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Original Article

The Role of Topical Thymoquinone in the Treatment of Acute Otitis Externa; an Experimental Study in Rats

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OBJECTIVE: The aim of this experimental study was to compare the dose-related effect of topical thymoquinone (TQ) with other topical agents used in the management of acute otitis externa (AOE) in a rat model.

MATERIALS and METHODS: Forty-eight male Wistar albino rats were divided into six groups each with eight rats per group. Group I was the control group with no external otitis, whereas external otitis were created in the other five groups (study groups). Dexamethasone, 0.1% TQ, 0.4% TQ, ciprofloxacin, and 0.9% saline (NaCl) drops was applied once daily in Groups II-VI, respectively. The treatment was administered regularly for 10 days. Pathologic and microbiologic evaluation were performed. Pathologically, the thicknesses of the stroma and the epithelium in the external auditory canal (EAC) were measured using an occulometer. Edema in the stroma, density of inflammatory cells and blood vessels, presence of fibroblasts, and changes in collagen fibers in the EAC were evaluated in five different areas to obtain the area of highest concentration and classified into four grades (0=no change, 1=mild, 2=moderate, 3=severe).

RESULTS: The higher concentration of TQ (0.4%) was more effective than dexamethasone and 0.1% TQ with respect to antibacterial and the anti-inflammatory properties.

CONCLUSION: TQ, particularly at a concentration of 0.4%, may be considered for topical application alone in the treatment of AOE, without any requirement for a combined treatment.

KEYWORDS: Thymoquinone, acute otitis externa, treatment, experimental study

INTRODUCTION

Acute otitis externa (AOE) is the most common infection of the external auditory canal (EAC). High temperatures, humidity, trauma, absence of cerumen, excessive sweating, alkaline pH, and the use of a hearing aid are all risk factors in the development of AOE [1]. Although AOE is primarily a local disease, it may be more severe and invasive in cases where the patients' immune system is suppressed. Edema and sensitivity in the EAC are noted in otoscopic examinations [2].

The most frequently isolated microorganism in cases of AOE is *Pseudomonas aeruginosa*, which has gram-negative properties and reproduces easily on a moist base [3]. Topical treatment is generally used in the treatment of AOE. When the infected area can be reached by topical drops, systemic antibiotics are not required [4]. Aminoglycosides, polymixin B, quinolones, and acetic acid are generally used as topical antimicrobial agents. The anti-inflammatory effects of these agents reduce edema and pain and can be used alone or combined with corticosteroids [4-6].

Thymoquinone (TQ) is an active constituent isolated from the *Nigella sativa* plant [7]. Previous studies have shown that the biological activity of *Nigella sativa*, which is used in traditional medicine, originates from the high ratio of TQ in the content [7]. Since it was first isolated, studies have shown anti-inflammatory, antioxidant, and anticarcinogenic activity [7, 8]. The aim of this experimental study was to compare the dose-related effect of TQ with other topical agents on AOE created in a rat model.

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MATERIALS and METHODS

Study Population and Animals

The approval for the study was granted by the Experimental Animal Research Ethics Committee of Mustafa Kemal University. The study was conducted in accordance with the items of the Helsinki Declaration relevant to experimental studies. All the animals were transported to the Experimental Animal Research laboratory of Mustafa Kemal University and were kept in cages with standard conditions and nutrition.

The study included a total of 48 Wistar albino rats, aged 12-16 weeks, weighing 300-400 g, with a healthy EAC, tympanic membrane, and middle ear bilaterally confirmed by an otomicroscopic examination.

External Otitis Model

To create the external otitis model, firstly, the rats were intraperitoneally administered anesthesia of 0.1 mL (90 mg/kg) ketamine hydrochloride and 0.2 mL (10 mg/kg) xylazine. Then, under microscopic guidance, both EACs were traumatized with a plastic micropipette at 80 rotations/minute for 5 minutes. Approximately 1 minute after the trauma, 0.1 mL *P. aeruginosa* (1.5×10^7 colony-forming units (CFU)/mL] drops were administered. The standard strain of *P. aeruginosa* ATCC 27853 was used [9]. The external otitis model was applied to 40 of the 48 rats in the study. After 24 hours, a microscopic view confirmed the development of external otitis in both ears of the 40 rats. Reproduction of the bacteria in the samples taken from the infected ears was also observed [10].

Smear samples were taken for ear cultures at 24 hours after inoculation and on Days 4, 7, and 10 of the treatment. The samples were inoculated in blood agar and eosin methylene blue agar. The colonies produced at the end of 18-24 hours incubation at 37°C were identified using conventional methods. Exclusion criteria for the external otitis groups of rats were defined as the death of an animal during the experiment or no visualization of findings of otitis under the otomicroscopic view.

The rats were randomly separated into 6 groups each with eight rats. A solution of TQ was prepared at concentrations of 0.1% and 0.4% in saline solution. Group I was the control group with no external otitis and no treatment applied. In Group II, 0.1 mL (1 mg/mL) dexamethasone drops were applied once daily to the ears. In Group III, 0.1 mL (1 mg/mL) 0.1% TQ drops were applied once daily. In Group IV, 0.1 mL (4 mg/mL) 0.4% TQ drops were applied once daily. In Group V, 0.1 mL (3.5 mg/mL) ciprofloxacin drops were applied once daily. In Group VI, 0.1 mL 0.9% saline (NaCl) drops were applied once daily. In the total 40 rats in Groups II-VI, where external otitis was created, the treatment was administered regularly for 10 days. No rat from any group died during the study period.

Pathologic Evaluation

Following completion of the treatment, ketamine and xylazine anesthesia was administered intraperitoneally and the rats were euthanized through cardiac exsanguinations. Both temporal bones were excised including the EAC and tympanic membrane and placed in 10% buffered formaldehyde solution. After fixation for 24 hours, the samples were decalcified for 5 days in a decalcification solution (formic acid 98%100%).

After the decalcification procedure, transverse and longitudinal sections were taken to show full layer of the EAC and placed on cassettes; after passing through different degrees of alcohol and xylene solutions, the sections were embedded in paraffin blocks. Sections of 4 µm in thickness were cut, stained with hematoxylin and eosin, and evaluated in a random order by a pathologist blinded to the groups using an Olympus BX53 microscope ((ZA 3262, U-OCMC, 24 mm diameter, 10/100X); the modified Emgard et al. [9, 10] classification was used. The thicknesses of the stroma, keratin layer, and epithelium in the EAC were measured using an occulometer. Edema in the stroma, density of inflammatory cells, density of the blood vessels, presence of fibroblasts, and changes in collagen fibers in the EAC were evaluated in five different areas to give the area of highest concentration, and the results were evaluated using four grades (0=no change, 1=mild, 2=moderate, and 3=severe).

Statistical Analysis

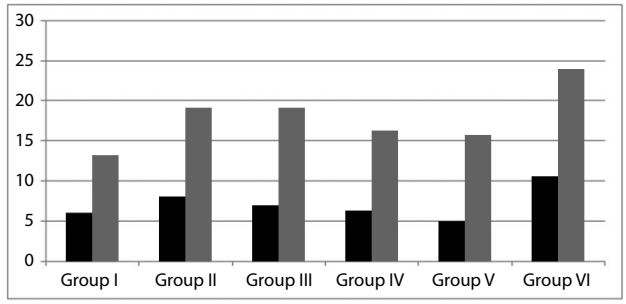
The Statistical Package for Social Sciences version 21.0 (IBM Corp.; Armonk, NY, USA) software was used for statistical analyses. The defined numerical values were compared statistically. For the multiple group comparisons of epithelial and stroma thicknesses measured in the EAC, one-way ANOVA analysis was applied, and in the paired comparisons, the post-hoc Tukey test was applied. A p value of <0.05 was accepted as statistically significant for all the statistical data.

The statistical analysis of the parameters edema, inflammatory cells, fibroblasts, blood vessels, and collagen and the results of the cultures taken on Days 1, 4, 7, and 10 of the study were analyzed using Chi-square analysis and paired comparisons were made for significant parameters.

RESULTS

The mean epithelial and stroma thicknesses of the groups are shown in Figure 1. No statistically significant difference was determined between Groups IV and V with respect to EAC epithelial and stroma thicknesses (p=0.867).

The EAC epithelial and stroma thicknesses of the rats in Groups IV and V were determined to be lower compared to Group VI with statistically significant differences (p=0.009, p=0.008, p<0.001, p=0.004, respectively, Table 1).



Group I: Control, Group II: Dexamethasone+Otitis, Group III: 0.1% Thymoquinone-Otitis, Group IV: 0.4% Thymoquinone+Otitis, Group V: Ciprofloxacin+Otitis, Group VI: Saline+Otitis

Figure 1. Mean epithelial and stroma thicknesses of the groups

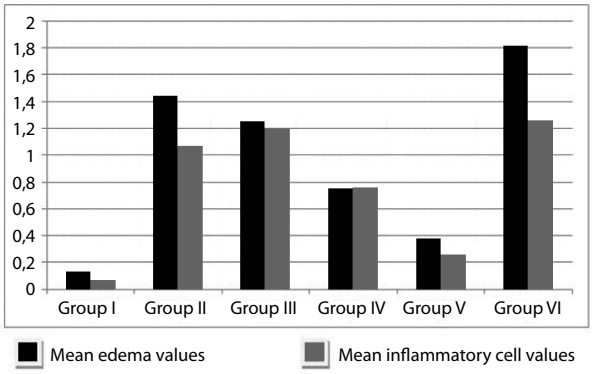


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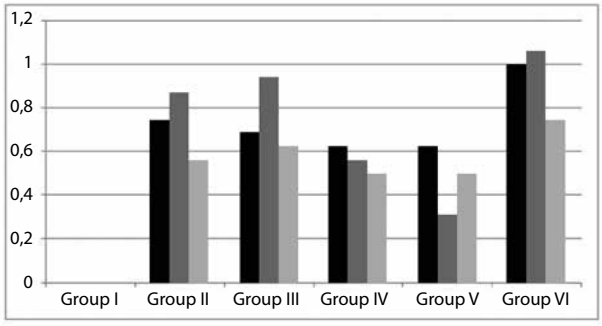
Figure 2. Mean edema and inflammatory cell values of the groups



Group I: Control, Group II: Dexamethasone+Otitis, Group III: 0.1% Thymoquinone-Otitis, Group IV: 0.4% Thymoquinone+Otitis, Group V: Ciprofloxacin+Otitis, Group VI: Saline+Otitis

Figure 3. Mean rates of fibroblast distribution, extent of blood vessels, and presence and distribution of collagen

When the groups were compared with respect to edema occurring in the EAC stroma, the rates of edema in Groups III, IV, and V were lower compared to Group VI with statistically significant differences (p=0.018, p<0.001, p<0.001, respectively, Table 1). In the comparison of the rates of inflammatory cells between the groups, no statistically significant difference was determined between Groups II, III, and IV. The rates of inflammatory cells in Groups IV and V were determined to be lower compared to Group VI with statistically significant differences (p=0.009, p<0.001, respectively, Table 1; Figure 2).

The rates of fibroblast distribution, extent of vascular infiltration, and presence and distribution of collagen in the groups were compared separately between the groups. The values of the control group were lower compared to those of all the other groups with statistically significant differences, and no significant difference was determined in the other groups with statistically significant differences (Figure 3). The spread of blood vessels was determined to be low in Group V compared to Groups II, III, and VI with statistically significant differences (p=0.017, p=0.001, p=0.007, respectively; Table 2).

In the evaluation of the culture results on Day 1 of the treatment, with the exception of the control group (Group I), reproduction was determined in all the ears in which the external otitis model was created.

Table 1. Comparison of the groups with respect to epithelial and stroma thickness, edema, and inflammatory cells

	p			
Groups	**Epithelial thickness**	**Stroma thickness**	**Edema**	**Inflammatory cells**
I-II (Control - Dexa)	p=0.571	p=0.090	p<0.001	p<0.001
I-III (Control - 0.1% TQ)	p=0.979	p=0.090	p<0.001	p<0.001
I-IV (Control - 0.4% TQ)	p=1.000	p=0.763	p=0.001	p=0.001
I-V (Control - Cip)	p=0.963	p=0.876	p=0.164	p=0.333
I-VI (Control - Saline)	p=0.003	p<0.001	p<0.001	p<0.001
II-III (Dexa - 0.1% TQ)	p=0.939	p=1.000	p=0.378	p=0.592
II-IV (Dexa - 0.4% TQ)	p=0.764	p=0.763	p=0.05	p=0.108
II-V (Dexa - Cip)	p=0.145	p=0.623	p<0.001	p=0.002
II-VI (Dexa - Saline)	p=0.267	p=0.252	p=0.158	p=0.434
III-IV (0.1% TQ + 0.4% TQ)	p=0.998	p=0.763	p=0.005	p=0.012
III-V (0.1% TQ - Cip)	p=0.638	p=0.623	p<0.001	p<0.001
III-VI (0.1% TQ - Saline)	p=0.031	p=0.252	p=0.018	p=0.749
IV-V (0.4% TQ - Cip)	p=0.867	p=1000	p=0.060	p=0.032
IV-VI (% 0.4 TQ - Saline)	p=0.009	p=0.008	p<0.001	p=0.009
V-VI (Cip - Saline)	p<0.001	p=0.004	p<0.001	p<0.001

Dexa: dexamethasone; TQ: thymoquinone; Cip: ciprofloxacin
Group I: control
Group II: dexamethasone+otitis
Group III : 0.1% TQ+otitis
Group IV: 0.4% TQ+otitis
Group V: ciprofloxacin+otitis
Group VI: saline+otitis
*Post-hoc Tukey test (p<0.05 significance)

When the results of the cultures taken on Days 4, 7, and 10 of the treatment were compared between the groups, the rate of culture positivity on Day 4 in Group V was determined to be lower than those in Groups II, III, IV, and VI with statistically significant differences. The rate of culture positivity on Day 7 in Group V was determined to be lower than those in Groups II, III, and VI with statistically significant differences (p<0.001, p<0.001, p<0.001, respectively) and similar to that of Group IV (p=0.101).

The rate of culture positivity on Day 10 in Groups IV and V was determined to be lower than those in Groups II, III, and VI with statistically significant differences. The culture positivity rates of Groups IV and Group V were similar. No statistically significant difference was determined between the culture positivity rates of Group III and Groups II and VI (p=0.273, p=0.273, respectively). The positivity rates of the culture results on Days 1, 4, 7, and 10 are detailed in Table 3.

DISCUSSION

The results of the current study showed that the thickness of the epithelial cells, stromal thickness, and the rates of inflammatory cells were lower in the Groups IV and V compared to Group VI with statistically significant differences. This indicates that a 0.4% dose of TQ has a strong anti-inflammatory effect.

Table 2. Comparison between the groups of FB, BV, and CL

	p		
Groups	**FB**	**BV**	**CL**
I-II (Control - Dexa)	p=0.001	p<0.001	p=0.006
I-III (Control - 0.1% TQ)	p<0.001	p<0.001	p=0.001
I-IV (Control - 0.4% TQ)	p<0.001	p=0.002	p=0.002
I-V (Control - Cip)	p<0.001	p=0.043	p=0.002
I-VI (Control - Saline)	p<0.001	p<0.001	p<0.001
II-III (Dexa - 0.1% TQ)	p=0.796	p=0.764	p=0.790
II-IV (Dexa - 0.4% TQ)	p=0.583	p=0.196	p=0.776
II-V (Dexa - Cip)	p=0.583	p=0.017	p=0.776
II-VI (Dexa - Saline)	p=0.317	p=0.498	p=0.417
III-IV (0.1% TQ + 0.4% TQ)	p=0.746	p=0.062	p=0.531
III-V (0.1% TQ - Cip)	p=0.746	p=0.001	p=0.531
III-VI (0.1% TQ - Saline)	p=0.159	p=0.599	p=0.551
IV-V (0.4% TQ - Cip)	p=1.000	p=0.210	p=1.000
IV-VI (0.4% TQ - Saline)	p=0.073	p=0.070	p=0.201
V-VI (Cip - Saline)	p=0.0733	p=0.007	p=0.201

Dexa: dexamethasone; TQ: thymoquinone; Cip: ciprofloxacin; FB: fibroblast distribution; BV: blood vessels; CL: collagen presence and distribution

Group I: control,

Group II: dexamethasone+otitis

Group III: 0.1% TQ+otitis

Group IV: 0.4% TQ+otitis,

Group V: ciprofloxacin+otitis

Group VI: saline+otitis

*Chi-square test (p<0.05 significance)

Table 3. Rates of positivity of the cultures taken on Days 1, 4, 7, and 10 of the treatment

	Day 1	Day 4	Day 7	Day 10
Group I	0/16	0/16	0/16	0/16
Group II	16/16	16/16	13/16	12/16
Group III	16/16	16/16	14/16	8/16
Group IV	16/16	16/16	[D] 4/16	[E], C 0/16
Group V	16/16	[A] 3/16	[D, B] 0/16	[E], C 0/16
Group VI	16/16	16/16	13/16	12/16

A: GV vs. GII, GIII, GIV, and GVI (p<0.001)

B: GV vs. GII, GIII, and GVI (p<0.001)

C: GIV, GV vs. GII, GIII, GVI (p<0.001)

D: GV vs. GIV (p=0.101)

E: GV vs. GIV (p=1.000)

In a rat model study by Emgard et al. [9], 100% efficacy of budesonide was determined on Days 10 and 20 of treatment with respect to bacteria eradication. Budesonide alone was stated to have cured the experimental AOE more effectively than a weak steroid combined with an antibiotic hydrocortisone acetate combined with oxytetracycline and polymixin B (HCPB), and this was attributed to the fact that inflammation is a major mechanism in the development of AOE, irrespective of the presence of either bacteria or fungi. However, it was emphasized that the inflammatory reaction of the EAC skin in this animal model of AOE cannot be directly extrapolated to the human situation.

In the current study, bacterial eradication of the topical steroid was determined as 18.75% and 25% on Days 7 and 10 of the treatment, respectively. Similar rates of bacteria eradication were determined in the group administered with saline. In our study, the bacteria eradication rates of the group administered with topical steroid were lower than those reported in the Emgard et al. [9] study with the use of budesonide, which may be explained by topical dexamethasone not exhibiting as potent anti-inflammatory property as budesonide and that the content did not have isopropanol with which the environment developed an acidic pH. In the current study, the similar rates of culture positivity obtained in Groups II and VI can be attributed to the steroid showing a similar effect to that of saline.

In the current study, as the culture positivity of Group V was lower than that of Groups II, III, IV, and VI on Day 4 of treatment and lower than that of Groups II, III, and VI on Days 7 and 10 of treatment both with statistical significance and as a similarity was determined with Group IV in terms of bacterial eradication on Days 7 and 10 in particular, this indicates that a dosage of 0.4% TQ has an antibacterial and anti-inflammatory effect as strong as that of ciprofloxacin. The stronger antibacterial and anti-inflammatory properties of the 0.4% dose of TQ than that of dexamethasone and 0.1% TQ suggest that the antibacterial and anti-inflammatory effect of TQ is dose-dependent.

In a study by Pistorius et al. [11] of 239 patients diagnosed with acute external otitis and treated with 0.2% ciprofloxacin for 7 days, the bacteria eradication rate was 92%. In the same study, 236 patients with AOE were treated with 0.2% ciprofloxacin+0.1% hydrocortisone for 7 days, and the bacteria eradication rate was 95%. Drehobl et al. [12] treated 319 external otitis patients with 0.2% ciprofloxacin for 7 days and 87.5% bacterial eradication was determined for *P. aeruginosa*. In the evaluation of previous studies, ciprofloxacin topical solution has been shown to have high eradication rates, such as 83.3%-95.7%, against *P. aeruginosa* [13]. In the ciprofloxacin-treated group in the current study, reproduction was determined in all the smear samples taken on Day 1 of the treatment, and the bacteria eradication rates were found to be 81.25%, 100%, and 100% on Days 4, 7, and 10 respectively. The bacteria eradication rates obtained in this study with the treatment of topical ciprofloxacin for a period of 10 days were consistent with those reported in literature.

Various studies have shown that TQ has anti-inflammatory, antibacterial, antiviral, antiallergic, antioxidant, analgesic, immunomodulator, and anticancer activity [7, 8, 14-18]. In the current study of the external otitis model, that inflammation was lower in the TQ group than that in the saline group with statistically significant difference, and although not significant, the epithelial and stroma thicknesses were lower in the TQ group than those in the dexamethasone group, suggest that TQ could be a promising molecule in the treatment of AOE.

In the current study, that the bacteria eradication rates of the 0.4% TQ on Days 7 and 10 were higher than those of the saline, dexamethasone, and 0.1% TQ groups with statistically significant difference shows that the anti-inflammatory and antibacterial properties were more effective than other topical treatments. The antibacterial effica-

cy of 0.4% TQ is also supported by the finding of antibacterial properties similar to those of ciprofloxacin from the seventh day onwards.

CONCLUSION

In conclusion, the results of the current study showed that in terms of bacteria eradication and the anti-inflammatory property, 0.4% TQ was more effective than dexamethasone and 0.1% TQ, thereby indicating that the anti-inflammatory and antibacterial effects of TQ are dose-dependent. In addition to the antibacterial and anti-inflammatory effects of TQ, as various studies have determined analgesic and antihistaminic properties, it is thought that TQ, particularly at a concentration of 0.4%, could be used topically alone in the treatment of AOE, without any requirement of a combined treatment [17]. However, there is a need for further clinical studies to confirm these findings.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Mustafa Kemal University (Approval No: 2014-11/4).

Informed Consent: Informed consent was obtained from the patient who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - H.D., C.A.; Design - C.A., T.Ö.; Supervision -R.D., M.İ., C.A.; Resource - R.D., C.A.; Materials - H.D., C.A.; Data Collection and/or Processing - H.D., C.A., T.Ö.; Analysis and/or Interpretation - R.D., M.İ., T.Ö, C.A.; Literature Search - H.D., C.A.; Writing - C.A., R.D.; Critical Reviews - R.D., C.A., M.İ.

Conflict of Interest: No conflict of interest was declared by the authors.

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