could reduce these undesired effects. Thus, the combination may be beneficial in third-degree burn wounds in mice compared to routinely-used silver sulfadiazine therapy.

Acknowledgments

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Conflicts of interest

None.

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نشریه جراحی دامپزشکی ایران
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چکیده

مطالعه آسیب شناسی اثرات روغن زیتون به همراه آب اهک بر روی سوختگی درجه سوم مدل موش

سیامک کاظمی درآبادی[۱*]، قاسم اکبری[۲]، سید حسین جارالمسجد[۱]، امیرعلی شهبازفر[۳]

[۱] گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه تبریز، تبریز، ایران
[۲] گروه علوم پایه، دانشکده دامپزشکی، دانشگاه تبریز، تبریز، ایران
[۳] گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه تبریز، تبریز، ایران

هدف- هدف این مطالعه مقایسه اثرات ترکیب روغن زیتون و آب آهک با سیلور سولفادیازین بر التیام سوختگی درجه سوم بود.

طرح- مطالعه تجربی تصادفی

حیوانات- شصت و سه موش سوری بالغ نژاد بالب/سی با وزن ۵±۲۵ گرم

روش کار- موش‌ها با تزریق داخل صفاقی کتامین ۱۰٪ و زایلازین ۲٪ بیهوش شده و با استفاده از دستگاه ابداعی الکتریکی سوختگی درجه سوم به ابعاد ۱×۱ سانتی‌متر در پشت آنها ایجاد گردید. موشها به سه گروه ۲۱ تایی به صورت زیر تقسیم شدند: گروه ۱- کنترل منفی؛ که محلول نرمال سالین را به صورت موضعی دریافت کردند، گروه ۲- کنترل مثبت؛ که روزانه پماد سیلور سولفادیازین موضعی دریافت نمودند، و گروه ۳- تیمار؛ که ترکیب روغن زیتون و آب آهک را روزانه به صورت موضعی دریافت کردند. هر گروه به سه زیرگروه تقسیم شده و استعمال داروها یا سالین در هر زیرگروه به ترتیب به مدت ۷، ۱۴، و ۲۱ روز صورت گرفت. از هیچ پانسمان دیگری روی زخم‌ها استفاده نشد. موش‌های هر زیرگروه در روزهای ۷، ۱۴، و ۲۱ آسان کشی شده و مقاطع رنگ آمیزی شده با هماتوکسیلین و ائوزین از ناحیه سوختگی تهیه گردید. ارزیابی‌های آسیب شناسی شامل ضخامت اپیدرم، عفونت ثانویه، و درصد کلاژن، ماده زمینه ای، فیبروبلاست و عروق خونی بود.

نتایج- گروه ۲ به صورت معناداری عفونت ثانویه کمتری نشان داد، و میزان عفونت در گروه ۳ در مقایسه با گروه ۱ کمتر بود. ضخامت اپیدرم در گروه ۳ اختلاف معناداری با گروه ۲ در انتهای هفته دوم داشت. در هر دو گروه ۲ و ۳ سنتز کلاژن در پایان هفته دوم بیشتر از گروه ۱ بود. این امر در مورد ماده زمینه ای نیز صادق بود. گروه ۳ رگ‌زایی بیشتری نسبت به گروه های دیگر در پایان هفته دوم داشت، ولی در نهایت در پایان هفته سوم این تفاوت کاهش پیدا کرد.

نتیجه گیری و کاربرد بالینی- با وجودی که آب آهک دارای برخی اثرات سایتوتوکسیک است، ترکیب آن با روغن زیتون این اثرات ناخواسته را کاهش می‌دهد. بنابراین، این ترکیب می‌تواند در درمان سوختگی‌های درجه سوم در موش در مقایسه با درمان رایج سیلورسولفادیازین، مفیدتر باشد.

کلمات کلیدی- آب آهک، روغن زیتون، سیلور سولفادیازین، سوختگی، موش