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ORIGINAL RESEARCH CONTRIBUTION

Crocodile Oil Enhances Cutaneous Burn Wound Healing and Reduces Scar Formation in Rats

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Abstract

Objectives: This study was performed to evaluate the burn wound–healing efficacy of crocodile oil from *Crocodylus siamensis* by employing deep second-degree burns in a Wistar rat model.

Methods: Twenty-four rats were assigned equally into four groups using a random-number table, and two burns were created on the dorsum of each animal except for the sham group. The three treatment groups received with saline solution (12 burns, served as negative control), silver sulfadiazine (12 burns, served as positive control), or crocodile oil (12 burns). Silver sulfadiazine cream was used as standard care, and the treatments were repeated twice daily for 28 days. After day 28 the animals were euthanized and the wounds were removed for quantitative real-time polymerase chain reaction, histologic, and immunohistochemical study.

Results: Crocodile oil accelerated the wound-healing process as indicated by a significant decrease in wound closure time in comparison to the burn control and silver sulfadiazine treatment groups. Histologic results showed well-organized and distributed skin structure and collagen deposition in the animals treated with crocodile oil. Transforming growth factor-β1 (TGF-β1), a key cytokine promoting scarring, was also observed to play a role in the burn wound healing. Immunohistochemical staining results showed the negative expression of TGF-β1 and Smad3 in the 28-days-postburn skin of crocodile oil group versus positive in the epidermis of burn controls. Compared to the burn control group, expressions of TGF-β1 and Smad3 mRNA decreased significantly ($p < 0.01$) in the 28-days-postburn skin of the crocodile oil group.

Conclusions: Our results showed that crocodile oil could enhance cutaneous burn wound healing and reduce scar formation in rats, which might be related to TGF-β1/Smad3 signaling.

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Burns are one of the most widespread injuries in accidents and remain a global public health issue.[1,2] The burn wound is a continuous and severe threat against the rest of the body due to invasion of infectious agents, antigen challenge, and repeated additional trauma caused by wound cleaning.[3] Although many advances have been made in our understanding and care of burn injuries, there are still many burns healed with scar formation, resulting in significant aesthetic disfigurement and dysfunction.[4,5]

The sequence of events repairing the wound is categorized into three overlapping phases: inflammation, proliferation and tissue remodeling, and scar maturation.[6] Burn wound healing involves a sequence of molecular and cellular events including inflammation, cell migration, angiogenesis, extracellular matrix synthesis, and reepithelialization.[7] Some studies have demonstrated that a number of cytokines are involved in the progression of the wound healing.[8,9] Transforming growth factor-β (TGF-β), known as a potent stimulus

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of connective tissue accumulation, is implicated in the pathogenesis of fibrotic disorders.[10] In terms of repair, TGF-β1 and TGF-β2 are known to promote scar tissue, while TGF-β3 may reduce scar formation.[11,12] The Smad signal transduction pathways are crucial in mediating several TGF-β responses in fibroblasts,[10] such as stimulation of collagens[13,14] and α-smooth muscle actin.[15,16] Smad3 is a key intracellular signal transducer in profibrotic TGF-β responses. Some researchers have found that targeted disruption of TGF-β/Smad3 signaling could modulate skin fibrosis.[10]

Oils extracted from plants or animal fats that are abundant with fatty acids are used to treat burn injuries.[1,17,18] Crocodile is a general term of animals of Crocodilia, Reptilia, which have high economic and medical value.[19] Crocodile oil extracted from the fatty tissues of crocodiles is rich in monounsaturated and polyunsaturated fats.[20] Crocodile oil and its products are used as ointments for burns and scalds in the traditional medicines, such as traditional Chinese and Southeast Asia medicine.[19] However, few studies on the role of crocodile oil in burn wound healing have been reported.

The aim of this study was to investigate the therapeutic effect of crocodile oil on experimental cutaneous burn wounds in Wistar rats. The mechanism of reducing scar formation was also evaluated by analyzing the expression of TGF-β1 and Smad3 in rat skins.

METHODS

Study Design

This was a randomized controlled trial to compare the burn wound–healing efficacy of deep second-degree rat burns treated with crocodile oil, silver sulfadiazine (positive control), and saline solution (negative control). The animal experiments were performed in accordance with the ''Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals'' formulated by Xiamen University's Animal Ethics Committee. The study was approved by the institutional review board of Xiamen University.

Animal Handling and Preparation

This study was conducted in the Laboratory Animal Center of Xiamen University. Twenty-four SPF-class male Wistar rats were kept on 12-hour-light/12-hour-dark cycle with a constant temperature of 21 to 22°C and 60% to 65% humidity. The rats were caged individually with free access to a commercial balanced rat diet and tap water. The animals were assigned equally into four groups using a random-number table: sham (normal group, no burn), burn control group treated with saline solution (12 burns, served as negative control), silver sulfadiazine–treated group (12 burns, served as positive control), and crocodile oil–treated group (12 burns). Silver sulfadiazine cream (1% wt/wt) was used as standard care.

Preparation of Crocodile Oil

Oil from *Crocodylus siamensis* was extracted using a previous described method.[21] Briefly, the fats were extracted with petroleum ether (boiling process 30–60°C) in an ultrasonic apparatus at room temperature (three times, 2 hours each). The extractions were collected together and concentrated in vacuum at 50°C by a rotary evaporator. The oil was then evaporated by a vacuum dryer until it reached a constant weight.[22]

Gas Chromatography Analysis of Crocodile Oil

The gas chromatography analyses were accomplished using a Varian 1200 gas chromatograph equipped with a mass spectrometer and CP-8713 capillary columns (Varian Medical Systems, Palo Alto, CA; 30 m × 0.25 mm, i.d. × 0.25 μm) following a temperature program of: 50°C for 5 minutes, rising at 10°C/minute to 170°C, holding for 10 minutes, rising 2°C/minutes to 210°C; holding for 25 minutes, then rising at 10°C/minutes again to 225°C; and injector, ion, and transfer line temperatures of 230, 200, and 250°C, respectively. The carrier gas was nitrogen (1 mL/min). The percentage composition was obtained from electronic integration measurements using flame ionization detection.[23]

Study Protocol

The rats were acclimated to the laboratory for 1 week prior to beginning the study and had free access to water and food at all times. The dorsal hair was shaved and depilated with 10% sodium sulfide solution 24 hours before the burn wound experiment. The rats were anesthetized by ether, and 70% alcohol was employed to sterilize the dorsal area. A deep second-degree burn wound of a surface 2.5 cm in diameter was created by using a 40-g glass full of water. The glass was preheated in 100°C boiling water for 10 minutes and then applied perpendicularly to the shaved area with gravity alone, without pressure, on one side of the back for a period of 10 seconds.[24] Two burns were created on the dorsum of each animal except the sham group (Figure 1).

In a preliminary study, the dose–response properties of crocodile oil and silver sulfadiazine were examined to determine the optimal dose, and the most effective in the wound healing was 0.3 g per wound (data not shown). Hence, to enhance efficacy, crocodile oil or

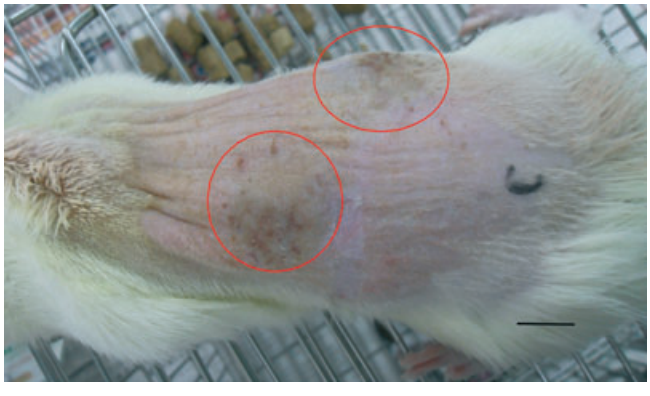

Figure 1. A deep second-degree burn wound of a surface (2.5 cm in diameter) was created; two burns were created on the dorsum of each animal except the sham group. Scale bar = 1.0 cm.

silver sulfadiazine was administrated at the dose of 0.3 g per wound in the experiment. The 0.3 g of crocodile oil or silver sulfadiazine was applied slowly to the burn wound area and extended slightly outside the wound area to ensure inclusion of the wound edges. The rats in the burn control group were treated with 0.3 g of saline solution per wound under the standard conditions. Treatments were repeated twice daily for 28 days. The first application was done directly after the wound injury.

Macroscopic Analysis of Wound Closure

Optical photographs were taken from the burn wound area at an equal distance from the wound and right angle to the wound surface on days 3, 7, 10, 17, 21, and 28. The wound surface areas were measured by tracing their contours using a transparent paper to evaluate wound contraction.[1] The area (mm^2) within the boundary was measured planimetrically.[25] Wound closure time, exudation, and the firmness of the wound surface were also observed.

Histologic and Immunohistochemical Analysis

On the 28th day, the experiment was terminated and the granulation tissues were excised for histologic examination. The excisional skin biopsies were fixed in 4% neutral buffered formaldehyde solution for 24 hours. To determine the structure and collagen deposition of the 28-days-postburn skin, deparaffinized sections were processed for Van Gieson stain. Five–micrometer-thickness sections were observed for histopathologic changes under microscope, such as the thickness of the newly formed epidermis and dermis.

For immunohistochemistry, 5-μm-thickness cross-sections from 28-days-postburn skin samples were deparaffinized, rehydrated in descending alcohol dilutions, and immersed in phosphate-buffered saline (PBS). The sections were treated with 0.01 mol/L, pH 6.0, citrate buffer solution at 95°C for 15 minutes and then with serum-free blocking agent (MaiXin Bio., Fuzhou, China) for 10 minutes, followed by incubation with polyclonal rabbit anti-human primary antibody anti-TGF-β1 (the identity of protein sequences between *Rattus norvegicus* and *Homo sapiens* is 99%; dilution, 1:50 [MaiXin Bio.]) and monoclonal rabbit anti-rat primary antibody anti-Smad3 (dilution, 1:150; Epitomics, Inc., Burlingame, CA) in PBS overnight at 4°C. For detection of bound primary antibodies, UltraSensitive S-P kit (MaiXin Bio.) was used according to the manufacturers' instructions. Substitution of the primary antibody with PBS served as negative control. Peroxidase activity was visualized by incubation with AEC (3-amino-9-ethylcarbazole; Boster Biological Technology, Ltd., Wuhan, China). Nuclei were counterstained with Mayer's hematoxylin (Boster) and coverslipped.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from the granulation tissues using the RNAiso Plus kit (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions and reverse-transcribed in 20 μL total volume using the SYBR PrimeScript real-time polymerase chain reaction (RT-PCR) kit. The process of reverse transcription was 37°C for 15 minutes (reverse transcription reaction) and one cycle at 85°C for 5 seconds (denaturation of reverse transcriptase). The synthesized cDNA was stored at –80°C. The mRNA levels for TGF-β1 and Smad3 in each sample were determined by a quantitative RT-PCR. Primers were synthesized by Takara Bio, Inc. The primers used for RT-PCR were as follows: TGF-β1 primers (forward 5′-TGGCGTTACTTGGTAACC-3′ and reverse 5′-GGTGTTGAGCCCTTTCCAG-3′); Smad 3 primers (forward 5′-AGCACACAATAACTTGGACC-3′ and reverse 5′-TAAGACACACTGGAACAGCGGATG-3′);[26] and β-actin (internal standard) primers (forward 5′-TGGAATCCTGTGGCATCCAT-3′ and reverse 5′-TAAAACGCAGCTCAGTAACA-3′). Real-time quantitative PCR was performed using the SYBR PrimeScript RT–PCR kit in accordance with the manufacturer's instructions. Then, PCR was carried out in a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) according to the following protocol: 30 seconds at 95°C, one cycle; 5 seconds at 95°C; and 20 seconds at 57°C, 45 cycles. Fluorescence was detected at the annealing stage of each cycle. A melting curve was generated during the reactions to check for the possibility of primer–dimer formation. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative mRNA level of each gene.[27]

Measures and Outcomes

All morphometric parameters were done with Image Analyzer (Olympus Microscope BX51, Olympus, Center Valley, PA) by using an image analyzing computer program (Image-Pro Plus 6.0, Media Cybernetics, Inc., Bethesda, MD). The primary outcome was the percentage of wound contraction. The sizes of the wound areas on the 7th, 10th, 17th, 21st, and 28th days were determined by a balance, and the percentage of wound contraction was calculated by subtracting the surface area of individual burns from the surface area of the largest burn and dividing this value by the surface area of the largest burn multiplied by 100.[28] This outcome had an interobserver correlation of 0.99 on a subset of burns. A secondary outcome was the epidermal and dermal thickness of the granulation tissues in different groups at 28 days postburn. The thickness of the newly formed epidermis was measured at 1-mm intervals, and the mean was calculated.[1] Dermal thickness was determined by measuring the distance from the epidermal–dermal junction to the dermal–fat junction for eight randomly selected sites/fields from two or more skin samples in each animal.[29] The collagen was stained red using Van Gieson staining, and its deposition was observed. Another secondary outcome was TGF-β1 and Smad3 mRNA expression in the 28-days-postburn rat skins. Quantitative RT-PCR analysis was used to examine TGF-β1 and Smad3 mRNA levels. The net intensity values for TGF-β1 and Smad3 bands were normalized to the housekeeping gene β-actin, and the data were presented as the percentage difference in TGF-β1/β-actin or Smad3/β-actin gene expression from the age-matched unwounded values (mean ± SEM). We also measured the TGF-β1 and Smad3 protein expressions in the 28-days-postburn rat skins by using immunohistochemical staining.

Data Analysis

Descriptive statistics were used to describe outcomes. Continuous variables are presented as means with standard deviations (SDs). A one-way analysis of variance (ANOVA) was used to assess the differences in the burn wound healing time and percent wound contraction of the groups. Additionally, we used a one-way ANOVA to assess differences in epidermal and dermal thickness of crocodile oil treated and untreated burn wounds after 28 days of treatment. The percentage differences in TGF-β1/β-actin and Smad3/β-actin gene expression of the burn control group and crocodile oil group were also assessed with one-way ANOVA. Statistical analysis was performed using SPSS 14.0 for Windows (SPSS, Inc., Chicago, IL). Normality of distribution was assessed through the use of the Kolmogorov-Smirnov test, and for multiple group comparisons, homogeneity of variance was assessed by the Levene test. A p-value <0.05 was considered statistically significant. A sample size of 12 burns in each group provided a power of 80% to detect a 33-percentage-point difference in the percentage of wound contraction between the groups[30] and was determined a priori using PASS: Power Analysis and Sample Size for Windows (version 11, NCSS, LLC, Kaysville, UT).

RESULTS

Chemical Composition of Crocodile Oil

Crocodile oil was analyzed by gas chromatography, resulting in the identification of 10 compounds which represented 88.57% of the oil. The composition of fatty acids from the crocodile oil is shown in Table 1. Crocodile oil extracted from *C. siamensis* was rich in monounsaturated fatty acid and polyunsaturated fatty acid, and among them the main constitutes were oleic acid, linoleic acid, and palmitoleic acid. Saturated fatty acid and other unknown compositions account for the remainder of the oil.

Table 1
Composition of Fatty Acids

Fatty Acid	Lipid Numbers (C:D)	Content (%)
Lauric acid	12:0	4.60
Myristic acid	14:0	3.23
Palmitic acid	16:0	29.22
Stearic acid	18:0	1.84
Palmitoleic acid	16:1	5.67
Oleic acid	18:1	30.47
Linoleic acid	18:2	11.74
Linolenic acid	18:3	1.07
Erucic acid	22:1	0.42
Nervonic acid	24:1	0.31
Saturated fatty acid		38.89
Unsaturated fatty acid		49.67
Monounsaturated fatty acid		36.86
Polyunsaturated fatty acid		12.81
Others		11.43

C = number of carbon atoms in the fatty acid; D = number of double bonds in the fatty acid.

Effect of Crocodile Oil on Burn Healing

The burn healing time in the crocodile oil group was shorter than in the burn control group and silver sulfadiazine groups (Figure 2). In the first 3 days, the burn wounds progressed to necrotic eschars (Figures 3a, 3g, and 3m), and there were no measurable differences among the groups. On the 10th day, we observed a significant improvement in wound closure in the crocodile oil–treated group (Figure 3o) when compared to the burn control group (Figure 3c and 3s) and the silver sulfadiazine group (Figure 3i and 3s). After 17 days of treatment, the animals in the crocodile oil group (Figure 3p) had smaller wound areas than those in the silver sulfadiazine group (Figures 3j and 3s). The results of morphologic evaluation on the 28th day showed that the best result was obtained with crocodile oil, in which the surface of the skin was smoother and the color was close to the normal skin (Figure 3r). The surface of the skin in the burn control group (Figure 3f) and the silver sulfadiazine group (Figure 3l) was rough, and it was much harder than the normal skin.

Effect of Crocodile Oil on Skin Structure and Collagen Deposition

Histologic examination on the 28th day showed that the epidermis in the sham groups was thin, and skin structure and collagen deposition (red staining) were well organized and distributed (Figure 4a). In the burn control and silver sulfadiazine groups, the epidermis was disorganized, and the boundary between the epidermal and the dermis was blurred, few hair follicles structures existed, and a mass of disorganized red collagen was present in the dermal layer of the skin (Figures 4b and 4c). In the crocodile oil group, there was full-thickness reepithelialization, in which the epidermis was thin and well organized, the hair follicle structure was well formed, and most area was covered with hair. The 28-days-postburn skin of the crocodile oil group showed lower synthesis and well-distributed accumulation of collagen compared to the other groups (Figure 4d).

Quantitative analysis showed that on the 28th day, the epidermis thickness of the skin in crocodile oil group was 86 and 67% (Figure 4e) thinner than in

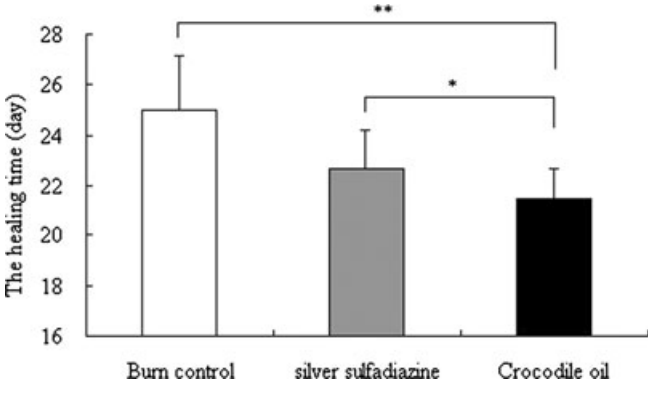

Figure 2. Healing time on Wistar rat dorsal wound. Values are mean ± SEM of 12 burns; values in brackets represent statistical differences: *p < 0.05; **p < 0.01.

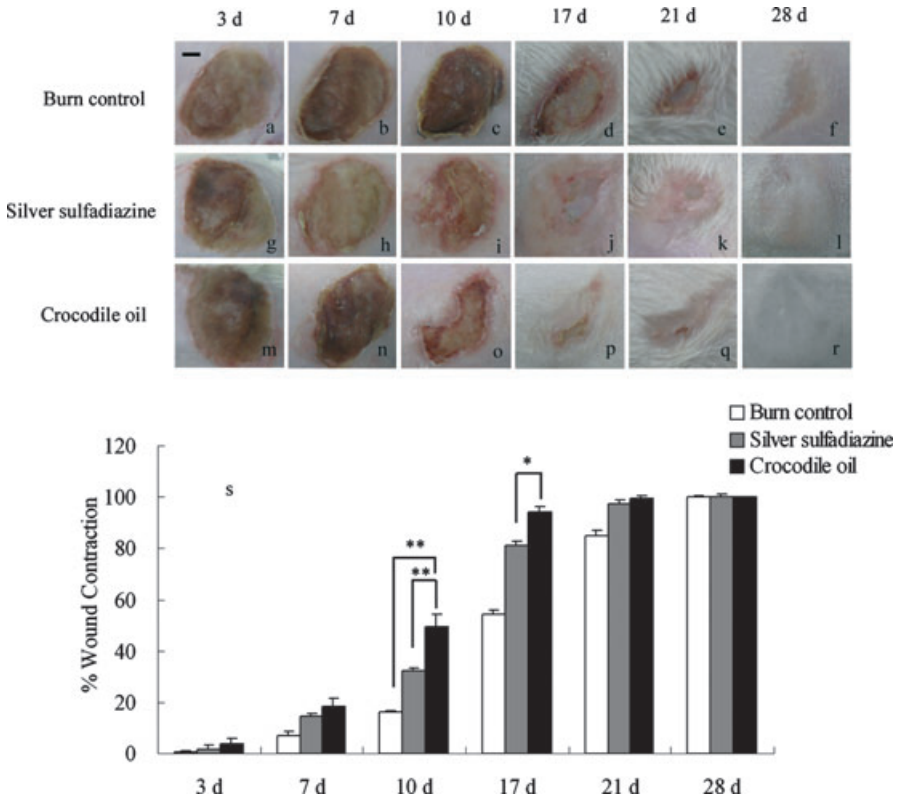

Figure 3. Wound contraction in different groups. Rat dorsal burn wound area photograph on the 3rd, 7th, 10th, 17th, 21st, and 28th days after application of different treatment: burn control group (a–f), silver sulfadiazine group (g–l), and crocodile oil group (m–r). Photographs were taken from a representative animal of each group. Scale bar = 0.5 cm. The percentage of wound contraction in different groups on the 3rd, 7th, 10th, 17th, 21st, and 28th days: burn control group, silver sulfadiazine group, and crocodile oil group. Values are mean ± SEM of 12 burns. *$p < 0.05$; **$p < 0.01$.

the skin of burn control group and silver sulfadiazine group, respectively. Also, crocodile oil treatment resulted in the formation of augmented dermis (34%) compared with the burn control (Figure 4f).

Crocodile Oil Reduced TGF-β1 and Smad3 Expression in Protein and mRNA Levels

To investigate the effect of crocodile oil on the TGF-β1/Smad3 signaling in scar formation, the expressions of TGF-β1 and Smad3 in the 28-days-postburn skin were detected. Immunohistochemical analysis showed that TGF-β1 and Smad3 were negatively expressed in the epidermis and low levels in the dermis in the crocodile oil group versus positively expressed in the burn control group (Figure 6).

The effects of crocodile oil on TGF-β1 and Smad3 mRNA expressions in the 28-days-postburn rat skin were assessed by quantitative RT-PCR (Figure 5). The analysis showed that crocodile oil significantly decreased the mRNA expressions of TGF-β1 and Smad3 ($p < 0.01$), compared to the burn control group.

DISCUSSION

In this animal model, crocodile oil demonstrated possible activity in the burn wound healing. The gas chromatography results showed that crocodile oil contained high concentrations of palmitic acid, oleic acid, and linoleic acid; previous research has demonstrated the efficacy of fatty acid agents in accelerating wound healing.[21,31] Membrane fluidity plays an important role in the process of wound healing.[32] The n-3 and n-6 polyunsaturated fatty acids can alter the composition and function of membrane rafts through eicosanoid-independent mechanisms.[33] Lipid peroxidation,[34] prostaglandin production,[35] and regulation of expression of some inflammation related genes[36] are also involved in wound healing. The proinflammatory effect of oleic and linoleic acids may speed up the wound-healing process.[37] Both fatty acids may also generate other lipoid mediators, such as intermediate hydroperoxides and lipoxins, which can alter the immune response and thereby alter tissue repair.[31,38] Moreover, some researchers have found that palmitic acid and oleic acid have antibacterial activity.[39,40]

As an early response to burn injury, resident dermal fibroblasts in the neighborhood of the wound begin to proliferate, and then 3 or 4 days after the wound insult they begin migration into the provisional matrix of the wound clot.[8] A combination of contraction of the wound mediated by myofibroblasts and reepithelialization results in wound closure.[11,41] Our results showed macroscopic differences of wound closure among the treatment groups in the proliferative phase, suggesting

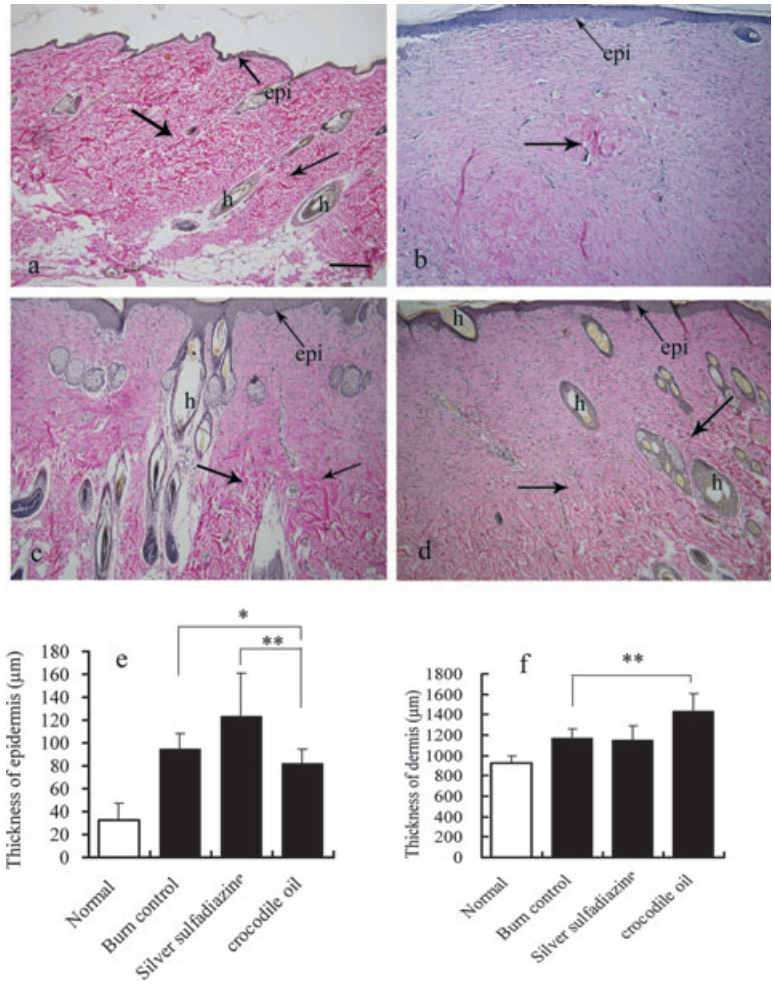

Figure 4. Van Gieson's staining in 28-days-postburn rat dorsal biopsies. (a) Normal group; (b) burn control group; (c) silver sulfadiazine group; (d) crocodile oil group. epi = epidermal; h = hair follicle. Morphometric analysis of the histologic sections showing (e) thickness of epidermis and (f) thickness of dermis of crocodile oil–treated and untreated burn wounds after 28 days of treatment. Scale bar = 200 μm; original magnification ×10. Data are mean ± SEM of 12 burns. Values in brackets represent statistical differences: *p < 0.05; **p < 0.01.

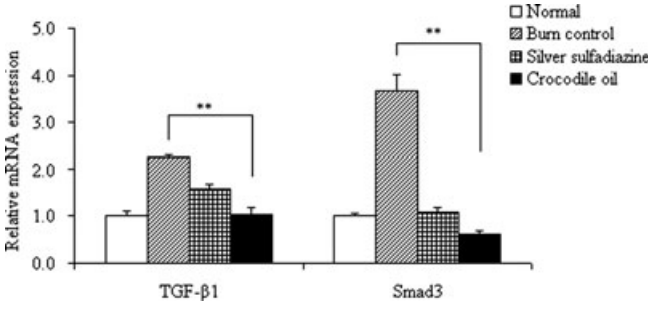

Figure 5. TGF-β1 and Smad3 mRNA expressions in the 28-days-postburn rat skins. Quantitative RT-PCR analysis was used to examine TGF-β1 and Smad3 mRNA levels. The net intensity values for the TGF-β1 and Smad3 bands were normalized to the housekeeping gene β-actin, and the data are presented as the percentage difference in TGF-β1/β-actin or Smad3/β-actin gene expression from the age-matched unwounded values (mean ± SEM). Values in brackets represent statistical differences: *p < 0.05, **p < 0.01. RT-PCR = real-time polymerase chain reaction; TGF-β1 = transforming growth factor-β1.

a specific effect of crocodile oil on wound closure in the first 10 days after burn induction. Longitudinal evaluation of the wound area of the animals in the crocodile oil group on the 28th day showed the structures of epidermis and dermis were well organized, the hair follicle structure was well formed, and most area was covered with hair.

Collagen is a major extracellular matrix protein that confers strength and integrity to the tissue matrix and plays an important role in homeostasis and in epithelialization at the later phase of healing.[6] But dysregulation in certain stages of the healing process could result in excessive deposition of collagen and formation of abnormal scar, as seen in hypertrophic scars and keloids.[9] In this study, lower synthesis and well-distributed accumulation of collagen were observed in the postburn skin of crocodile oil treatment, which might lead to the reduction of scar formation.

TGF-β1 is implicated in pathogenic fibrotic conditions in kidney, liver, and lung disease and now in scarring of skin wounds as well.[8,42] Some researchers have found

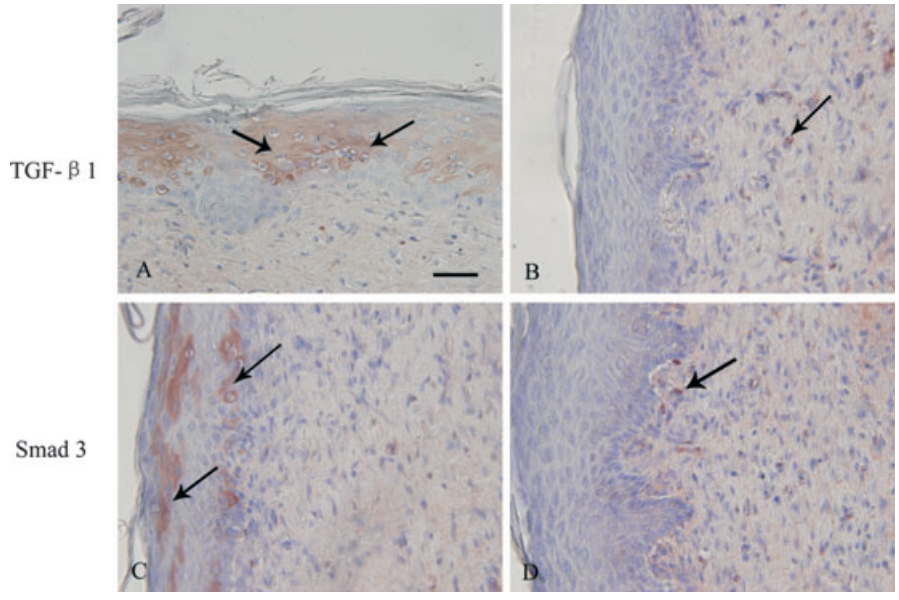

Figure 6. Immunohistochemical detection of TGF-β1 and Smad3 in the 28-days-postburn rat skins. In the burn control group (A, C), epidermis (*arrows*) were positively stained by anti-TGF-β1 and anti-Smad3 antibody using AEC (3-amino-9-ethylcarbazole) as a chromogenic substrate. In the crocodile oil group (B, D), TGF-β1 and Smad3 were negatively expressed in the epidermis and low levels in the dermis. Nuclei were counterstained with hematoxylin, which appear in blue. Scale bar = 50 μm; original magnification ×40. TGF-β1 = transforming growth factor-β1.

that neutralizing TGF-β1 at the wound site could reduce scarring[43] and exogenously added TGF-β1 was able to amplify its own expression and induce scar in a normally scarless human model of fetal skin repair.[44] Among the Smad family, Smad3 seems to be a key player in wound healing, fibrogenesis, and scarring[45] and is a key mediator for regulation of collagen synthesis and other profibrotic responses to TGF-β.[10] In this study, TGF-β1 and Smad3 in protein level were expressed negatively in the 28-days-postburn skin of the crocodile oil group compared with the burn control group. Furthermore, the mRNA expressions of TGF-β1 and Smad3 in the crocodile oil group were lower than in the other groups. These results agree with the results in the study by Soo et al.,[44] indicating that crocodile oil might reduce scarring via TGF-β1/Smad3 signaling pathway.

LIMITATIONS

This study has several limitations that merit discussion. The therapeutic effect of crocodile oil was studied for macroscopic analysis of wound closure. The burn wound–repairing mechanism of crocodile oil remains unclear and needs to be further investigated. The present data also suggest that the effect of crocodile oil on scar formation might be related to a TGF-β1/Smad3 signaling pathway. Our study was performed in an animal model, and the findings might not be applicable to humans.

CONCLUSIONS

Our study showed that crocodile oil from *C. siamensis* can enhance the burn wound healing in a deep second-degree burn rat model and can promote skin regeneration and collagen deposition. In addition, crocodile oil can reduce scar formation, possibly due to the down-regulating TGF-β1 and Smad3 expressions.

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