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Original Article PLASTIC & RECONSTRUCTIVE SURGERY

Experimental investigation of the effect of thymoquinone on the viability of random patterned skin flaps

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ABSTRACT

OBJECTIVE: The purpose of this study is to demonstrate the potential effects of thymoquinone, a well-known antioxidant agent, on random flap viability and thus to improve the clinical use of these flaps.

METHODS: In this study, 24 Sprague Dawley female albino rats weighing between 200–250 grams were used. Three groups consisting of randomly selected 8 rats were formed, as study, sham and control groups. Modified McFarlane flap model sized 3x9 cm. was used. In control group, only local wound care was carried out for ten days after flap elevation. In sham group, daily intraperitoneal dimethyl sulfoxide (DMSO) solution was injected in the post-operative 10-day period. In the study group thymoquinone and DMSO solution were injected intraperitoneally together. 10 days later, the study was terminated and flaps were photographed for necrotic area measurements, tissue and blood samples were taken out for biochemical and histopathological studies. In biochemical studies, tissue and serum total antioxidant capacity (TAC) and thiobarbituric acid reactive substrates (TBARS) levels were measured. Histopathological studies included inflammatory cell infiltration, collagen, fibroblast formation and angiogenesis.

RESULTS: There was significant decrease in the necrotic areas of flaps in the study group. Serum and tissue antioxidant levels were significantly high and TBARS levels were significantly low. Histopathological examination showed no significant difference in inflammatory cell infiltration, fibroblast formation or angiogenesis between the groups. However, collagen density in the study group was found to be more than the other groups and the structure was better formed.

CONCLUSION: Thymoquinone increases the flap viability due to its antioxidant properties and it has a positive contribution to wound healing, although it has no significant anti-inflammatory or anjiogenetic activity. In the future, we think that it can be clinically useful for preventing distal flap necrosis in patients with high risk.

Keywords: Random flap; reconstructive surgery; thymoquinone.

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With the rapid development of flap surgery techniques since the late 1970s, there have been major changes in reconstruction approaches. The reconstructive ladder [1], defined in 1982, was useful in choosing the safest repair method in soft tissue repair. With the ability to isolate vessels down to the muscle

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and fasciocutaneous level and the introduction of the surgical microscope, free tissue transplants came to the fore and a new era opened in reconstructive surgery. It was to increase flap sizes and primary closure of the donor areas thanks to the identification of fasciocutaneous and musculocutaneous vascular zones by Taylor et al. [2, 3]. Recently, with the definition and development of the perforator flap concept and the development of the "free-style" flap concept and supermicrosurgical techniques, it has become possible to transfer the most suitable tissue for aesthetic and functional repair in the appropriate size and thickness, creating minimal donor site morbidity.

Although flaps are generally transplanted to defect areas with insufficient blood supply (deperioste bone, cartilage without perichondrium) due to their own feeding vessels, they are also preferred in cases where functional reconstruction is intended (total lower lip defect, facial paralysis) as they allow the transfer of structures such as muscle, bone and nerve. Random pattern skin flaps are frequently used to cover skin defects with local tissues due to their easy applicability. However, distal flap necrosis is a significant problem, especially when removing long skin flaps, and therefore they must be removed by adhering to a certain aspect ratio. This limits the clinical use of random pattern flaps. Many experimental and clinical studies have been conducted to increase the viability of random flaps [4, 5].

Thymoquinone is obtained from the nigella sativa plant, which has been used as a natural medicine in the treatment of many diseases for thousands of years. Nowadays, it has been widely used in experimental studies on cancer treatment, especially due to its antioxidant capacity, and positive responses have been received. It shows its antioxidant properties, especially through the inhibition of enzymatic lipid peroxidation [6]. There is no study on the effect of the substance on random flaps, which has previously been shown to have a positive effect on ischemia reperfusion injury in muscle flaps due to its anti-oxidant effect. The aim of this experimental study is to demonstrate the possible positive effect of thymoquinone on the survival of random skin flaps and thus to increase the clinical use of these flaps.

MATERIALS AND METHODS

Approval for this study was received from the Istanbul University Animal Experiments Local Ethics Committee with decision number 2014/88 dated 02.09.2014. The study follows the ethical standards of the Helsinki Declaration, as revised in 2013. In the study, 24 female, adult Sprague Dawley rats, weighing between 200–250 g, obtained from Istanbul University Aziz Sancar Institute of Experimental Medicine, were used. The rats were housed at an average temperature of 20–25 degrees, with one animal in each cage, in a 12-hour day and night rhythm. All animals were fed with tap water and standard rat chow. Surgical interventions were performed at the Animal Experiments Laboratory of Istanbul University Aziz Sancar Institute of Experimental Medicine. The animals were divided into three groups of 8 rats each: experimental group, control group and sham group.

Highlight key points

- It was found that thymoquinone significantly reduced the area of distal necrosis in dorsal random pattern flaps created from rats and contributed positively to wound healing.
- Serum and tissue antioxidant levels were significantly high and MDA levels were significantly low in the experiment group. Collagen density in the experiment group was significantly higher.
- Further studies with various dose ranges and application routes are necessary before clinical use.

Anesthesia in all rats was provided intramuscularly with 35 mg/kg ketamine hydrochloride (Ketalar® –Pfizer) and 10 mg/kg xylazine hydrochloride (Rompun® 2% –Bayer). When necessary, a maintenance dose of 1/3 of this dose was administered.

All rats were anesthetized after being divided into three randomly selected groups of 8 rats each. Following anesthesia, the surgical area was shaved with a shaver and cleaned with povidone iodine. The rats were placed face down on the surgical table and fixed with patches on the tail and four extremities. Before the surgical procedure, prophylaxis was administered to all animals with a single dose of 150 mg/kg subcutaneous ampicillin injection. A 3x9 cm modified McFarlane flap [7] was drawn on the back of all animals in the experimental and control groups, with a caudal basis, with the help of a previous template (Fig. 1).

The flaps were lifted over the deep muscle fascia, including the panniculus carnosus layer, with the help of a size 15 scalpel. The flaps were adapted into place with 3.0 sharp polypropylene sutures. The rats were placed in separate cages in the postoperative period. Each rat received daily wound care with povidone-iodine in the postoperative period. Groups were created as follows according to drug treatment after the surgical procedure.

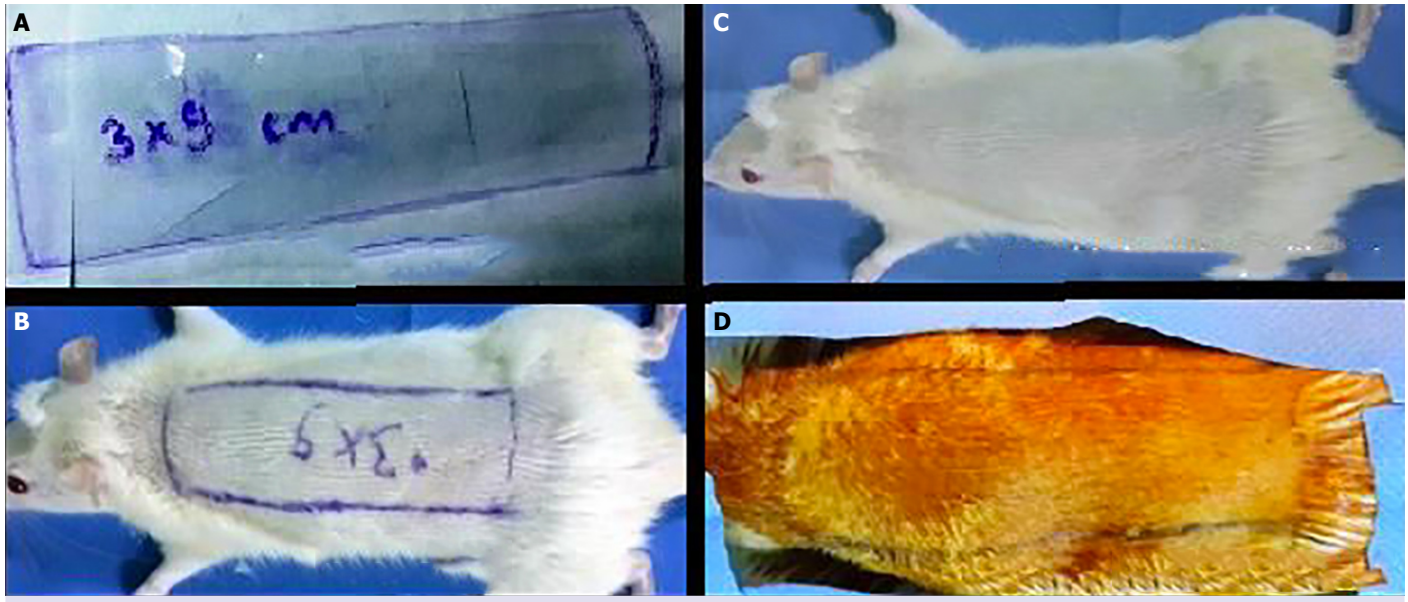

Figure 1. Preparation of the flap outline. **(A)** Preparation of temple **(B)** Flap design **(C)** Shaving prior to surgery **(D)** Preparation of the surgical site.

1. Control group: The group that was followed up with only wound care after the operation.
2. Sham group: The group in which only dimethyl sulfoxide (DMSO) solution was injected after the operation.
3. Experimental group: The group injected with Thymoquinone® (Sigma Aldrich) dissolved in DMSO solution after the operation.

In the postoperative period, 5 mg/kg thymoquinone dissolved in 0.1 cc DMSO solution was injected intraperitoneally to the experimental group with the help of a 28G insulin syringe, and 0.1 cc DMSO solution was injected to the sham group. The same injections were repeated once a day, at the same time every day, for 10 days after the operation.

On post-operative day 10, subjects received intramuscularly 30 mg/kg ketamine hydrochloride (Ketalar®, Eczacıbaşı) and xylazine hydrochloride (Rompun®, Bayer,) 10 mg/kg. Under anesthesia, 1x1 cm tissue samples were taken from the distal and proximal 1/3 parts of the back flap areas for histopathological examination, and from the middle part for biochemical examination. The experiment was terminated by taking 4 cc of blood by cardiac puncture. The subjects were euthanized by cervical dislocation under anesthesia. Death was confirmed by checking the heartbeat.

Tissue samples taken for histopathological examination were delivered to the pathology laboratory in 10% formaldehyde solution. Blood samples were centrifuged at 5000 rpm for 5 minutes and serum was separated. Tissue samples and serums taken for biochemical analysis were delivered to the biochemistry laboratory in dry ice.

Evaluation

Flap Area Measurements

The circulation of the dorsal flaps in all rats was monitored by daily inspection. Demarcation line formation was observed in the distal flaps from the 4th day. On the 10th day, all flaps were photographed from an equal distance with the help of a Nikon D5100 camera. The photographs were transferred to the Image J program on a computer and live tissue, necrotic area and necrotic area percentage measurements were made.

Biochemical Evaluation

TBARS Measurement in Tissue and Serum

Lipid peroxidation is a well-studied mechanism frequently used to indicate cell damage, and lipid peroxides are unstable indicators that form complex forms and reactive compounds such as malondialdehyde (MDA). Measuring the end product of lipid peroxidation is a frequently used method to demonstrate oxidative damage.

The measurement of thiobarbituric acid reactive substrates (TBARS) method is also used for lipid peroxidation, and according to this method, MDA in the samples forms a compound with TBA in a ratio of 1:2. This resulting com-

pound is determined by colorimetric or fluorometric methods (OxiSelect™ TBARS Assay Kit (MDA Quantitation)).

Measurement of serum and tissue TBARS amounts was made according to the following method at Istanbul Medeniyet University Faculty of Medicine, Department of Medical Biochemistry. Skin tissues taken from rats were washed with physiological saline and then passed through liquid nitrogen. The blood samples taken were centrifuged at 5000 rpm for 5 minutes to separate the serum.

As a result of weighing, tissues weighing between 0.08–0.340 g were used to determine MDA using the TBARS method. According to the method, it was homogenized to 10% homogenate in cold 1.15% KCl, 20% acetic acid and 8.1% sodium dodecyl sulfate solution. 250 µl of homogenate and serum were taken and kept in a hot water bath for 15 minutes in 1.5 ml of 0.75% TBA, 1 ml of 30% TCA and 0.2 ml of 5N HCl solution. The color formed after the hot water bath in the samples centrifuged at 3000 rpm for 10 minutes was read at 532 nm against the blank created from the same solutions in equal amounts (Schimadzu® UV 20000). 1,1,3,3 – tetraethoxy propane (TEP) was used as the standard for TBARS. TBARS concentrations were calculated using the molar extinction coefficient of the standard. TBARS concentrations were given as nmol/mL homogenate.

Total Antioxidant Capacity Measurement

Serum and tissue Total Antioxidant Capacity (TAC) measurement was performed at Istanbul Medeniyet University Faculty of Medicine, Department of Medical Biochemistry, using the Sunred brand (Catalog No: 201-11-1187) Rat T–AOC enzyme-linked immunosorbent assay (ELISA) kit.

The kit measures according to the double antibody sandwich Elisa method. Serum or tissue samples are loaded into wells pre-coated with monoclonal rat total antioxidant antibody and left for incubation. The second TAK antibody labeled with biotin is then added and incubated with Streptavidin-Horse radish peroxide for immune complex formation.

After the unadhered antibodies and substrates are removed by washing, the reaction is terminated by adding stop solution to the kit incubated with chromogens A and B, and absorbance reading is made at 450 nm wavelength (Sunred Assay Kit TAK).

Standards of 32 U/mL, 16 U/mL, 8 U/mL, 4 U/mL and 2 U/mL were prepared from the stock solution by serial dilution and added to the wells. 40 µl samples were taken from serum samples and 10% tissue homogenates prepared with 1.15% KCl and added to the wells. Add 50 µl to the wells containing only the samples. Biotin-labeled TAK antibody was added and incubated at 37°C for 1 hour. After 5 washes with a micro-ELISA washer (Bio-Tek, ELx50), 50 µl of chromogen A and B were added to all wells, including the blank, and incubated for 10 minutes. Concentrations were calculated from the standard curve created according to the standards by adding 50 µl stop solution and reading at a wavelength of 450 nm on the micro-ELISA reader (Bio-Tek, FL 600) device. Results were given as U/mL.

Histopathological Evaluation

On the 10th day after the operation, 1x1 cm tissue samples were taken from the proximal and distal necrosis-solid tissue transition areas of the flaps for histological study. The tissue samples taken were fixed in 10% neutral formaldehyde. Following fixation, all tissues were dehydrated using routine histological follow-up methods and embedded in paraffin. 5 µm thick sections were taken from paraffin blocks. All sections were subjected to routine histological staining and immunohistochemical staining methods. Necrosis areas in the epidermis and dermis were evaluated with Hematoxylin + Eosin (H + E) staining in terms of PNL (polymorphous nucleated leukocyte), lymphocyte, fibroblast and collagen formation. For angiogenesis evaluation, the number of vessels in the sections was counted with VEGF (Vascular Endothelial Growth Factor, VEGF Antibody (A-20), 200 µg/ml, Santa Cruz sc-152, SensiTek HRP Anti-Polyvalent Lab Pack, ScyTek SHP125) dye. Cell density was evaluated with a scoring system numbered from 1 to 3 (1: low, 2: moderate, 3: high). Number of cells per mm^2 for VEGF: below 50 was scored as 1, between 50–100 was scored as 2, and above 100 was scored as 3. For each subject, the data obtained from the proximal and distal parts were averaged and compared between groups.

The obtained histological preparations were examined with an Olympus BX61 research microscope and photographed with an Olympus DP72 model microscope camera.

Statistical Evaluation

NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size) 2008 Statistical Software (NCSS LLC, Kaysville, Utah, USA) program was used for statistical analysis. While evaluating the study data, in addition to descriptive statistical meth-

ods (mean, standard deviation, median, frequency and ratio), the Kruskal Wallis test was used to evaluate the field and biochemical variables according to the three groups, and the Mann Whitney U test was used to determine the group causing the difference. Fisher Freeman Halton (Monte Carlo) Exact test was used to compare histopathological variables. Result significance was evaluated at the $p<0.05$ level within the 95% confidence interval.

RESULTS

The study was completed on the 10th day after the operation. No complications related to anesthesia or surgical procedures were observed in the subjects. All rats survived until the end of the study. The results obtained on 8 rats each in the control, sham and experimental groups were evaluated.

Flap Area Measurements

All flap areas and necrosis areas in the groups were measured one by one, and the percentages of necrotic area/flap area were calculated.

Areas of necrosis show statistically significant differences between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, the necrosis areas of the experimental group were found to be significantly lower than the control and sham groups ($p<0.01$). The average necrosis area of the sham group cases was found to be significantly lower than the control group ($p<0.01$).

The percentage of necrosis shows a statistically significant difference between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, the necrosis percentage of the experimental group was found to be significantly lower than the control and sham groups ($p<0.01$). The average necrosis percentage of the sham group cases was found to be significantly lower than the control group ($p<0.01$) (Table 1, Fig. 2, 3).

Biochemical Analyzes

The amount of tissue TBARS differs statistically significantly between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, the tissue TBARS amount of the experimental group was found to be significantly lower than the control and sham groups ($p<0.01$). The mean tissue TBARS amount of the sham group cases was found to be significantly lower than the control group ($p<0.01$).

Table 1. Area measurements by groups

Group	Flap area (millimeter square)	Necrosis area	Necrosis percentage (%)
Control			
Mean	805975.13	393870.50	48.98
SD	23184.53	34794.24	3.51
Median	800752.50	390236.50	50.0
Minimum	782151	342979	42.10
Maximum	849721	452802	53.20
Experiment			
Mean	811114.13	93415.38	11.46
SD	16538.65	20829.26	2.40
Median	813727.50	94088.50	11.65
Minimum	783840	61750	7.80
Maximum	834417	128551	15.40
Sham			
Mean	797578.50	265461.75	33.06
SD	32011.25	58899.54	7.45
Median	791169.50	263309.00	31.70
Minimum	761303	169210	21.60
Maximum	865860	348052	43.40
[a]p	**0.277**	**0.001***	**0.001***
[b]Post hoc			
Control vs. experiment	**0.529**	**0.001***	**0.001***
Control vs. sham	**0.345**	**0.001***	**0.001***
Experiment vs. sham	**0.115**	**0.001***	**0.001***

SD: Standard deviation; a: Kruskal Wallis Test; b: Mann-Whitney U Test; *: P<0.01.

Serum TBARS amount shows a statistically significant difference between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, the serum TBARS amount of the experimental group was found to be significantly lower than the control group ($p<0.01$). No statistically significant difference was detected between the serum TBARS measurements of the sham group and the experimental and control group cases ($p>0.05$).

TAC serum measurements show statistically significant differences between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, TAC serum measurements of the experimental group were found to be significantly higher than the control and sham groups ($p<0.01$). No significant difference was detected between the sham group and the control ($p>0.05$).

FIGURE 2. Appearance of necrotic areas on the flaps on the 10th day. **(A)** Control **(B)** Sham **(C)** Experiment.

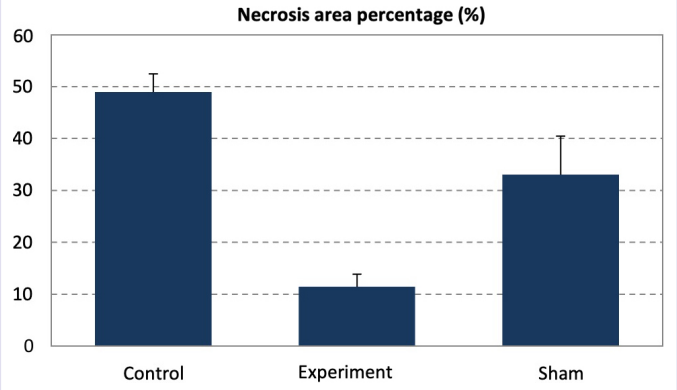

FIGURE 3. Necrosis percentage distribution by groups (mean and standard deviation).

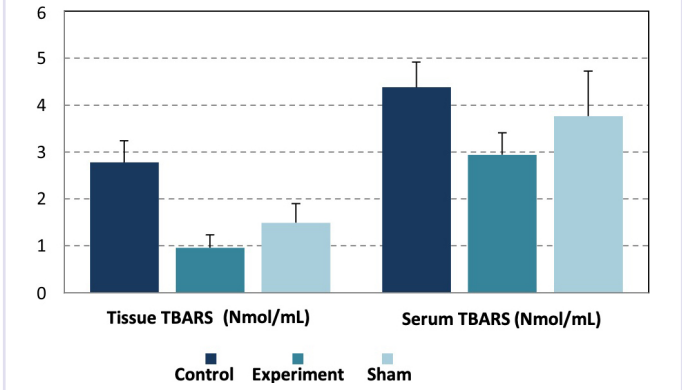

FIGURE 4. Tissue and serum TBARS distribution by groups (mean and standard deviation).

TAC tissue measurements show statistically significant differences between groups ($p<0.01$). In the pairwise comparisons made to determine which group caused the significance, TAC tissue measurements of the experimental group were found to be significantly higher than the control and sham groups ($p <0.01$). The TAC tissue average of the sham group cases was also found to be significantly higher than the control group ($p<0.01$) (Table 2, Fig. 4, 5).

Histopathological Evaluation

Fibroblast scores in proximal and distal samples did not differ significantly according to groups ($p>0.05$).

PNL scores in proximal and distal samples did not differ significantly between groups ($p>0.05$).

Lymphocyte scores in proximal and distal samples did not differ significantly between groups ($p>0.05$).

Proximal collagen formation; the number of medium and high score cases in the experimental group was determined to be significantly higher than in the control and Sham groups ($p <0.05$). Distal collagen formation in control group cases was found to be significantly higher than the experimental and sham groups ($p<0.05$). No significant difference was detected between the distal collagen formation rates in the experimental and sham groups ($p>0.05$) (Table 3, Fig. 6).

VEGF scores in proximal and distal samples did not differ significantly according to groups ($p>0.05$).

DISCUSSION

Tissue defects occur for a variety of reasons such as trauma, tumor, and infection. While most of these defects can be repaired by primary, secondary repair and grafting methods, flap surgery is preferred in defects where tissues with poor vascularity such as vessels, tendons and bones are exposed and in cases where functional and aesthetic recovery is a priority. While flaps can be removed from areas adjacent to the defect area, flap transplants can also be performed from distant areas using microsurgical techniques. Repair options vary depending on the content of the defect and the anatomical region where it is located. Based on the principle of repairing the lost tissue with a similar one, repair with local tissue options with a similar structure should be preferred. However, since the viability of the flap depends on the arteriovenous structures on which it is carried, reasons such as insufficient vascularization in the areas adjacent to the defect and lack of sufficient tissue restrict the use of local tissues. Many studies have been conducted on many

Table 2. Thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) evaluations by groups

	Tissue TBARS (Nmol/mL)	Serum TBARS (Nmol/mL)	TAC-Serum (U/mL)	TAC-Tissue (U/mL)
Control				
Mean	2.78	4.38	4.54	2.12
Standard deviation	0.46	0.54	1.31	0.94
Median	2.85	4.30	4.17	2.26
Minimum	1.9230	3.82681	3.28	1.06
Maximum	3.3461	5.36521	7.40	3.37
Experiment				
Mean	0.96	2.94	6.94	6.09
Standard deviation	0.27	0.47	1.25	1.51
Median	0.96	2.79	6.63	5.62
Minimum	0.5769	2.42290	5.59	4.65
Maximum	1.4807	3.52000	9.09	9.52
Sham				
Mean	1.49	3.77	3.92	3.99
Standard deviation	0.41	0.96	0.92	1.00
Median	1.32	3.73	4.11	3.78
Minimum	1.1153	2.38452	2.28	3.00
Maximum	2.1400	5.20000	4.99	5.61
[a]p	**0.001***	**0.001***	**0.001***	**0.001***
[b]Post hoc				
Control vs. experiment	**0.001***	**0.001***	**0.006***	**0.001***
Control vs. sham	**0.002***	**0.208**	**0.529**	**0.005***
Experiment vs. sham	**0.005***	**0.141**	**0.001***	**0.009***

a: Kruskal Wallis Test; b: Mann-Whitney U Test; *: P<0.01.

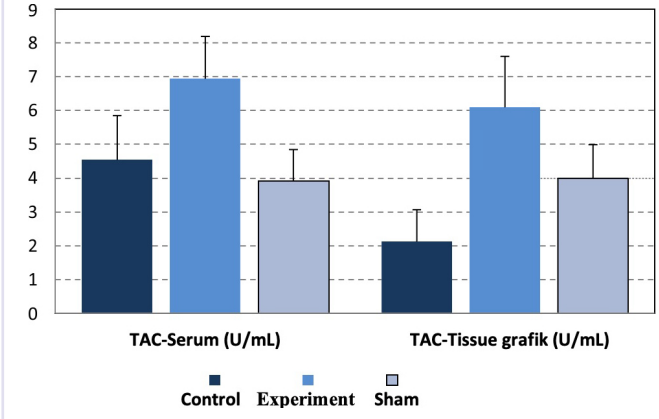

Figure 5. Serum and tissue TAC distribution according to groups (mean and standard deviation).

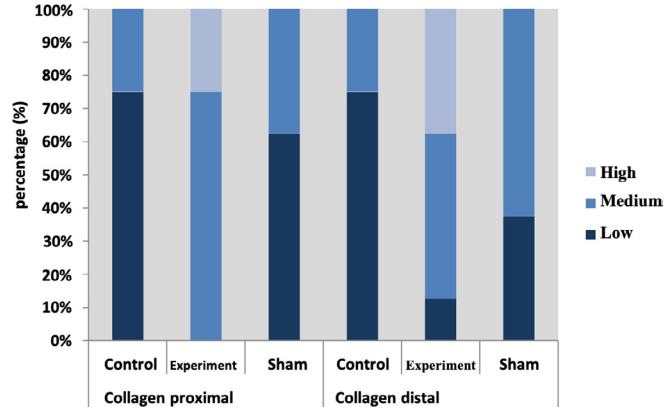

Figure 6. Distribution of collagen formation findings according to groups.

techniques such as tissue expansion, surgery, and medical preconditioning to close defects with maximum survival rate with existing vascular resources using local flaps.

Despite the studies carried out to date, distal flap necrosis still remains a problem. It has been shown in many studies that the primary etiology of flap necro-

Table 3. Histopathology evaluations according to groups

	Control (%)	Exp. (%)	Sham (%)	p
PNL** proximal				
Low	100	87.5	75.0	**0.745**
High	0	12.5	25.0	
PNL** distal				
Low	87.5	75.0	100.0	**1.000**
Moderate	12.5	12.5	0	
High	0	12.5	12.5	
Lymphocyte proximal				
Low	87.5	75.0	62.5	**0.836**
Moderate	12.5	25.0	37.5	
Lymphocyte distal				
Low	37.5	87.5	75.0	**0.163**
Moderate	62.5	12.5	25.0	
Fibroblast proximal				
Low	75.0	37.5	37.5	**0.111**
Moderate	25.0	62.5	25.0	
High	0	0	37.5	
Fibroblast distal				
Low	75.0	37.5	50.0	**0.387**
Moderate	25.0	25.0	37.5	
High	0	37.5	12.5	
Collagen proximal				
Low	75.0	0	62.5	**0.013***
Moderate	25.0	75.0	37.5	
High	0	25.0	0	
Collagen distal				
Low	75.0	12.5	37.5	**0.037***
Moderate	25.0	50.0	62.5	
High	0	37.5	0	
VEGF*** vessel proximal				
Low	75.0	50.0	62.5	**0.870**
Moderate	25.0	37.5	37.5	
High	0	12.5	0	
VEGF*** vessel distal				
Low	62.5	12.5	50.0	**0.051**
Moderate	37.5	37.5	50.0	
High	0	50.0	0	

Fisher Freman Halton (Monte Carlo) test; *: P<0.05; **: PNL: Polymorphonuclear leukocyte; ***: VEGF: Vascular endothelial growth factor; Exp: Experiment; PNL: Polymorphonuclear leukocyte; VEGF: Vascular endothelial growth factor.

sis in the postoperative period is perfusion failure. The process leading to flap necrosis begins with the separation of the vessels feeding the flap and sympathetic fibers from the flap after flap removal. With sympathetic innervation, blood flow in the distal part of the flap decreases with the release of vasoconstrictor substances such as Endothelin 1, thromboxane A2, PG F2 α. Flap perfusion returns to normal as sympathetic neurotransmitters are depleted within 12–24 hours. When circulation is restored after 6–12 hours, reperfusion injury can occur, which is the secondary pathogenetic factor in flap necrosis [8]. Free oxygen radicals are released through different mechanisms in ischemic tissue, and these radicals cause tissue damage. The xanthine dehydrogenase/xanthine oxidase enzyme system, neutrophilic nicotinamide diphosphate, neutrophil endothelial adhesion through the myeloperoxidase enzyme system, and intracellular calcium overload are the main mechanisms involved in this process.

Many studies have been conducted to increase flap viability. The most effective method in these studies is delay methods [9]. Delay can be done surgically or vascularly, and it increases flap viability by reducing the release of vasoconstrictor and thrombotic agents observed in acute flap surgery, usually in two or three-stage operations, giving the flap time to adapt to ischemia, increasing distal blood flow, increasing neoangiogenesis and vasodilation in the flap. Pang et al. [10] showed that there was an increase in blood flow in surgically delayed random-pattern porcine skin flaps, especially in the distal part of the flap. Taylor et al. [3] and Callegari et al. [11] have shown that pre-existing "choke" anastomoses are opened by vascular delay in pigs, rabbits, dogs and humans. Lineaweaver et al. [12] showed that vascular retardation increased the viability of the skin island of the TRAM flap and this resulted in vasodilatation and enhancement by increasing VEGF and FGF gene expression. They showed that this occurs through induction of angiogenesis. Although surgical delay methods have been proven to be clinically effective, the fact that they require multi-stage surgery and are expensive and time-consuming limits the clinical use of delay procedures. Today, studies are being conducted on the use of pharmacological agents to increase flap viability as an alternative to delaying procedures. For this purpose, vasodilator, antithrombotic agents, agents to prevent neutrophil aggregation and adhesion, hemorheological agents, anti-inflammatory agents, and antioxidant agents have been tried [8].

Although the effectiveness of these drugs has been demonstrated experimentally in animals, their clinical effectiveness in increasing flap viability has not been surpassed by delaying methods. Recent studies have focused

on local angiogenic cytokine protein (VEGF, PDGF) therapy, and these treatments have been shown to increase flap viability [13, 14]. Free oxygen radicals formed as a result of ischemia/reperfusion are effective in the pathogenesis of many diseases such as the aging process, cardiovascular diseases, cancer, lung diseases, inflammatory and rheumatic diseases. There are different antioxidant systems in the body against oxyradicals.

Antioxidants can be divided into two groups: endogenous and exogenous. They exert their effects through 4 basic mechanisms. These are scavenging, quenching, repair and chain-breaking effects. Many studies have been conducted on how antioxidants increase flap viability through different mechanisms. Cetin et al. [15] showed that fucoidin reduces ischemia reperfusion damage in the skin flap by dose-dependently inhibiting the "rolling" phase, which is the first step in leukocyte adhesion. Many agents such as carnitine, desferoxamine, vitamins E, C, and coenzyme Q have been experimentally shown to increase flap viability with antioxidant activity [16]. Ozkan et al. [17] showed that methylene blue positively affected the viability of the rat epigastric island flap through xanthine oxidase inhibition.

Thymoquinone is obtained from the seeds of the Nigella sativa plant, popularly known as black cumin, which is used as a natural medicine in the treatment of joint pain, flatulence, dyspepsia, liver and lung diseases. Many experimental studies have been conducted especially on its anticarcinogenic effect. Ait Mbarek et al. [18] showed that intratumorally administered nigella sativa extract in mice with tumors resulted in a reduction in tumor size and a decrease in the rate of metastasis. In their in vitro and in vivo studies on squamous cell carcinoma and fibrosarcoma, Ivankovic and colleagues found that thymoquinone caused a dose-dependent cytotoxic effect and growth inhibition on tumor cells. Badary et al. [19] showed that thymoquinone reduces ifosfamide-induced renal toxicity and increases the antitumoral activity of ifosfamide. Peng et al. [20] reported that thymoquinone was used in osteosarcoma. They showed that it inhibits proliferation and angiogenesis. Its inhibitory effect on tumor proliferation has been demonstrated in ovarian adenocarcinoma [21], uterine sarcoma, human osteosarcoma [22], fibrosarcoma, and prostate cancer [23]. Apart from these, it has been stated that thymoquinone has antimicrobial, antihepatotoxic, antinephrotoxic, antidiabetic and antiplatelet effects on the vascular system through arachidonic acid metabolism. Studies have attributed the anticarcinogenic, antihepatotoxic and antinephrotoxic properties of thymoquinone to its existing antioxidant capacity. Houghton and colleagues demonstrated that thymoquinone strongly inhibits non-enzymatic lipid peroxidation in cell culture. In the study conducted by Meral et al. [24], they showed that thymoquinone reduced the level of superoxide radicals and reduced lipid peroxidation in diabetic rabbits. In the study conducted by Sayed-Ahmed and Nagi [25] on the effect of thymoquinone on gentamicin nephrotoxicity in rats, they examined the changes in tissue glutathione peroxidase, catalase, glutathione, and TBARS, which is an indicator of lipid peroxidation, and found a significant decrease in the thymoquinone group. In the study conducted by Hosseinzadeh et al. [26] on the effect of thymoquinone on ischemia reperfusion injury in rat skeletal muscle, after systemic intraperitoneal thymoquinone administration at doses of 20 mg/kg, 40 mg/kg, 80 mg/kg, a significant decrease in ischemia reperfusion injury was observed in the total sulfhydryl group, in the MDA level, which is the end product of lipid peroxidation. They found a significant decrease and an increase in total antioxidant capacity. It was also stated in the study that the antioxidant effect of thymoquinone increased dose-dependently. In their study, Kara et al. [27] found that thymoquinone applied in the rapid maxillary expansion procedure in rats caused an increase in new bone formation, capillary number and inflammatory cell density in the maxillary sutures.

Selcuk et al. [28] studied the effect of thymoquinone applied intraperitoneally at a dose of 2 mg/kg/day and topically as a 0.5% solution for 21 days in a second-degree burn model created in a rat. As a result, they stated that minimum necrosis, lower total oxidative stress level and minimum bacterial count were measured in the group where thymoquinone was applied combined systemically and topically. As a result, they stated that thymoquinone accelerates wound healing and reepithelialization by reducing inflammation and oxidative stress in systemic or topical application.

In a study conducted by Abla et al. [29] with acetylcysteine on rat skin flaps, they found that acetylcysteine reduced flap necrosis by 14%. In the study conducted by Angel et al. [30] with the antioxidant desferoxamine, a significant decrease in flap necrosis areas was detected. In our study, there were also statistically significant differences in necrotic area measurements and necrosis/flap area percentages in the thymoquinone-injected group compared to the control and sham groups.

A significant decrease in necrosis percentages was detected in the sham group, which was applied only with DMSO, compared to the control group. This suggests that thymoquinone reduces distal flap necrosis thanks to its antioxidant properties. This result is parallel to other studies on flap viability in the literature.

In our study, to demonstrate the antioxidant activity of thymoquinone, TAC and TBARS change, which is an indicator of lipid peroxidation, were examined in serum and tissue samples taken from flaps, and it was found to be significantly lower in the thymoquinone group than in the control group and sham group.

While a significant change was detected in tissue TBARS and tissue TAC measurements in the sham group, where DMSO109, which is known to have antioxidant activity, was applied alone, compared to the control group, no significant difference was detected in serum measurements. This shows that thymoquinone increases antioxidant capacity at serum and tissue levels. These results are consistent with other studies in the literature on the antioxidant activity of thymoquinone [19, 31].

Although there are studies in the literature showing that thymoquinone has anti-inflammatory properties [32], in our study, no significant difference was detected in PNL and lymphocyte levels in the proximal and distal flaps between the groups and no significant anti-inflammatory activity was demonstrated.

Although there is no statistically significant difference in fibroblast levels between the groups, it is noteworthy that the proliferation in the distal flaps in the experimental group was higher than in the other groups. This suggests that thymoquinone contributes slightly positively to fibroblast proliferation.

When the collagen levels in the sections were examined, it was observed that the collagen density was significantly higher in the experimental group in the flap distals, and fiber structures began to appear and formed bundles in some places. This shows that thymoquinone stimulates collagen synthesis.

Peng et al. [20] showed in their in vivo and in vitro studies that thymoquinone has antitumor and antiangiogenic effects in osteosarcoma. There is no study in the literature on the neoangiogenetic effect of thymoquinone on flaps. In our study, although staining with anti-VEGF antibody showed more intense vascularization in the distal flaps, especially in the experimental group, no statistically significant difference was observed between the groups. This suggests that thymoquinone does not have a significant neoangiogenetic effect on the flaps.

Our study has several shortcomings. Since this is an experimental study on rats, the anatomical differences between the human and rat skin are an important point that has to be taken into account when interpreting the results of this study. Unlike human skin, rat skin has an additional skin layer called panniculus. It is a layer of special kind of muscle in terms of the structure of muscle fibers. Due to these limitations, results of this study should be carefully interpreted when translated into human subjects. Future studies on human subjects are required in this matter. Another limitation is that, despite our histological and biochemical findings, the exact mechanism of thymoquinone and biochemical pathways involved is not clear. Further studies with the addition of advanced DNA/RNA sequencing and western blot techniques can shed light on the exact mechanisms of thymoquinone on cellular level.

Conclusion

The results obtained from our study are parallel to many studies conducted with other antioxidant and anti-inflammatory agents in the literature. Since each antioxidant shows its effectiveness through different and diverse mechanisms, it is not possible to standardize their effectiveness and since there are no other studies on the effect of thymoquinone on flap survival, there is no information about the effects of thymoquinone at different doses, the doses at which minimum and maximum effectiveness is observed, and its effectiveness in topical or oral application.

Ethics Committee Approval: The Istanbul University Animal Experiments Local Ethics Committee granted approval for this study (date: 02.09.2014, number: 2014/88).

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