Evaluation of the Effect of Thymoquinone Treatment on Wound Healing in a Rat Burn Model

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Thymoquinone (TQ) is a plant extract that has been shown to have antimicrobial, anti-inflammatory, and antioxidant effects. Because of these activities, the authors hypothesized that TQ would reduce inflammation and oxidative stress and accelerate wound closure in a rat model of deep second-degree burns. For the purposes of this study, 40 Sprague-Dawley rats were divided into five groups of eight rats each. Group 1 was the control group, group 2 was the silver sulfadiazine group, group 3 was treated with systemic TQ, group 4 received topical TQ, and group 5 was administered topical and systemic TQ. After the deep second-degree burn damage was created, daily dressing changes and TQ administration were continued in the study groups for a period of 21 days. Systemic TQ was administered intraperitoneally at a dose of 2 mg/kg/day, whereas the topical treatment was applied using a 0.5% solution. The changes in the wound site were observed macroscopically, histopathologically, microbiologically, and biochemically in all groups. The smallest necrotic areas were observed at the end of the study in the groups that were administered a combination of systemic and topical TQ, or solely topical TQ ($6.1 \pm 1.6\,cm^2$ and $6.7 \pm 0.4\,cm^2$, respectively), whereas the largest necrotic areas were observed in the control group ($11.2 \pm 1.2 cm^2$). The total antioxidant state levels in the control group were significantly lower than in the other groups ($P < .05$), whereas the total oxidative stress levels were lower in the TQ groups compared with the control group ($P < .05$). The lowest bacterial counts were observed in the groups treated with both topical and systemic TQ ($P < .05$). TQ given systemically and/or topically reduced inflammation and oxidative stress and accelerated the rate of wound closure or reepithelialization. (J Burn Care Res 2013;34:e274–e281)

In cases such as burn wounds in which the protective barrier function of the skin is disturbed, agents accelerating wound healing are desirable. These agents should debride the unhealthy dead tissue, reduce bacterial growth, and result in more rapid wound closure and healing. Numerous topical and systemic agents have been described for this purpose in the literature.[1–4] However, recent studies have increasingly focused on herbal extracts and antimicrobial-epithelializing agents.[5–8]

Thymoquinone (TQ) is a biologically active constituent of black cumin seed (*Nigella sativa*) oil, and its antimicrobial,[9–11] antiallergic,[12] antidiabetic,[13] anti-inflammatory,[14] antioxidant,[15] and antineoplastic[16] properties have been demonstrated in various studies. Gökçe et al[17] have showed on an experimental testicular torsion model that TQ significantly reduces the histological tissue damage caused by ischemia-reperfusion. Kouidhi et al[9] demonstrated that TQ has antibacterial and resistance-modifying activities against oral pathogens. Because of these known properties, TQ has been thought to possibly positively influence wound healing, similar to certain other extracts of plant origin.[5–8]

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We hypothesized that TQ given topically and/or systemically would reduce inflammation and oxidative stress and accelerate the rate of healing of deep second-degree burns in a rat model.

METHODS

The study was conducted at Dicle University in the Medical Sciences Application and Research Centre with the permission granted by the Dicle University School of Medicine Ethics Committee to conduct research on animal subjects. In this study, 40 randomly selected Sprague-Dawley rats weighing 250 to 300 g were used. The rats were divided into five groups with eight rats in each group. Group 1 was the control group and nothing was applied, group 2 was the burn plus silver sulfadiazine group, group 3 was the burn plus systemic TQ group, group 4 was the burn plus topical TQ group, and group 5 was the burn plus topical and systemic TQ group.

Chemicals

The TQ powder (2-isopropyl-5-methylbenzoquinone) was purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) is a widely used solvent and the amount used can be quantified.[18,19] For the purposes of the present study, 2 mg/kg TQ was dissolved in 1 ml DMSO for the systemic administration, and the 0.5% topical solution was prepared by dissolving 500 mg TQ in 100 ml DMSO. The TQ prepared using this method was administered at a dose of 2 mg/kg for the systemic treatment and as a 0.5% solution for the intraperitoneal and topical applications.

Experimental Protocol

Anesthesia was given under aseptic conditions, using 50 mg/kg ketamine hydrochloride (Ketalar®, Parker Davis, Eczacibasi, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun®, Bayer AG, Leverkusen, Germany) administered intramuscularly to all the rats. After the animals were placed on a level surface, their backs were shaved and a standard 4 × 5 cm (20 cm^2) deep second-degree burn wound area was created. The deep second-degree burn area was created by pressing a 4 × 5 cm hot plate heated to a constant temperature of 110°C for 10 seconds on the skin where the burn wound was to be formed.[6] The burning device was applied to the skin with the force of gravity alone. After the burn damage was created, daily changes of the dressing and TQ administration were continued in the study groups for a period of 21 days. The systemic TQ was administered intraperitoneally at a dose of 2 mg/kg/day, whereas the topical treatment was applied as a thin layer of a 0.5% solution. After irrigating the wound site with saline, the topical treatment was performed using 1% silver sulfadiazine (Silverdin, Deva Holding, Istanbul, Turkey) in group 2 and using TQ in groups 4 and 5. At the end of the study, the changes in the wound site were observed macroscopically, histopathologically, microbiologically, and biochemically in all the groups.

Histopathological Analysis

All the rats were euthanized at the end of day 21, and 3- to 5-mm full-thickness tissue samples were obtained from the edges of the burn wounds, together with the adjacent healthy tissue. After the samples were fixed in 10% formalin for 24 hours, they were subjected to routine histological tissue assessments. The cross-sections 3- to 5-μm thick and embedded in paraffin, were cut with the microtome and then stained with hematoxylin eosin according to the standard protocol. The preparations were examined under the light microscope by a qualified pathologist blinded to the groups. The tissue samples were histologically evaluated in terms of the vascularization and granulation tissue formation, and the data for every group were assigned scores (Table 1). To quantify the histological changes noted, we used the scoring system described by Mohajeri et al[6] and Schlager et al.[20]

Biochemical Analysis

For the biochemical analyses, the tissue samples were homogenized in 2 ml of phosphate buffer at pH 7.0. After centrifuging at 4000 rpm, 0.5 ml of

Table 1. Histopathological scoring of the burn wounds

	Histopatologic Scoring			
	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**
Vascularization	None	Small number capillaries	Well-differentiated capillary system	Wide neovascularization
Granulation	None	Thin layer	Thicker layer	Uniformly thick layer

the supernatant was removed and diluted with 4.5 ml phosphate buffer. The total antioxidant state (TAS) and total oxidative stress (TOS) values in the tissues were measured.

The TAS and TOS levels were spectrophotometrically measured in the autoanalyzer (Aeroset®, Abbott®, IL) using the method described by Erel.[21] This method is based on the discoloration of the ABTS (2.2 ′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]) radical cation by antioxidants in proportion to the concentration and antioxidant capacity of the antioxidants. This change in the color of the ABTS radical cation is measured as a change in the absorbance measured at 660 nm. The TAS levels were expressed as micromolar Trolox equivalents per liter (μmol Trolox Eq/L), whereas the TOS levels were expressed as micromolar hydrogen peroxide equivalents (μmol H_2O_2 Eq/L) per liter.

Microbiological Assessment

After all the animals were anesthetized on the 21st day after the creation of the burn wounds; 9-mm full-thickness punch skin biopsies were taken from the center of the burn eschars using an aseptic technique. The tissue biopsy samples were placed in sterile glass vials containing sterile brain–heart infusion broth and incubated at 35 ± 2°C for 2 hours. The vials were reweighed, and the tissue homogenates were prepared in 2 ml of the brain–heart infusion broth using a sterile mortar and pestle. A portion (0.1 ml) of each homogenate was cultured on blood agar, chocolate agar, eosin methylene-blue agar and Sabouraud-dextrose agar (Oxoid Ltd., Basingstoke, United Kingdom). All plates were examined 24 and 48 hours after incubation at 37°C. The last dilution was calculated as 10^{-5}. Individual colonies were identified and quantified as colony-forming units per gram of tissue. The results of all the colony-forming units were averaged and expressed as the mean Log_{10}.

Macroscopic Evaluation

On the 5th, 7th, 10th, 15th, 18th, and 21st days after creation of the burn wounds, all burned areas were macroscopically evaluated in terms of the viable and necrotic zones. The viable zones were pink–white, soft, and bled when cut with a scalpel, but the necrotic zones were dark and stiff, and did not bleed when cut. The necrotic zones in the burned areas were marked using millimetric cross-sectional transparent film. After the film was scanned, the necrotic areas were calculated using an image analysis program (Soft Imaging System Analysis, version 3.0, Munster, Germany).

Statistical Analysis

Statistical analyses were done using SPSS (version 11.5, SPSS Inc., Chicago, IL) and power analyses was performed by PASS 11 (NCSS Inc. Kaysville, UT) software. With eight burns in each study group, our study had 80% power to detect a 3.9% difference in wound closure at 21 days with a P value of .05. The groups were compared using the Kruskal–Wallis test. For the parameters that pointed to significant results, the reciprocal groups were compared by means of the Mann–Whitney U test. A $P < .05$ value was considered as significant.

RESULTS

At the end of the 21-day study period, none of the wounds in any of the study groups were completely closed. The size of the remaining wounds was smallest in the groups treated with systemic and topical TQ (6.1 ± 1.6 and 6.7 ± 0.4 cm, respectively; $P < .05$

Table 2. Histopathological and macroscopic findings

	Vascularization (Mean ± SD)	Granulation (Mean ± SD)	Epithelialization, % (Mean ± SD)	Wound Size, cm2 (Mean ± SD)
Control group	0.63 ± 0.5	0.88 ± 0.6	29.9 ± 7.5	11.2 ± 1.2
Silver sülfadiazine group	1.5 ± 0.5*	1.75 ± 0.7†	45.6 ± 5.4‡	8.7 ± 0.9†
Systemic TQ group	1.88 ± 1.0*	1.87 ± 0.8†	46.5 ± 6.9‡	9.0 ± 0.9‡
Topical TQ group	2 ± 0.7‡	2 ± 0.8*	58 ± 2.5‡§||	6.7 ± 0.4‡§||
Systemic and topical TQ group	2.13 ± 0.8‡	2.25 ± 0.7‡	61.6 ± 10‡§||	6.1 ± 1.6†§||

TAS, total antioxidant state; *TOS*, total oxidative stress.

*Different from control group ($P = .01$).

†Different from control group ($P < .05$).

‡Different from control group ($P \leq .001$).

§Different from silver sülfadiazine group ($P \leq .001$).

||Different from systemic TQ group ($P \leq .001$).

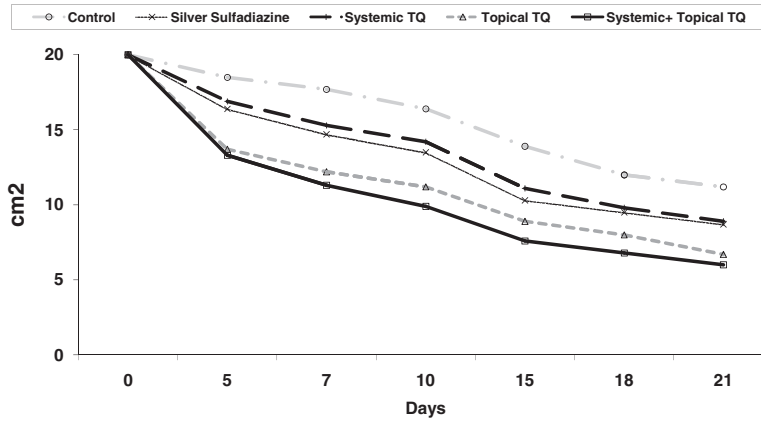


Figure 1. Changes in the wound surface area.

for both when compared with controls). The area of the remaining necrotic area was greatest in the control group ($11.2 \pm 1.2\,cm^2$). The rates of wound closure were similar in the silver sulfadiazine (SSD) and systemic TQ groups (Table 2; Figure 1).

The tissue samples from all the groups were histopathologically compared in terms of the vascularization and granulation tissue formation. The granulation tissue formation and vascularization were significantly lower in the control group compared with the other groups ($P < .05$), (Figures 2 and 3; Table 2). The inflammatory cell response and epithelialization observed among the groups was the highest in the control and silver sulfadiazine groups; it was lower in the groups that were treated with TQ (Figures 2 and 3).

When all the groups were biochemically compared, the TAS levels in the control group were significantly lower than in the other groups. Although the TOS levels were lower in the TQ groups, they were significantly higher in the control and silver sulfadiazine groups (Table 3).

The isolated microorganisms in the eschar tissue cultures were *Staphylococcus aureus* (45%), *Staphylococcus epidermidis* (30%), *Pseudomonas aeruginosa* (15%), and *Escherichia coli* (10%). During the microbiological assessments of all the groups, the lowest microbiological count was observed in the groups that were administered a combination of systemic and topical TQ, whereas the highest growth was observed in the control group ($P < .05$) (Tables 3 and 4; Figure 4).

DISCUSSION

Although medical substances still form the basis of burn and wound care, recent studies have also reported the positive effects of substances of plant origin on wound healing in animal models.[5,7,8,22] Mohajeri et al[6] demonstrated the angiogenic and antibacterial effects of fresh kiwifruit and the accelerating effect of its topical use on the healing of burn wounds. Starley et al[22] have reported that topical papaya application may help the sloughing of

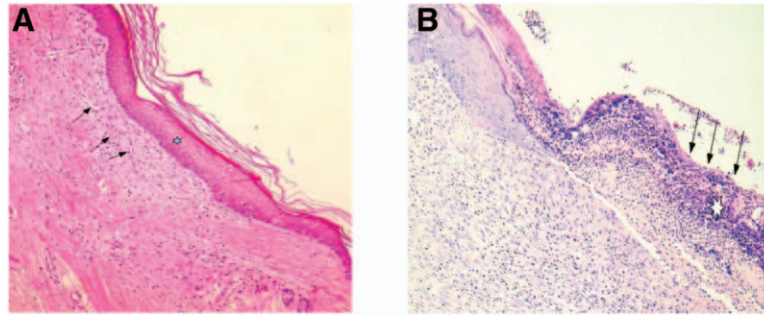


Figure 2. Cross-sectional histopathology of the burned skin on postburn day 21. A. Systemic and topical thymoquinone treatment group; a thick layer of surface epithelium is visible on the surface area (asterisk); below it, there is minimal inflammatory cell response within the well-differentiated capillary system in the granulation tissue (arrow) (hematoxylin eosin stain, × 200). B. Control group; the lack of epithelialization is indicated (arrow); the moderate inflammatory cell response is more intensely visible at the surface (asterisk) (hematoxylin eosin stain, × 200).

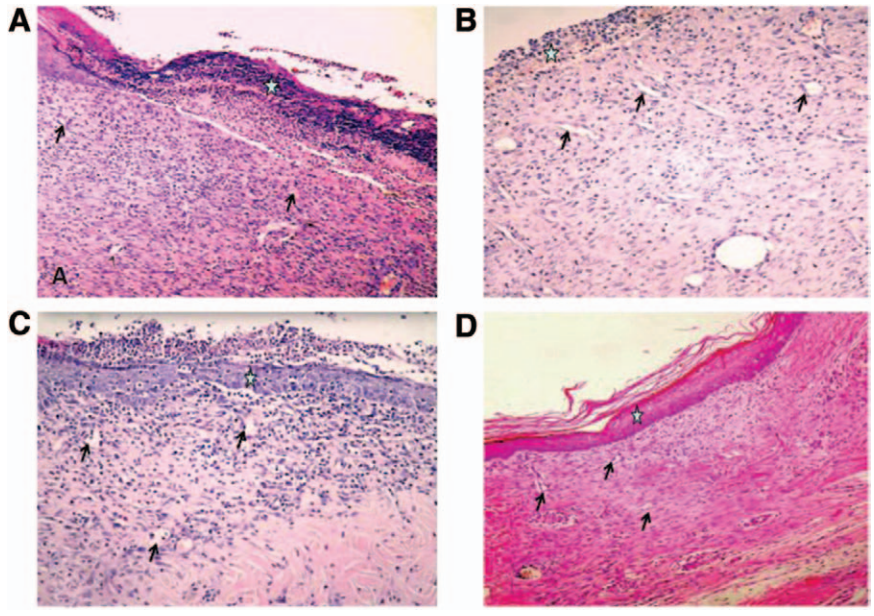


Figure 3. Cross-sectional histopathology of burn skin on postburn day 21. A. Control group; there was no epithelization shown by an arrow, severe inflammatory cell response (asterisk), and small number of capillaries in granulation tissue (arrow) (hematoxylin eosin, ×200). B. Silver sulfadiazine treatment group; mild inflammatory cell response at the surface where no epithelialization has occured (asterisk), and well-differentiated capillary system in dermal granulation tissue (arrow) (hematoxylin eosin, ×200). C. Topical thymoquinone treatment group; a thin epithelialized layer at the surface showing intense inflammation (asterisk), and the well-differentiated capillary system within the granülation tissue in the dermis (arrow) (hematoxylin eosin, ×200). D. Systemic and topical thymoquinone treatment group; a thick layer of surface epithelium on the surface area (asterisk), and the well-differentiated capillary system in the dermis (arrow) (hematoxylin eosin, ×200).

necrotic tissue in burn wounds, have an antimicrobial effect, and provide the necessary granulation tissue formation for skin grafting. TQ is a biologically active component of the oil of *N. sativa* (black cumin) and its various antimicrobial,[9–11] anti-inflammatory,[14,15] and antioxidant[16] features have already been demonstrated. Given these known properties, TQ has been thought to have the potential to positively influence wound healing similar to certain other extracts of plant origin.[5–8,22]

Because burn wounds disturb the physical barrier function of the skin, they render the individual prone to infections and delay the healing of the tissue because of bacterial contamination.[9,10,13–16] Therefore, the use of topical antibacterial agents is important in the care of burn wounds. Silver sulfadiazine has been one of the most commonly used broad-spectrum topical antibacterial agents in the treatment of burn wounds around the world for a long time.[23–25] On the basis of these known properties and its use in numerous experimental studies

Table 3. Biochemical and microbiological findings

	TAS (Mean ± SD)	TOS (Mean ± SD)	Eschar Culture (Mean ± SD)
Control group	0.38 ± 0.17	102.23 ± 31.2	3.95 ± 1.6 × 104
Silver sulfadiazine group	0.57 ± 0.12*	103.44 ± 42.5	1.41 ± 1.55 × 104 †§
Systemic TQ group	0.63 ± 0.23*	58.1 ± 22.25†‡	1.35 ± 1.47 × 104 †§
Topical TQ group	0.57 ± 0.13*	58.08 ± 26.9*‡	1.31 ± 1.42 × 104 †§
Systemic and topical TQ group	0.77 ± 0.26†	64.28 ± 19.35†‡	0.004 ± 0.01 × 104†

TAS, total antioxidant state; *TOS*, total oxidative stress.
*Different from control group ($P < .05$).
†Different from control group ($P < .001$).
‡Different from silver sulfadiazine group ($P < .05$).
§Different from systemic and topical group ($P < .05$).

Table 4. The frequency of positive cultures on postburn day 21

Groups	Control	Silver sulfadiazine	Systemic TQ	Topical TQ	Systemic + Topical TQ
Eschar culture*	7/8 (88%)	4/8 (50%)	4/8 (50%)	4/8 (50%)	1/8 (13%)

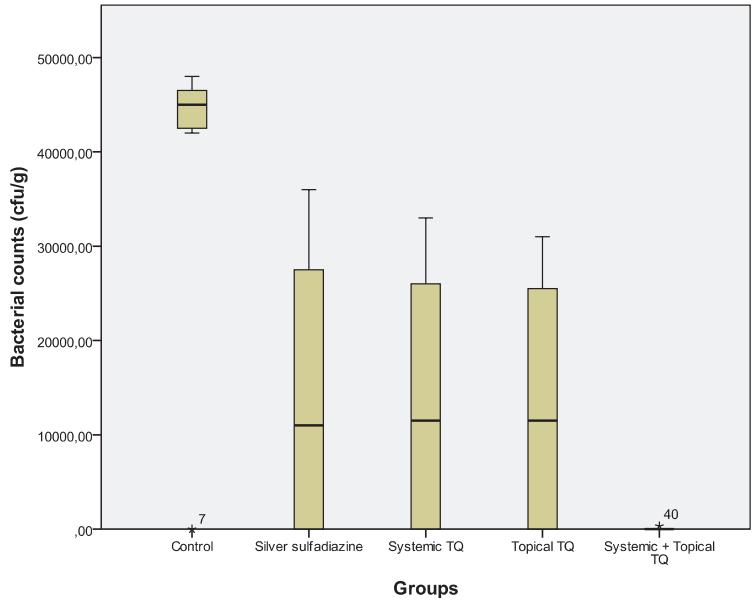
TQ, thymoquinone.
*Data were given as positive cultures/total number of rats.

on burn wounds, silver sulfadiazine was selected for comparison with TQ in our study. At the end of the study, the greatest antibacterial effect was observed in group 5, which was administered a combination of systemic and topical TQ ($P < .05$). No significant difference was observed between the silver sulfadiazine group and groups 3 and 4, in which TQ was administered systemically or topically, respectively ($P > .05$).

In various studies, burn damage has been demonstrated to increase the oxidative stress and lipid peroxidation rates.[22,26] LaLonde et al[26] demonstrated that the use of antioxidants significantly reduces burn mortality. In their clinical study, Yan et al[27] have showed the positive effects of oral antioxidants on morbidity in burn damage. Vorauer-Uhl et al[28] demonstrated that the topical use of superoxide dismutase reduces the necrotic area and accelerates epithelialization in burn wounds. The TAS and TOS levels provide reliable data about the tissue antioxidant levels and oxidative stress. Under conditions causing oxidative stress, the TAS levels decrease, whereas the TOS levels increase.[21] In our study, the TAS levels were higher and the TOS levels were lower in the TQ groups compared with the control groups.

In our study, macroscopic evidence of wound closure was significantly greater in all the study groups in compared with the control group ($P < .05$). When the study groups were compared among themselves, the fastest rate of wound closure was observed in groups 4 and 5, in which the TQ was topically administered (wound size: $6.7 \pm 0.47\,cm^2$ and $6.05 \pm 1.5\,cm^2$, respectively). Additionally, the inflammatory cell response was the lowest and the epithelization was the highest in these groups.

TQ is applied topically and systemically at various doses, as seen in the literature.[10,29–31] In these studies, intraperitoneal administration is usually carried out in 1 to 10 mg/kg doses,[17,32] whereas doses of 0.5 to 100 mg/kg are preferred for the oral



cfu/g: colony-forming units per gram
Box-plots with the middle bars representing the medians and the boxes representing the interquartile ranges.

Figure 4. Comparison of the quantitative cultures from the burn wounds on postburn day 21.

administration.[17,31–33] Because Al-Ali et al[34] demonstrated that systemic administration has a stronger effect compared with oral administration, we also preferred systemic administration to the oral route. Topical administration is generally preferred in ophthalmological cases, and the preferred doses are between 0.1 and 0.05%.[12,35] In our study, the systemic dose of TQ was 2 mg/kg, and the topical dose was a 0.5% solution. Future studies are required to determine the optimal timing and dosing of TQ.

Our study has several limitations. First, the study ended before any of the wounds were completely closed. Thus it is not possible to determine the long-term effects of TQ on wound healing and scarring. Second, the results in rats may not generalize to larger animals and humans. Third, SSD was used as the control because it is still the most commonly used topical agent to treat second-degree burns. However, there is evidence that SSD may delay healing, which would have biased our results in favor of the study agent.[36,37] The lack of a comparison with other topical antioxidants is another limitation of our study, and further studies are needed to clear these points.

On the basis of our data, TQ appears to accelerate the rate of wound closure both in topical and systemic administrations, and this effect is stronger for the topical administration. In light of these findings, we believe that TQ may constitute an alternative treatment option or provide a different perspective in the care of burn wounds.

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