



COMPARATIVE STUDY OF THE EFFECTS OF ZOUSH OINTMENT AS A NATURAL PRODUCT AND SILVER SULFADIAZINE ON THE SECOND-DEGREE BURN WOUNDS HEALING IN MICE: ROLE OF ANTIOXIDANTS AND THE GENE EXPRESSION OF MATRIX METALLOPROTEINASE-9

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Despite progress in the treatment of burn wounds, effective wound healing remains a severe challenge. In present study, the efficacy of ZOUSH ointment on second-degree burn wound in an animal model has been investigated through antioxidant analysis. The expression of Mmp-9 as a repair factor was measured as well. After induction of second-degree burn, eighty female BALB/C mice were randomly divided into four groups. The first group was treated with ZOUSH, the second group received Silver Sulfadiazine (SSD), the third group received Eucerin, and the control group no medication. Every five days, skin and blood samples of five sacrificed mice were collected and stored at -80°C for further biochemical and histological analyses. Results showed a significant increase in Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione (GSH.); however, Malondialdehyde (MDA) concentration significantly decreased in both samples of the ZOUSH treated groups in comparison with the control group ($p \leq 0.05$). Evaluating the wound healing process during the treatment showed that ZOUSH accelerates the wound healing process compared to other groups. Gene expression analysis showed increased Mmp-9 expression level more than ten times compared to the untreated group. According to present study results, ZOUSH ointment proved highly effective in the wound healing of the second-degree burn. Thus, ZOUSH ointment seems to be a reliable alternative to current burn wound therapies.

INTRODUCTION

Burn is a skin injury mostly caused by high temperature, caustic chemicals, or chemical warfare¹. Burn injury is a type of inflammation that relies on the treatment approach can either continue to recover or

degenerate to further necrosis². The thermal injury causes a systemic inflammatory response in which cytokines, growth factors, matrix metalloproteinases (MMPs), and reactive oxygen species (ROS) are produced to recruit cells to the wound tissue^{3&4}. Skin thermal injury is an oxidation process associated with

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the generation of ROS from various cellular populations. Oxidant changes are manifested in local and systematic dimensions⁵. Burn injury upsets the physiological balance between the production of free radicals and the antioxidant system. The overproduction of free radicals leading to lipid peroxidation (LPO) causes damage to tissue. Malondialdehyde (MDA.), as the end product of LPO, is considered the leading indicator of oxidative stress and tissue damaging⁶. Enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), Glutathione S-transferase (GST), and glutathione (GSH) play a crucial role in combination with oxidative stress and tissue injury⁷. Therefore, the triggering of the produced ROS with antioxidants and alterations in the antioxidant status can be important for pharmacological burn treatment⁵.

Matrix metalloproteinase (MMPs) released by keratinocytes and inflammatory cells play main roles in the wound healing process⁸. Dysregulation of MMPs led to prolonged inflammation and delayed wound healing⁹. Mmp-9 is expressed at the leading edges of migrating keratinocytes during the woundhealing process, and Mmp-9 knockout (KO.) mice lead to delayed wound closure indicating the primary role of Mmp-9 in wound healing¹⁰⁻¹³.

Despite many treatment discoveries, wound healing remains a challenge to modern medicine. Many local and systemic medications have been used, but none of them has proved entirely satisfactory. The most common treatment is Silver Sulfadiazine (SSD) which is mostly administered due to antimicrobial features¹⁴. However, long-term use of SSD might develop microbial resistance, renal dysfunction, and leukopenia^{15&16}.

Studies have shown that some herbal ointments can accelerate burn wound healing¹⁷⁻¹⁹. ZOUSH as a herbal ointment comprises extracts of some herbal plants including *Satureja khuzestanica*, Thyme, Oregano plant, honey and polyurethane²⁰. The antioxidant activity of all ingredients has been reported in different studies^{19&21-24}. It appears that ZOUSH has antibacterial effects that promote the healing process of second-degree thermal burn^{20&25}, but further studies are required to find out its mechanism on the burn

healing process. Present study was designed to evaluate the antioxidative and molecular effects of ZOUSH ointment on second-degree burns healing in mice compared with SSD. O.

MATERIALS AND METHODS

Burn wound induction

This interventional-experimental study was approved by the Research Council of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1396.589). Eighty 6-9-week female BALB/C mice weighing 18.5±1.2 were under study. They were kept under the same light, darkness and humidity conditions. Mice were given ad libitum access to food and water. All procedures were performed based on protocols approved by the Animal Care and Use Committee of Virginia Commonwealth University (IACUC AM 10154) based on the Steritz and Holder burn mouse model (BMM)²⁶⁻²⁸. Briefly, the mice were anesthetized with xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg). In this model, the burn wound is infected by *seudomonas aeruginosa*. At first, the back's fur was shaved with an electrical cropper in a dimension of 3×3 cm² and disinfected with 70% alcohol. A deep second-degree burn wound was made with a hot plate (diameter 2×2 cm²) at nearly 97°C (heated 5 min and put on the skin for 10 seconds under a similar pressure). Finally, five minutes after burning, the wound was infected by 20 µl suspension of *pseudomonas aeruginosa* with a sub-lethal half Mcfarland concentration at the site of the wound²⁰.

Grouping of mice and treatment

Mice were divided into four groups of mice ($n= 20$ each), and treatment began 24 hours after burning for 20 days. Group 1 received topical ZOUSH, Group 2 was treated with topical SSD, Group 3 was administered topical Eucerin as a base ointment and Group 4 was considered as the control group without medication. Treatment was performed twice a day in the morning and afternoon at certain times. All animals received the same amount of ointments (0.5 g). During treatment, the size of the wounds was measured by caliber every five days; and the percentage of the wound area and wound healing was calculated by the following formula²⁹⁻³³.

The same researcher did all measurements.

Percent of wound area = wound area in day x/
wound area on the first day.

Percent of wound healing = 100 – percent of
the wound area.

In parallel with wound healing process analyses, every five days, five mice were randomly collected from each group and sacrificed for biochemical and histological studies. The blood and scar tissue samples were collected and stored at -80°C for the assessment of biochemical factors. The frozen tissue samples were homogenized in PBS (1:10 w/v) and centrifuged at 12000 g for 15 minutes. The tissue supernatant and serum samples were used for biochemical experiments.

Preparation of alcoholic extracts

The alcoholic extracts of plants such as *Satureja khuzestanica*, *Organum Vulgare* and *Zataria Multiflora* were obtained from Barij Essence Pharmaceutical Company in the form of powder. The extracts' components were measured by gas chromatography (GC-MS) in Barij Company^{20&34-36}.

Formulation of ZOUSH Ointment

ZOUSH ointment was made with the 5 percent concentration of extracts, honey and polyurethane with equal ratio and the other 95% with Eucerine as a base²⁰.

Histological analysis

Hematoxylin and Eosin (H&E) staining were used to stain tissue sections. Microscopic images of tissue were taken under X100 magnification.

Superoxide dismutase (SOD) activity

The SOD activity of the sera and tissue homogenates was measured using the Winterbourne method²⁸. Slightly, 0.2ml of (0.1mol/l EDTA and 0.3mmol Sodium Cyanide), 0.1ml of (1.5mM NBT), and 200µl of homogenized tissue (or buffer for control) were added to the mixture and mixed spontaneously. The mixture was kept at 37°C for 5 minutes. After that, 0.05 ml of 0.12 mM riboflavin, and 0.067 M potassium phosphate buffer of pH 7.8 were added, and the

absorption was measured at 560 nm, after 12 minutes of incubation at room temperature. The SOD activity was reported as U/mg protein.

Catalase (CAT) activity

Catalase activity of the samples was assayed as previously described³⁷. Briefly, 0.1 ml of serum or tissue homogenates was added to 1.0 mL of 20 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7.0). H₂O₂ decomposition was monitored at 240 nm.

Assay of glutathione (GSH) concentration

The GSH concentration was measured using the TIETZ method in the sera and tissue homogenate³⁸. Tissue samples were homogenized, mixed with 5% sulfosalicylic acid, and incubated at 4°C for 10 minutes. The test mixture, including 100 µl of supernatant and 810 µl of 0.3 M disodium phosphate and 90 µl of (0.4% DTNB in 1% sodium citrate), was added and changes in absorption were measured at 412 nm. The GSH concentration was reported in µmole/mg protein.

Assay of malondialdehyde (MDA) concentration

Thiobarbituric acid-reactive substances (TBARS) were measured by a slightly modified method of Uchiyama and Mihara. After protein precipitation through TCA assay, 1.5 ml of supernatant and 2 ml of TBA (0.67%) were mixed and placed in boiling water for 30 min. After that, 1.25 ml of *n*-butanol was added to the cooled sample, vortexed and centrifuged at 2000 g for 5 min. The absorbance of the supernatant was read at 532 nm. 1,1,3,3-tetraethoxy propane was used as standard³⁹.

Real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). After checking the quality of RNA samples by Nanodrop (ND-1000) spectrophotometer, 1 µg of total RNA was used for cDNA synthesis according to the manufacturer's protocol (Fermentas Inc., MD). Applied Biosystems 7900 instrument was used for quantitative real-time PCR (qRT-PCR) by the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Matrix metalloproteinase-9 Mmp-9 and B-actin primers were designed by

Primer3web (version 4.1.0) taking into account essential components, including primer binding temperature, G and C nucleotide content, lack of primer, primer size, and being synthesized with the Pishgam company. Table 1 displays Primer sequences.

Table 1: Sequence of primers.

Gene name	Sequence	Size
Mmp-9	F TAAAGGCCGCTCGGATGGTT R CCACTCGGGTAGGGCAGAAG	89bp
B-actin	F GCCGGGACCTGACAGACTAC R TGGCCATCTCCTGCTCGAAG	141bp

Statistical analysis

All data were analysed based on mean \pm SE using SPSS 22. One-way ANOVA was performed with Tukey's test. In all the tests, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Results

Wound healing analyses

Second-degree burns involving the epidermis and varying thickness of the dermis⁴⁰ are shown in the present study (Fig. 1a). For

wound healing analysis, the percentage of wound healing was measured using the caliber every 5 days. Results showed that the healing effect of ZOUSH Ointment is greater than that of the other treatments (Fig. 1b&c). The ZOUSH ointment receiving group got more wound healing effect than the control group on the 5th, 10th, 15th, and 20th days, respectively ($p < 0.05$). Furthermore, results showed that there was insignificant difference between the wound healing effect of ZOUSH and SSD ($p > 0.05$).

It is worth noting that the size of the wound in the control groups reduced as well, but it was much more slowly than the ZOUSH and SSD treated groups.

Re-epithelialization analyses

The presence of re-epithelialization was studied by H& E staining. Loss of the epidermis was still evident in all groups on the 5th day. Re-epithelialization was more prominent in ZOUSH group through the treatment period in comparison to SSD and Eucerin group. In the control group, the dermis was repaired, but it was not as complete as the other groups (Fig. 2).

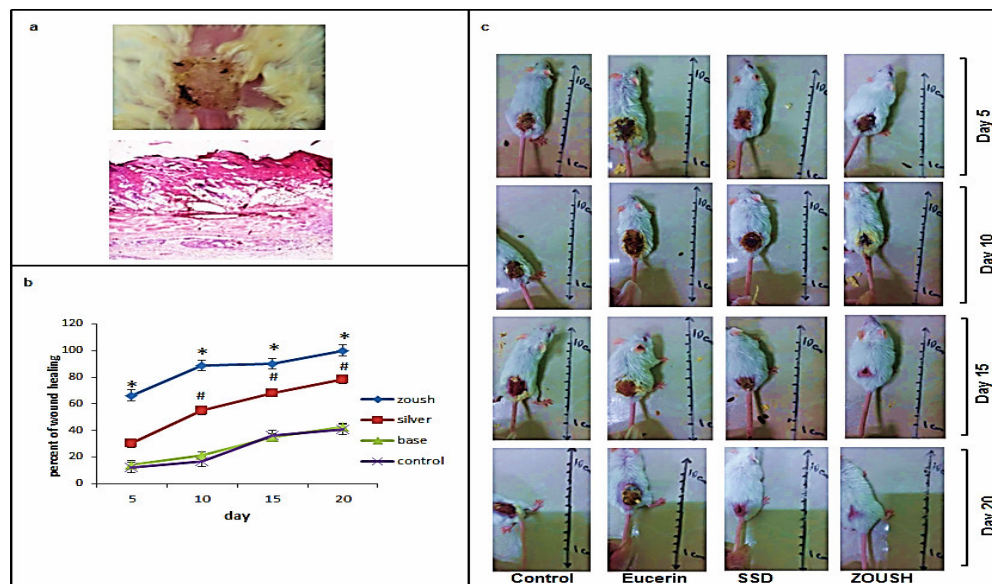


Fig. 1: The wound healing process. a) Histological analyses of burn wounds indicated that second-degree burn had been developed. b,c) wound area measurement during the treatment showed that ZOUSH can accelerate the wound healing process significantly in comparison to control groups on the 5th, 10th, 15th and 20th day of treatment. SSD can improve the process on 10th, 15th and 20th day of treatment.

* : $p < 0.05$ ZOUSH ointment versus control on the same day of the experiment.

: $p < 0.05$ Silver ointment versus control on the same day of the experiment.

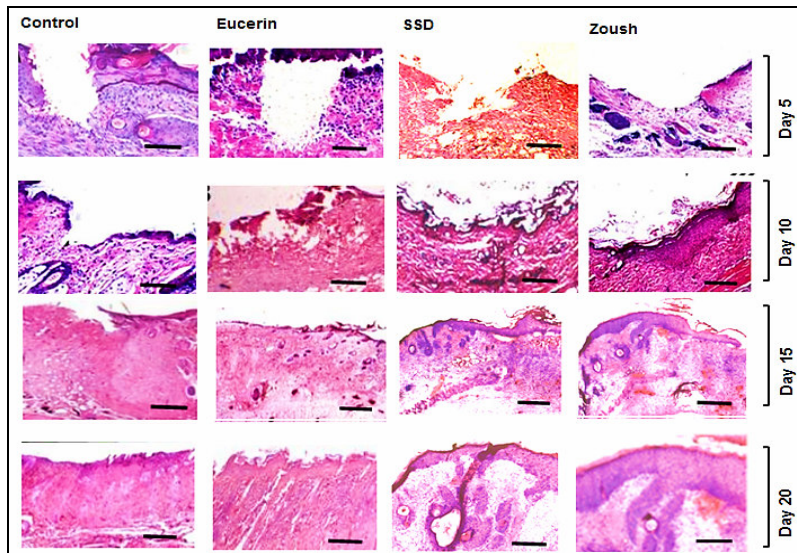


Fig. 2: Microscopic images of skin tissue samples of 5th, 10th, 15th and 20th day (magnification of X100) in ZOUSH, SSD, Eucerin and control groups.

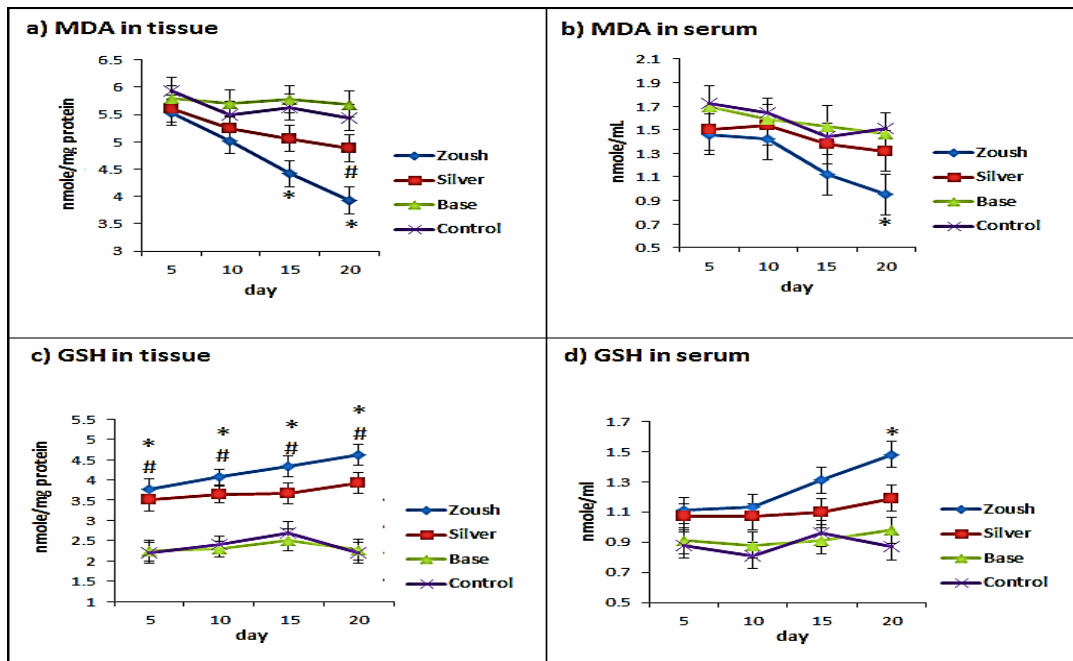


Fig. 3: The effects of ZOUSH and SSD on MD and GSH concentration in the second- degree burn in comparison to control and base groups. a) MDA level decreased significantly in tissue samples in ZOUSH group compared the control groups on 20th day. On the 15th and 20th day, SSD group showed significant increases. b) MDA level also showed a considerable decrease in ZOUSH group in sera on the 20th day. c) GSH concentration increased in tissue samples in ZOUSH and SSD group compared to control groups during the treatment and d) GSH only showed a significant increase in sera in ZOUSH group on the 20th day.

* : $p < 0.05$ ZOUSH ointment versus control on the same day of the experiment.

: $p < 0.05$ SSD versus control on the same day of the experiment. There was not significant difference between ZOUSH and SSD group.

Antioxidant analyses

The effect of ZOUSH Ointment was evaluated on MDA, CAT, SOD, and GSH in serum and tissue samples then compared with other groups. MDA analysis results in serum and tissue samples of the treated animal versus the control group are illustrated (Fig. 3a&b). Results showed that burn can cause a significant increase in the MDA level both in scar tissue and serum. The increase in MDA associated with the reduction in GSH level indicates the intensity of oxidative stress (Fig. 3). ZOUSH treatment via a time-dependent manner improved the tissue GSH level and

caused a substantial decrease in MDA levels in comparison with the control (Fig. 3). Except for the SSD group, SOD tissue activity increased in ZOUSH and SSD treated groups compared to the control and base groups on the 20th day (Fig. 4a & b). Serum catalase activity = increased in ZOUSH and SSD treated groups during the treatment period. There was not a significant increase in catalase activity in tissue samples treated with ZOUSH and SSD. In all experiments, ZOUSH ointment was proved to be more effective (Fig. 4c&d). During the treatment, all the biochemical parameters showed gradual changes in weekly intervals.

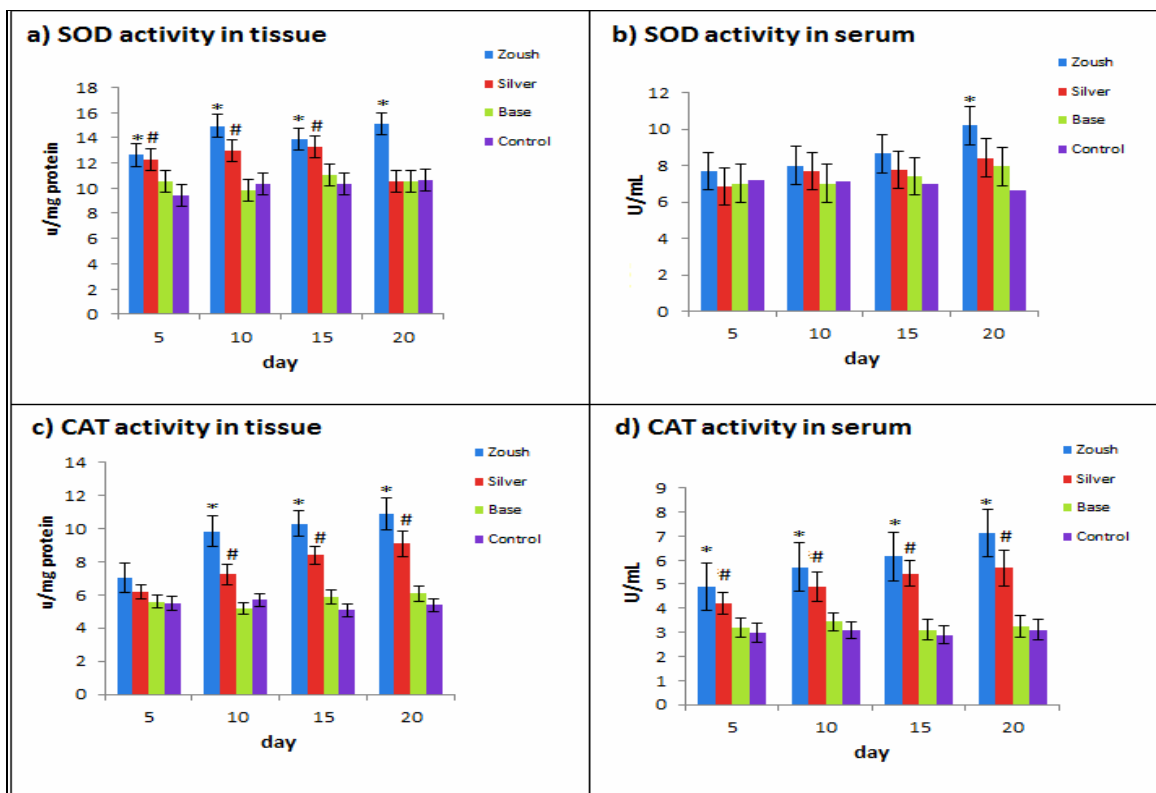


Fig. 4: The effects of ZOUSH and SSD ointment on SOD and CAT activity in the second -degree burn in comparison to control groups. a) In tissue samples, the activity of SOD enzyme significantly increased in ZOUSH and SSD groups during the treatment. b) On the 20th day, SOD. activity significantly increased in sera in ZOUSH group. c) Catalase activity showed significant differences on 10th, 15th and 20th in tissue samples in ZOUSH and SSD. groups. d) Catalase activity changes were substantial during the treatment in ZOUSH and SSD in sera.

* : $p < 0.05$ ZOUSH ointment versus control on the same day of the experiment.

: $p < 0.05$ Silver ointment versus control on the same day of the experiment, there were no significant differences between ZOUSH and SSD group.

Real-time PCR Analyses

The expression of Mmp-9 was measured in tissues treated by SSD compared to the untreated group on the 20th day. Results showed that although both ZOUSH and SSD treatment cause an increase in Mmp-9 expression, ZOUSH had greater effect (Fig. 5).

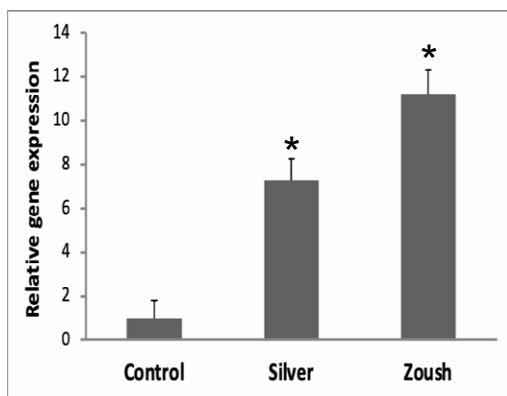


Fig. 5: The expression of Mmp-9 was analyzed in ZOUSH and SSD. treatment groups relative to untreated group on 20th day of treatment.

Discussion

Evidence from animal models and human studies demonstrated oxidative stress in burn injuries⁴¹. A severe burn is associated with the release of inflammatory factors, including Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). The activation of toxic inflammatory mediators, oxidants, proteases may further damage the skin and capillary endothelial cells and cause ischemic tissue necrosis⁴². Ischemia and consecutive reperfusion causing oxidative stress are characterized by an imbalance between ROS and the antioxidant defense system⁴³. This imbalance in the antioxidant defense system leads to lipid peroxidation which is a leading cause of oxidative damage in cellular membranes and cell death^{44&45}. Typically, cells can protect themselves from the harmful effects of oxygen radicals by antioxidant systems, including antioxidant molecules such as glutathione⁴⁶. During oxidative stress, antioxidant defense enzymes and glutathione decrease, but the MDA concentration increases. A suitable indicator of oxidative stress is the formation of MDA as the end product of lipid peroxidation. Thus, clinical response to burn can depend on the balance

between the production of free radicals and antioxidant molecules^{47&48}. Therefore, antioxidant treatment may probably be a suitable treatment for burn injuries. Recently, different researchers have focused their attention on medicinal plants and oxidative stress. In comparison with SSD, the efficacy of ZOUSH treatment on skin burn lesion, was assessed by monitoring oxidative stress and antioxidant factors such as MDA, CAT, SOD and GSH concentrations in both serum and tissue samples of treated animals. ZOUSH Ointment including the same ratio of *S. Khuzestanica*, Thyme, Oregano extract and honey with Eucerin as a base of the Ointment. The effects of ZOUSH constitutive on oxidative stress, antioxidant systems and virulence gene silencing of *Pseudomonas aeruginosa* have been investigated in previous studies⁴⁹⁻⁵⁵. Our results showed that CAT and SOD activities and GSH concentration increase significantly in ZOUSH treated group compared to SSD and control, but MDA levels reduced.

The level of MDA concentration in ZOUSH treated group was more than that in SSD group. At the same time, wound closure was measured based on the size of the wounds. The wound healing effects in the ZOUSH treated group were greater than that in SSD and control groups. The effect of ZOUSH was more significant on the 5th day, but the rate of wound healing decrease during the treatment in ZOUSH group. Wound healing is a multistep and complicated process that consists of four phases: hemostasis, inflammation, proliferation, and tissue remodeling. This process is affected by many factors impacting different stages and leading to impaired or accelerated wound healing⁵⁶. Workouts accelerates wound healing because it can reduce the inflammation phase⁵⁷. Anti-inflammatory effect of ZOUSH has been reported in a recent study²⁰. It can justify the sharp slope of the wound healing curve during the first days of ZOUSH treatment.

ZOUSH ointment is rich in antioxidant compounds such as terpenes, polyphenols, *M. Piperita*, *M. Rotundifolia*, *M. Pulegium*, *M. longifolia*, flavonoids, and phenolic acids. Inhibiting lipid peroxidation, scavenging free radicals, and enhancing the activities of SOD and CAT are mechanisms that can be applied to

accelerate wound healing process⁵⁴. The iron ion in honey has antioxidant properties which inhibit the reaction of Fenton⁵⁸. The phenolic elements of honey and the decrease in ROS levels can help heal wounds⁵⁹.

The results of the present study are consistent with those of this study, emphasizing the relationship between the antioxidant activity of the plant extracts and wound healing. Stimulatory effects of ZOUSH Ointment on the reversible activity of fibroblasts emphasize dermal wound healing feature of ZOUSH Ointment. Fibroblasts play a momentary role in dermal wound heal and cell colonization^{20, 60, 61}.

Even though ZOUSH and SSD mainly influenced the antioxidant activity and wound healing, ZOUSH proved to be more effective than SSD. It is associated with the strength of ZOUSH Ointment in ROS scavenging and preventing oxidative stress, having anti-inflammatory effects, and restoring the normal function of the skin.

In present study, gene expression analyses indicated an increase in the expression of the Mmp-9 and the effectiveness of the ZOUSH ointment for the treatment of burn wounds. Expression of Mmp-9 plays a role in separating the keratinocytes from the membrane, rebuilding the extracellular matrix, and potentially increasing cell migration and angiogenesis. The protein was expressed by this gene digest collagen type IV and V, and other extracellular matrix proteins biomolecules. Mmp-9 plays a significant role in wound healing, as if the gene is inhibited, the wound healing process is delayed^{62&63}.

It is observed that the high amount of Mmp-9 in chronic ulcers, triggers inhibition of the normal recovery process in the wound²⁰. Fray *et al.* reported that Mmp-9 is essential in wound healing and the repair of damaged tissues, it increases until the inflammation in the area, and then go through a decreasing trend to reach a normal. Mmp-9 expression plays a vital role in the onset of re-epithelialization, and is considered a useful measure for wound healing⁶³.

In a study conducted by our researchers, expression of the Mmp-9 gene in the group treated by ZOUSH and silver ointment increased.

Conclusions

Our researchers concluded that in the burn wound model of mice, ZOUSH Ointment can improve the healing process compared to SSD and the control groups. Owing to its evident antioxidant effects, ZOUSH can be used as an alternative agent for wound therapy. However, wound healing is a multi-factorial process involving different groups of enzymes, receptors, and others. Therefore, further studies are needed to understand the exact molecular mechanism of ZOUSH Ointment in wound healing process.

Abbreviations

SOD: Superoxide Dismutase

CAT: Catalase

GSH: Glutathione

MDA: Malondialdehyde

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of the Baqiyatallah University of Medical Sciences.

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نشرة العلوم الصيدلانية جامعة أسيوط



دراسة مقارنة لتأثيرات مرهم ZOUSH كمنتج طبيعي وسلفاديازين الفضة على التئام جروح الحروق من الدرجة الثانية في الفئران: دور مضادات الأكسدة والتعبير الجيني لمصفوفة ميتالوبروتيناز-9

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على الرغم من التقدم المحرز في علاج جروح الحروق، إلا أن التئام الجروح الفعال لا يزال يمثل تحديًا كبيرًا. في الدراسة الحالية، تم التحقق من فعالية "ZOUSH" مرهم ZOUSH على جرح حرق من الدرجة الثانية في نموذج حيواني من خلال تحليل مضادات الأكسدة. كما تم قياس التعبير عن مصفوفة ميتالوبروتيناز-9 (Mmp-9) كعامل إصلاح. بعد تحريض الحرق من الدرجة الثانية، تم تقسيم ثمانين أنثى من الفئران BALB / C بشكل عشوائي إلى أربع مجموعات. المجموعة الأولى عولجت بـ ZOUSH، المجموعة الثانية تلقت سلفاديازين الفضة (SSD)، المجموعة الثالثة تلقت Eucerin، المجموعة الضابطة لم تأخذ أي دواء. كل خمسة أيام، تم جمع عينات من الجلد والدم من خمسة فئران تم التضحية بها وتخزينها في درجة حرارة -80 درجة مئوية لمزيد من التحليلات الكيميائية الحيوية والنسجية. أظهرت النتائج زيادة معنوية في إنزيمات الأكسيد الفائق (SOD)، الكاتالاز (CAT)، والجلوتاثيون (GSH). ومع ذلك، انخفض تركيز مالونديالدهيد (MDA) بشكل ملحوظ في كلتا عينتي ZOUSH المعاملتين مقارنة مع مجموعة التحكم ($P \leq 0.05$). أظهر تقييم عملية التئام الجروح أثناء العلاج أن ZOUSH يسرع عملية التئام الجروح مقارنة بتسريع عملية التئام الجروح مقارنة بالمجموعات الأخرى. أظهر تحليل التعبير الجيني زيادة مستوى تعبير Mmp-9 أكثر من عشر مرات مقارنة بالمجموعة غير المعالجة. وفقًا لنتائج الدراسة الحالية، أثبت مرهم ZOUSH فعالية عالية في التئام جروح الحروق من الدرجة الثانية. وبالتالي، يبدو بديلًا موثوقًا به للعلاجات الحالية لجروح الحروق.