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The Effects of Argan Oil in Second-degree Burn Wound Healing in Rats

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Abstract

Argan oil, produced from the kernels of the argan tree (Argania spinosa), has been shown to have antioxidant properties. To examine the effect of argan oil in second-degree burn wound healing, an *in vivo* experiment was conducted among 30 adult male Wistar rats divided into 5 equal groups: a sham group, a control group (burned but no topical agent), a group in which argan oil was applied once a day, a group in which argan oil was applied twice a day, and a group treated with 1% silver sulfadiazine once a day.Second-degree burns were created by scalding hot water (85˚ C for 15 seconds). Treatment began 24 hours after the burn injury; in the argan oil groups, 1 mL of argan oil was administered via syringe to the wound. The rate of wound healing was quantified by wound measurements on days 1, 7, and 14 after burn injury. Tissues were analyzed for molecular and histologic changes in TGF-β expression and fibroblast activity. Percent contraction of burned skin tissue was determined using the stereo investigator program, which calculated the burn field to the

millimeter. Means (SD) were calculated and compared using Duncan's multiple comparison test. The group receiving argan oil twice daily showed significantly increased mRNA levels of TGF-β1 from 39.66- to 58.70-fold compared to the burn control group on day 14 (P <0.05). Both argan oil-treated groups showed significantly increased contraction compared to the burn control group at all 3 timepoints; the group receiving argan oil twice daily had a greater contraction rate (31% on day 7, 76% on day 14) than the silver sulfadiazine group (22% on day 7, 69% on day 14), (P <0.05). Histopathological assessments on days 3, 7, and 14 showed greater healing/contraction in both argan oil and silver sulfadiazine groups compared to the control group. These results suggest argan oil is effective in healing experimentally created second-degree burns in rats. Prospective, randomized, controlled clinical studies are needed to evaluate the safety, efficacy, and effectiveness of this treatment modality for patients with second-degree burn wounds.

Burn injuries rank fourth among all other injuries after vehicle accidents, violence, and falls.[1] In a retrospective global study,[2,3]approximately 265,000 people were reported to die from burns. In the United States, approximately 1 million people are admitted to hospitals annually due to burns, and of these patients, approximately 45,000 are treated as inpatients.[4] Currently, the mortality rate of burn injuries shows a decline owing to improvements in the care and treatment of patients.[2,3,5]

Burn injury is directly related to the degree of burn. First-degree burns include superficial burns such as sunburn; only the epidermis is affected, causing itching and mild pain.[5,6] Second-degree burns can result from contact with hot liquids or surfaces (eg, an iron); they affect the entire epidermis. Partial destruction of the dermis[6,7] and edema[5-7] also may occur. If left untreated, a second-degree burn can become a third-degree burn with increased edema formation.[8,9] The damage in the necrotic tissue extends to the nerve endings and the patient loses sensation to pain in the burned area.[5-7] This necrotic tissue is called scarring, and closure without scarring is not possible in second- and/or third-degree wounds.[8,9]

Wound healing involves cellular events such as cell migration and angiogenesis along with epithelial tissue repair and extracellular fluid retention.[10] Many experimental studies[11,12] have demonstrated an array of inflammatory cytokines are involved in wound healing. Transforming growth factor-β (TGF-β), known to be a strong stimulator of connective tissue formation, also plays an important role in the pathogenesis of fibrotic disorders such as burns.[13] Although significant improvements have been achieved in wound healing, scar tissue formation cannot be avoided after the repair process of burn injury, and this can constitute a significant esthetic problem.

Many topical agents have been used in the treatment of burn injuries.[14,15] Silver sulfadiazine is a topical antimicrobial agent that has become the standard of care in burn treatment.[16] However, topical application of silver sulfadiazine creams has been shown clinically[16-18] to sometimes result in systemic complications such as neutropenia, redness of the skin, crystalluria, and methemoglobinemia. These treatments could prevent repair of burn injury and cause scar tissue formation. Therefore, burns and scar tissue formation after burn injuries have become one of the most extensively studied problems for which researchers have continuously developed new applications through experimental and clinical trials.

Argan oil is produced by the cold press of the kernels of the Argan tree (Argania spinosa), a plant endemic only to the drought lands of southwestern Morocco.[19] Argan oil traditionally has been used as a topical treatment of various conditions, including dry skin, psoriasis, eczema, wrinkles, point pain, and skin inflammation. When taken orally, clinical studies[20] have shown argan oil can protect against high cholesterol and atherosclerosis and is a protective agent for the liver. Experimental studies[21,22] have demonstrated the antioxidant and anticancer properties of argan oil. Argan oil is used to treat many conditions, and European, Asian and US cosmetic companies have made it readily available over the counter (OTC).

The aim of this *in vivo* study was to examine the effects of argan oil in the treatment of experimentally induced scalding water burns on TGF-β expression and fibroblast activity, healing, and contraction rates.

Materials and Methods

Chemicals. All reagents and chemicals were analytical grade and purchased from commercial suppliers. The argan oil used in this study originated from southwestern Morocco. It was extracted in February 2013 from the hard core of the fruit by a traditional hand-press method.[20] Argan oil was used in its rough state without any preliminary processing. It was preserved at room temperature in a brown glass bottle to protect it from light. Silver sulfadiazine was purchased from Deva Holding, Istanbul, Turkey.

Animals. Thirty (30) adult male Wistar rats weighing ~220–250 g were given standard rat pellet feed and tap water ad libitum. The rats were housed in stainless steel cages (360 mm x 200 mm x 190 mm), each containing 2 or 3 animals, from 15 days before the start of the experiment. All animals were housed under standard laboratory conditions (light period 7.00 am to 8.00 pm, 21 ± 2˚ C, relative humidity 55%) throughout the experimental period. The animal care and experimental protocols were approved by the Experimental Animal Ethics Committee, Ataturk University, Erzurum, Turkey (31/05/2013-14).

Experimental procedure. After 24 hours' acclimation, the animals were assigned at random to 5 groups of 6 each for the following treatment: Group 1 was the sham group and was not burned (no topical agent was applied), group 2 was the burn control group (no topical agent was applied), group 3 was treated with argan oil once a day, group 4 was treated with argan oil twice a day, and group 5 was treated with 1% silver sulfadiazine once a day.

All rats were anesthetized intraperitoneally with thiopental (30 mg/kg body weight). After their backs were shaved to create the second-degree burn injury, the animals were placed in the supine position on a hollow metal plate through which water was circulated from an 85˚ C water bath. To maintain equivalent skin-metal contact pressure, the glabrous plantar surface of the bottom was held in heated-metal contact for 15 seconds with a 10-g weight. This heat exposure caused a uniform second-degree burn on the back of the skin.[23] The animals were resuscitated with an intraperitoneal injection of 5 mL of normal saline solution.

Treatment began 24 hours after the burn injury. Argan oil (1 mL) was applied topically via syringe. In order to quantify the rate of wound healing, the size of lesions was determined via milimetric photography and then measured using a stereo investigator program (MBF Bioscience Company, Williston, Vermont, USA) at 1, 7, and 14 days after burn injury. Wound pictures were taken at each assessment.

After macroscopic wound healing examination, all rats were euthanized using a high dose (50 mg/kg) of tiopenthal sodium and the tissues were taken immediately from lesion sites and kept at -80˚ C for molecular analyses. The rest of the tissues were transferred immediately to 4% paraformaldehyde for histopathological analyses.

Contraction measurement. Percent contraction of burned skin tissue was determined using the stereo investigator program, enabling calculation of the burning field to the millimeter. The area of the wounds on the first day was considered as 100%, and wound areas on subsequent days were compared with the wound area on the initial days.

Results were calculated according to the formula[24]:

$$\text{Percantage of wound contraction} = \frac{\text{Initial wound size} - \text{spesific day wound size}}{\text{Initial wound size}} \times 100$$

Total RNA extraction and cDNA synthesis. Tissues (20 mg) were stabilized in RNA Stabilization Reagent (RNAlater, Qiagen, Hilden, Germany) and then disrupted using the TissueLyser II (2 x 2 minutes for liver and muscle; 2 x 5 minutes, Qiagen, Hilden, Germany). Total RNA was purified using RNeasy Mini Kit Qiagen according to the manufacturer's instructions in Qiaqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA using a high-capacity cDNA reverse transcription kit. RNA was treated with 2 µL 10 X RT Buffer, 0.8 µL 25 X dNTPs mix, 2 µl 10 X RT Random Primers, 1 µL MultiScribe Reverse Transcriptase, and 4.2 µL DEPC-H2O. Reverse transcription was carried out at 25˚ C for 10 minutes, followed by 120 minutes at 37˚ C, and finally 85˚ C for 5 minutes using Veriti 96 Well Thermal Cycler (Applied Biosystem, Singapoore). cDNA concentration and quality were assessed and quantified by using the Epoch Spectrophotometer System and Take3 Plate (Biotek, Epoch, Winooski, VT, USA).

Real-time quantitative polymerase chain reactin (PCR) analyses. Relative TGF-β1 expression analysis were performed with StepOne Plus Real Time Polymerase Chain Reactin System technology (Applied Biosystem, ABD) using cDNA synthesized from wound area epidermis and dermis tissue RNA. PCR amplification was achieved with TaqMan Gene Expression Assays Rn00572010_mL for rat TGF-β1, and Rn00667869 for rat β-actin (Applied Biosystems). Expression data of β-actin in each tissue were used as endogenous control. For each tissue, quadruplicate determinations were performed in a 96-well optical plate for both targets (TGF-β1 and β-actin) using 2.5 µL of cDNA (100 ng), 1 µL of TaqMan Gene Expression Assay, 10 µL of TaqMan PCR Master Mix (Applied Biosystems), and 6.5 µL of RNase free water in each 20-µL reaction. The plates were heated for 2 minutes at 50˚ C and 10 minutes at 95˚ C and subsequently 40 cycles of 15 seconds at 95˚ C and 60 seconds at 60˚ C were applied. All data are expressed as fold-change in expression compared to the expression in other animal groups, using the 2(-ΔΔCt) method.[25]

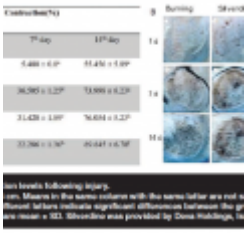
Histopathologic analyses. Samples were taken for histopathological studies with a small excision containing part of the wound area from skins. Tissue samples were fixed in 10% neutral formalin. Tissues were embedded to paraffin wax and sections were cut to 5-µm thickness and stained with hematoxylin and eosin. Histopathological changes were evaluated with light microscopy (Olympus BX 51, Japan).

Statistical analyses. Statistical analysis was performed according to 1-way analysis of variance (ANOVA). For wound contraction and molecular results, differences among the averages of groups were obtained using Duncan's multiple comparison test. Differences were considered statistically significant at $P < 0.05$. All data were expressed as mean ± standard deviations (SD).

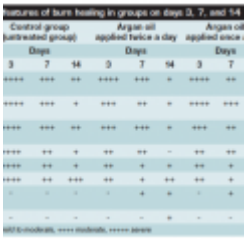
Results

Wound contraction. Percent of contraction of burned skin tissue in the rats was determined by stereo investigator. The average (SD) rates on all groups are shown in Figure 1. The burn control group showed low increased contraction levels of 0-, 5.488-, and 55.456-fold at days 3, 4, and 14, respectively ($P < 0.05$). Analysis also showed twice-a-day application of argan oil resulted in greater

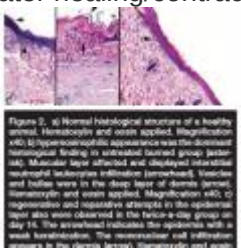
contraction levels of 0-, 31.428-, and 76.054-fold at days 3, 4, and 14, respectively compared to burn control group (P<0.05).



Histopathologic results. The parameters of histopathological lesions and assessment of healing are presented in Table 1. The healthy animals' skin tissues showed a normal structure (see Figure 2a). The epidermis and part of the dermis were injured severely in all groups on day 3 (see Figure 2b). The epidermis and dermis were hypereosinophilic; coagulation necrosis with acute inflammatory reaction of the epidermis and dermis also were noted (see Figure 2b).



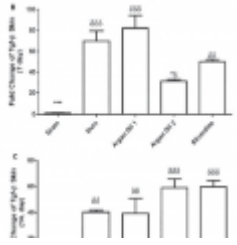
Histopathological assessments on days 3, 7, and 14 showed greater healing/contraction in the argan oil and silver sulfadiazine groups compared to the control group.



Regenerative and reparative characteristics in the epidermal layer also were observed (see Figure 2c–e). Inflammatory cells — specifically, infiltration of neutrophils without an epithelial layer — were noted in lower epidermis (see Figure 3c,e) and in the epidermis (see Figure 2d).

The eschar had fallen off in all groups by day 14, and the epidermis was observed to have epithelialized in the argan oil and silver sulfadiazine groups. Inflammatory reactions were still noted (see Figure 2c–e).

TGF-β1 mRNA levels. As shown in Figure 3, the TGF-β1 gene expression increased in the burn control groups on days 3, 7, and 14 by 61.63-, 69.76-, and 39.66-fold, respectively, compared with the sham groups (P <0.05).



On day 3, the increase in the TGF-β1 mRNA expression significantly decreased from 61.33-fold to 33.35-, 42.12-, and 24.29-fold in the argan once daily, argan twice daily, and silver sulfadiazine groups, respectively, when compared with the burn control group (P <0.05).

On day 7, while the increase in the TGF-β1 mRNA expression significantly decreased from 69.76-fold to 31.13- and 49.86-fold in the twice-daily argan and silver sulfadiazine groups, respectively, TGF-β1 mRNA expression increased from 69.76- to 81.94-fold in the once-daily argan group when compared with the control (P <0.05).

On day 14, TGF-β1 mRNA expression significantly increased from 39.66-fold to 58.70- and 59.52-fold in the twice-daily argan and silver sulfadiazine groups, respectively, when compared with the control group (P <0.05). The argan once-daily group (39.66) showed no significant difference in mRNA levels of TGF-β1 on day 14 when compared with the control group (39.18) (P <0.05).

Discussion

Argania spinosa grows endemically in Morocco and has been used topically and orally OTC in the treatment of many conditions. Traditionally, argan oil produced from Argania spinosa is used in the treatment of rheumatoid arthritis, gastritis, diarrhea, and headache. Among the many clinical and experimental scientific studies[21,22,26] conducted on argan oil in recent years, some of the more noteworthy indicate argan oil can have anticancer, antioxidant, antithrombotic, and antihypertensive effects. Manufacturers commonly use argania spinosa for cosmetic purposes due to its high flavonoid content (mainly quercetin and myricetin derivatives).[27] Preclinical and clinical studies[28] have demonstrated argan oil can prevent lipid peroxidation in rat and human plasma.

Delayed healing of burn wounds can result in infection and sepsis. Burn healing involves 3 main phases[29]: inflammation, tissue regeneration, and remodeling. Histopathological examination performed in the present study showed argan oil used twice a day had better anti-inflammatory effect and epithelial regeneration when compared to silver sulfadiazine. Argan oil also was a factor in remodeling (healing) in the present study; a significant difference was noted in skin remodeling between both single-dose and double-dose application of argan oil and silver sulfadiazine.

Histopathological examination showed, compared to the control group, the most significant histopathological improvement was noted at day 14, and ongoing healing was noted at days 3 and 7. Twice-daily application of argan oil was found to be more effective in healing than the other options studied.

Inflammation, cell migration, angiogenesis, and reepithelization are involved at the molecular and cellular level in wound healing.[10]The strong antioxidant effects of argan oil have been demonstrated *in vitro* in cancer cell studies; this research suggests argan oil may have anticancer activity via antioxidant activities.[21,22] Preclinical studies[30] have shown oxidative stress considerably increases during recovery from burn injury, and the use of medications reducing oxidative stress provides significant benefits in the wound healing process. Argan oil contains abundant amounts of tocopherol,[31] a strong antioxidant substance.[32,33]Furthermore, experimental studies[34] also showed alpha tocopherol exerted significant effects on cytokines, particularly IL-4, IL-5, IL-13, and TGF-β1. In addition, alpha tocopherol plays an important role in lipid peroxidation and the expression of various inflammatory genes, which have been shown in preclinical studies[35-37] to exhibit considerable changes during burn wound healing.

TGF-β1 is an important growth factor regulating various cellular functions at all stages of wound healing. During the wound healing process, TGF-β1 increases the formation of granulation tissue and collagen formation[38] and promotes wound contraction.[39] Collagen is an important extracellular matrix protein; it is responsible for the integrity of the tissue matrix and also is involved in tissue homeostasis and epithelization at the late stage of wound healing process.[29] Its failure in the wound healing process results in abnormal scar tissue formation through collagen deposition and erroneous collagen formation. The present study provided evidence argan oil therapy decreased fibroblast activity. A reduction of TGF-β1 over time was noted in the burn control groups at days 3, 7, and 14. In the argan oil and silver sulfadiazine groups, the reduced TGF-β1 levels increased on day 3.

Compared to the burn control group, twice-daily application of argan oil and silver sulfadiazine significantly increased TGF-β1 expression at day 14. Previous experimental studies[40] demonstrated a time-dependent increase in TGF-β1 levels during the wound healing process. As such, argan oil may promote wound healing by increasing reepithelialization during recovery from burn injury.

Conclusion

The results of this *in vivo* study showed hot water-induced, second-degree burns in rats treated with argan oil healed more expediently than wounds treated with silver sulfadiazine. Significant differences between argan oil and silver sulfadiazine were observed in TGF-β1 expression, wound contraction, and histopathological findings. Argan oil represents a potential therapeutic option in the future treatment of burn injuries. Prospective, randomized, controlled clinical studies are needed to examine the safety, efficacy, and effectiveness of argan oil for the treatment of burn wounds. n

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