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ARAŞTIRMA / RESEARCH

Efficacy of boric acid therapy in a *pseudomonas aeruginosa*-induced chronic otitis media model in rats

Sıçanlarda *pseudomonas aeruginosa* ile indüklenen kronik otitis media modelinde borik asit tedavisinin etkinliği

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Abstract

Purpose: Chronic otitis media (COM) is one of the common infectious diseases of the middle ear caused by bacterial or viral pathogens. The purpose of the present study was to investigate the efficacy of boric acid (BA) in the treatment of COM by comparing topical 4% and 8% BA applications with systemic ciprofloxacin in a *Pseudomonas aeruginosa* (*P. aeruginosa*) inoculation-induced model of COM in rats.

Materials and Methods: Forty-two Sprague Dawley rats were divided into seven equal groups. The COM model was created with *P. aeruginosa*. Clinical, histopathological and, immunohistochemical comparisons were performed between the control, COM, topical 4% and 8% BA treatment, a systemic ciprofloxacin treatment, and topical 4% and 8% BA contact groups.

Results: In the COM model, moderate edema, inflammation, degeneration and moderate tumor necrosis factor-alpha (TNF-α) expression were detected with the application of 4% BA. Mild edema, inflammation, degeneration, and mild TNF-α expression were detected with 8% BA application.

Conclusion: Both 4% and 8% BA treatment provided significant clinical, histopathological and immunohistochemical improvement. The application of 8% BA was found to exhibit greater therapeutic efficacy, with no harmful effect on the middle ear mucosa.

Keywords:. Boric acid, chronic otitis media, *pseudomonas aeruginosa*, rat, tumor necrosis factor-alpha

Öz

Amaç: Kronik otitis media (KOM), bakteriyel veya viral patojenlerin neden olduğu orta kulağın sık görülen enfeksiyöz hastalıklarından biridir. Bu çalışmanın amacı, sıçanlarda *Pseudomonas aeruginosa* (*P. aeruginosa*) ile indüklenen KOM modelinde topikal %4'lük ve %8'lik borik asit (BA) uygulamasını sistemik siprofloksasin ile karşılaştırarak KOM tedavisinde borik asidin etkinliğini araştırmaktır.

Gereç ve Yöntem: 42 Sprague Dawley cinsi sıçan 7 eşit gruba ayrıldı. *P. aeruginosa* ile KOM modeli oluşturuldu. Kontrol grubu, KOM grubu, topikal %4'lük ve %8'lik BA tedavi grubu, sistemik siprofloksasin tedavi grubu ve topikal %4'lük ve %8'lik BA temas grubu arasında klinik, histopatolojik ve immünohistokimyasal karşılaştırmalar yapıldı.

Bulgular: KOM modelinde %4'lük BA uygulaması ile orta derecede ödem, enflamasyon, dejenerasyon ve orta derecede tümör nekroz faktör-alfa (TNF-α) ekspresyonu tespit edildi. %8'lik BA uygulaması ile hafif ödem, inflamasyon, dejenerasyon ve hafif TNF-α ekspresyonu tespit edildi.

Sonuç: %4'lük ve %8'lik BA tedavisinin önemli klinik, histopatolojik ve immünohistokimyasal iyileşme sağladığı görüldü. %8'lik BA uygulamasının daha yüksek tedavi etkinliğine sahip olduğu ve orta kulak mukozasına zararlı bir etkisinin olmadığı gösterildi.

Anahtar kelimeler: Borik asit, kronik otitis media, *pseudomonas aeruginosa*, sıçan, tümör nekroz faktörü-alfa

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INTRODUCTION

Otitis media (OM), one of the most common infectious diseases, particularly in developing countries, is caused by bacterial or viral pathogens in the middle ear[1,2]. The prevalence in different regions and ethnic groups can range between 0.4% and 46%, and it can be seen in all age groups[3,4]. Chronic OM (COM) is characterized by a perforated tympanic membrane, a persistent discharge from the middle ear, and hearing loss[1,5]. Bacteria can be isolated from the purulent discharge in at least 70% of cases of COM[4]. These patients frequently seek medical assistance and use antibiotics[5]. Significant intra- and extracranial complications such as hearing loss, meningitis, brain abscess, and facial nerve paralysis may develop in patients treated inappropriately or left untreated[5,6].

COM generally derives from bacterial infections[7]. In refractory COM, otorrhea is thought to derive from a biofilm layer caused by bacteria[8]. *Pseudomonas aeruginosa* (*P. aeruginosa*) is reported to be the most widely isolated pathogen in COM[9]. *P. aeruginosa* adheres to the damaged epithelium caused by COM in the middle ear mucosa, and can produce a biofilm[5]. Local antibiotics and topical agents are employed to treat the condition. One of the most frequently used antibiotics in that context is ciprofloxacin[10]. Systemic antibiotics are also used in COM in case of unsuccessful treatment or intracranial compression[7]. Ciprofloxacin is the most effective and important of the systemic antibiotics against *P. aeruginosa* infections[9,11].

Pathogen resistance to antibiotics is an important problem in the treatment of COM infections[12]. Antiseptic and acidic topical agents are also employed in treatment, in addition to antibiotics[13]. Due to its mildly acidic nature, the outer ear canal is bacteriostatic in character. Lowering pH in COM may be beneficial to treatment by enhancing this bacteriostatic character[3]. The advantages of topical applications in otology include high concentrations being achieved in the area of application, the fact that no side-effects are caused, and lower bacterial resistance. However, topical agents can also give rise to vestibular complications by passing from the middle ear to the inner ear through the round window[13].

Boric acid (BA) is a weak acid with antiseptic properties used as an insecticide, preservative, lubricant, and industrial agent. It can be used in the treatment of small cuts and burns, aphthous lesions, in cases with discharge such as gonorrhea vaginitis, and cystitis, and to treat middle ear infections. It is frequently employed in the form of a 4% solution prepared with 70% alcohol or distilled water[13].

To the best of our knowledge, no previous studies have used an 8% BA solution and examined its effectiveness against *P. aeruginosa* causing refractory COM infection. As a result, this research hypothesizes that in the treatment of COM, 8% BA application may be more effective than 4% BA application without creating additional side effects.

The purpose of the present study was to investigate the efficacy of BA in treatment of COM by comparing topical 4% and 8% BA applications with systemic ciprofloxacin in a *P. aeruginosa* inoculation-induced model of COM in rats.

MATERIALS AND METHODS

Animals

Approval for the study was granted by the Ataturk University Animal Experiments Local Ethical Committee (date: 27.12.2018, decision no: 237). The research was conducted in conformity with the principles concerning laboratory animals set out in the Declaration of Helsinki[14]. The study was performed in the Ataturk University Medical Experimental Application and Research Center (ATADEM) Erzurum, Turkey. Forty-two healthy female Sprague Dawley rats weighing a mean 220-250 g were included in the study. All animals were treated in accordance with the applicable animal protection laws and experimental ethical principles. Throughout the study, the rats were housed in transparent plastic cages in a well-ventilated environment in a 12-h light: dark cycle, at a mean temperature of 23º C and mean humidity of 42%. The rats were fed with standard chow, and no weight loss or death were observed during the study. The animals were divided into seven groups of six rats each. All rats' otoscopic examinations were evaluated as normal.

Experimental protocol and drug administration

Following clinical evaluations, general anesthesia was administered with intraperitoneal (i.p.) administration of ketamine hydrochloride (Ketalar vial, Pfizer, Istanbul, Turkey) 40 mg/kg + xylazine hydrochloride (Rompun vial, Bayer, Istanbul) 10 mg/kg. Procedures

were performed under sterile conditions by the same surgeon. Rats were randomly assigned into seven groups of six animals each. Since alcohol has been shown to exhibit ototoxic effects in perforated ears in some studies, BA solutions prepared with distilled water were employed in the present research[15,16]. The tympanic membranes of 24 rats not belonging to the control or BA contact groups were perforated 50% bilaterally. Using a dental injector, 0.1 ml $1x10^8$ colony *P. aeruginosa* strains (ATCC-27853) were then inoculated into the middle ear via the perforation. The same procedure was repeated one week subsequently. The rats were then observed without treatment for the next three weeks. COM and purulent discharge from the outer ear canal were subsequently observed at otomicroscopy examinations. *P. aeruginosa* was isolated in cultures.

The study design was as follows:

Group 1, the control group: (n=6; 12 temporal bones): these six rats were defined as the control group.

Group 2, the COM group: (COM, n=6; 12 temporal bones): the tympanic membranes of these six rats were perforated by 50%, after which COM was induced through injection of *P. aeruginosa*.

Group 3, the COM and 4% BA treatment group: (COM-%4 BA, n=6; 12 temporal bones): six rats were treated with topical BA following COM induction; 1 ml 4% BA was applied topically to the middle ear through the outer ear canal for 14 days.

Group 4, the COM and 8% BA treatment group: (COM- %8 BA, n=6; 12 temporal bones): six rats were treated with topical BA following COM induction; 1 ml 8% BA was applied topically to the middle ear through the outer ear canal for 14 days.

Group 5, the COM and ciprofloxacin treatment group: (COM-ciprofloxacin, n=6; 12 temporal bones): six rats were treated with systemic ciprofloxacin following COM induction; 20 mg/kg i.p. ciprofloxacin was administered for 14 days.

Group 6, the group receiving 4% BA administration to the healthy middle ear: (Healthy middle ear-4% BA, n=6; 12 temporal bones): The tympanic membranes of the six rats in this group were perforated by 50%, after which 4% topical BA was applied to the middle ear. One milliliter of 4% BA was applied topically via the outer ear canal for 14 days.

Group 7, the group receiving 8% BA administration to the healthy middle ear: (Healthy middle ear-8% BA, n=6; 12 temporal bones): The tympanic membranes of the six rats in this group were perforated by 50%, after which 8% topical BA was applied to the middle ear. One milliliter of 8% BA was applied topically via the outer ear canal for 14 days.

At the end of the study the rats were euthanized under general anesthesia with thiopental sodium (80 mg/kg Pentothal; Abbott, Campoverde di Aprilia, Italy). The temporal bones were then removed, and the middle ear mucosa were dissected. The specimens were set aside for histopathological and immunohistochemical examination. Histopathological and immunohistochemical examination were performed by the same pathologist who was unaware of the rat groups.

Histopathological examination

Ear tissue specimens collected at necropsy for histopathological evaluation were fixed in 10% formalin solution for 48 h. The tissues were then decalcified. Following routine bone tissue procedures, the specimens were embedded in paraffin blocks, sections 4 μm in thickness being taken from each block. The preparates made ready for histopathological analysis were stained with hematoxylin-eosin (H&E) and examined under a light microscope (Olympus BX-51; Olympus, Tokyo, Japan). Histopathological findings in the studied sections were graded on the basis of their severity - none (-), mild (+), moderate (++), or severe (+++).

Immunohistochemical examination

All sections were placed onto slides containing adhesive (poly-L-Lysine) for immunoperoxidase were passed through xylol and alcohol series. After washing with PBS, the sections were kept for 10 min in 3% H_2O_2 for endogenous peroxidase inactivation. Following treatment with antigen retrieval solution in a microwave at 500 Watts for 2x5 min in order to reveal antigens in the tissues, the specimens were left to cool. The tissues were then incubated with tumor necrosis factor alpha (TNF-α) (Catalog no. sc-52746, diluent ratio: 1/100 Santa Cruz, EU) for 30 min at 37° C in order to detect inflammation. The tissues were treated in accordance with the immunohistochemistry kit procedure (Abcam HRP/DAB Detection IHC kit). 3-3' Diaminobenzidine (DAB) was used as the

chromogen. Background staining was performed using H&E. The sections were then graded depending on their immune positivity – none (-), mild (+), moderate (++), or severe (+++).

Statistical analysis

Statistical analyses were performed on Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM Corporation, New York, NY, USA). The non-parametric Kruskal-Wallis test was used to analyze differences in data obtained semi-quantitatively at histopathological examination between the groups, and the Mann Whitney U test was used for two-group comparisons. p values <0.05 were regarded as significant for all tests.

RESULTS

Clinical findings

The ears of all rats were examined after the study. The clinical findings were as follows:

Group 1 (Control): The external auditory canals were dry and the tympanic membranes were intact in the 12 ears of the six rats in this group.

Group 2 (COM): Purulent discharge was present together with tympanic membrane perforation in the 12 ears of the six rats in this group.

Group 3 (COM+4% BA): The tympanic membranes of the 12 ears were perforated. The external auditory canals were dry, and wetness was present in the middle ear cavity in eight ears, while purulent discharge was also present in the external auditory canal in four.

Group 4 (COM+8% BA): Tympanic membranes of the 12 ears were perforated. The external auditory canal was dry in 11 ears, with wetness being detected only in the middle ear cavity. Discharge reaching the external auditory canal was present in one ear.

Group 5 (COM+ ciprofloxacin): The tympanic membranes were perforated in all 12 ears. The external auditory canals were dry in seven ears, with wetness in the middle ear cavity. Purulent discharge also was also observed in the external auditory canal in five ears.

Group 6 (Healthy+4% BA): All tympanic membranes were perforated. Wetness was observed only in the middle ear cavities of all rats.

Group 7 (Healthy+8% BA): The tympanic membranes of all 12 ears were perforated. Wetness was present only in the middle ear cavities of all rats.

Histopathological findings

The histopathological findings are summarized in Table 1 and Figure 1, and the immunohistochemical findings in Table 1 and Figure 2.

Table 1. Scoring of histopathological and immunohistochemical findings in middle ear tissues

	Thickening of the tympanic membrane	Inflammation and edema in the submucosa	Degeneration and desquamation of the mucosa	Inflammation in the tympanic cavity	TNF-α expression
Group 1	-	-	-	-	-
Group 2	+++	+++	+++	+++	+++
Group 3	++	++	++	++	++
Group 4	+	+	+	+	+
Group 5	+	++	+	+	+
Group 6	-	-	-	-	-
Group 7	-	-	-	-	-

Group 1 (Control): Examination of the ear tissue specimens from the rats in this group revealed a normal histological appearance (Figure 1-A).

Group 2 (COM): Ear tissue specimens from this group exhibited revealed severe exudate in the tympanic cavity, debris consisting of exfoliated epithelium, neutrophils and leukocytes, and thickening caused by severe edema in the tympanic membrane (Figure 1-B). In addition, severe edema in the middle ear submucosa, inflammation, hyperemia in vessels, and desquamation and degeneration in the mucosal epithelia were also observed (Figure 1-C).

Group 3 (COM + 4% BA): Histopathological examination of the middle ear tissue specimens from

this group revealed thickening due to moderate edema and inflammation in the tympanic membrane, moderate inflammation and edema in the middle ear submucosa, and moderate degeneration in the mucosal epithelia (Figure 1-D). A statistically significant difference was determined compared to the COM group ($p<0.05$).

Group 4 (COM + 8% BA): The middle ear tissue specimens from this group exhibited mild thickening in the tympanic membrane, very mild inflammation in the submucosa, and mild degeneration in the mucosal epithelia (Figure 1-E). A statistically significant difference was determined compared to both the COM group and the 4% BA group ($p<0.05$).

Group 5 (COM + ciprofloxacin): Histopathological examination of the middle ear tissue specimens from this group revealed mild thickening in the tympanic membrane, mild degeneration in the middle ear mucosa, and mild edema and inflammation in the submucosa (Figure 1-F). A statistically significant difference was determined compared to the COM group ($p<0.05$).

Group 6 (Healthy ear + 4% BA): a normal histological appearance was observed in this group (Figure 1-G).

Group 7 (Healthy ear + 8% BA): Histopathological examination of the middle ear tissue specimens from this group also revealed a normal histological appearance (Figure 1-H).

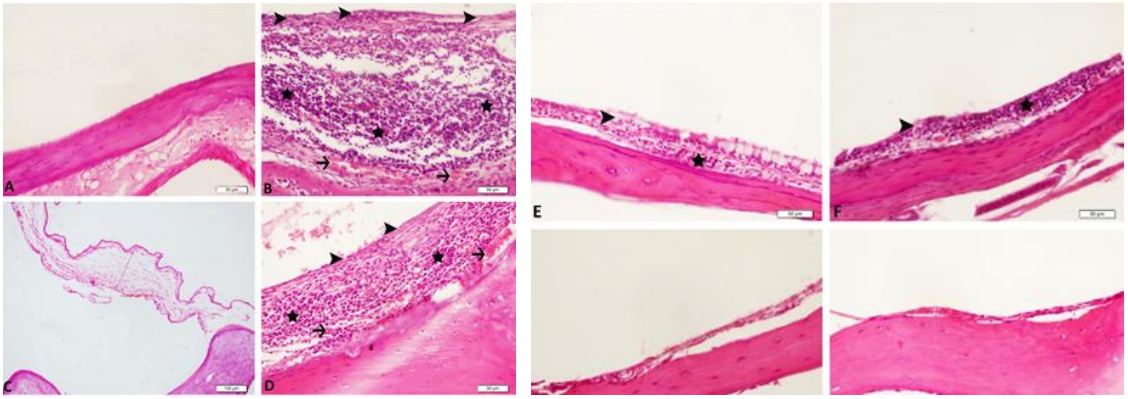

Figure 1. Middle ear, A: Control Group, normal histological appearance, B:Inflammation Group, severe degeneration at mucosal epithelium (arrow heads), severe inflammation at submucosa (asterisks), hyperemia (arrows), C: severe thickening of tympanic membrane, D:Inflammation+ 4% BA Group, moderate inflammation at submucosa (asterisks), moderate degeneration at epithelium of mucosa (arrow heads), hyperemia (arrows), E: Inflammation + 8% BA Group, mild inflammation at submucosa (asterisk), mild degeneration at epithelium of mucosa (arrow heads), F: Inflammation + Cipro Group, mild degeneration at epithelium of mucosa (arrow heads), mild inflammation at submucosa (asterisks), G: Healthy + 4% BA Group, normal histological appearance, H: Healthy + 8% BA Group, normal histological appearance, H&E, Bar: 50 µm.

Immunohistochemical findings

Group 1 (Control): Immunohistochemical examination of the middle ear tissue specimens from this group revealed negative TNF-α expression (Figure 2-A).

Group 2 (COM): Immunohistochemical examination of the middle ear tissue specimens from this group revealed severe TNF-α expression in inflammatory cells in the submucosa, mucosal epithelia, and debris (Figure 2-B).

Group 3 (COM + 4% BA): The middle ear tissue specimens from this group exhibited moderate TNF-α expression in neutrophils and leukocytes in the submucosa and mucosal epithelia (Figure 2-C). A statistically significant difference was determined compared to the COM group ($p<0.05$).

Group 4 (COM + 8% BA): Immunohistochemical examination of the middle ear tissue specimens from this group revealed mild TNF-α expression in neutrophils and leukocytes in the submucosa and mucosal epithelia (Figure 2-D). A statistically

significant difference was determined compared to both the COM group and the 4% BA group ($p<0.05$).

Group 5 (COM + ciprofloxacin): Immunohistochemical examination of the middle ear tissue specimens from this group revealed mild TNF-α expression in neutrophils and leukocytes in the submucosa and mucosal epithelia (Figure 2-E). A statistically significant difference was determined compared to the COM group ($p<0.05$).

Group 6 (Healthy ear + 4% BA): The middle ear tissue specimens from this group exhibited negative TNF-α expression (Figure 2-F).

Group 7 (Healthy ear + 8% BA): TNF-α expression was also evaluated as negative in this group (Figure 2-G).

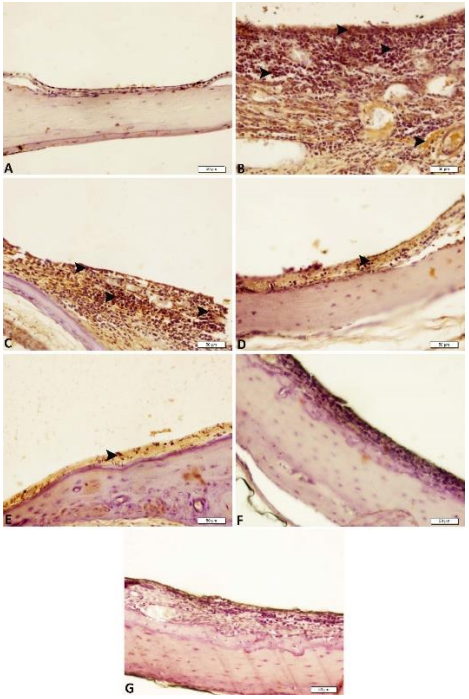

Figure 2. Middle ear, A: Control Group, Negative TNF-α expression, B: Inflammation Group, severe TNF-α expression at epithelium of mucosa and inflammatory cells at submucosa (arrow heads), C: Inflammation + 4% BA Group, moderate TNF-α expression at epithelium of mucosa and inflammatory cells at submucosa (arrow heads), D: Inflammation + 8% BA Group, mild TNF-α expression at inflammatory cells (arrow heads), E: Inflammation + Cipro Group, mild TNF-α expression at inflammatory cells (arrow heads), F: Healthy + 4% BA Group, Negative TNF-α expression, G: Healthy + 8% BA Group, Negative TNF-α expression, H&E, Bar: 50 µm.

DISCUSSION

To the best of our knowledge, no previous studies have employed an 8% BA solution in an experimental COM model. This study investigated the effectiveness of topical 4% and 8% solutions of the antiseptic agent BA against *P. aeruginosa* in an experimental COM model. The fact that bacteria such as *P. aeruginosa* exhibit different resistance patterns in COM, an important health problem, may result in failure of treatment.[5] Studies investigating the treatment of COM have examined the therapeutic options and have reported that topical agents are more effective than systemic ones[17,18]. Topical antiseptics can be as efficacious as topical antibiotics in the treatment of COM. In addition, topical antibiotics entail various disadvantages, such as high cost, requiring patient compliance, and the development of resistance. BA is a less costly alternative to topical antibiotics and exhibits bactericidal and bacteriostatic effects against *P. aeruginosa*[1].

BA solution has been shown to be safe and effective in the treatment of suppurative OM in children[18]. BA prevents the formation of the biofilm produced by bacteria that makes COM more refractory to antimicrobial agents[1]. A study evaluating the bactericidal effect of BA reported no bactericidal effect at concentrations of 0.5%, 0.75%, or 1%[19]. Another study showed that the use of 3% BA in patients with COM inhibited the growth of *P. aeruginosa*[20]. Research in which BA was employed at concentrations of 2%, 4%, and 6% showed that its antibacterial effectiveness increased in line with the degree of concentration[21]. The BA concentration has therefore been linked to its antibacterial efficacy[1]. Similarly in the present study, 8% BA emerged as more effective then 4% BA at clinical, histopathological and immunohistochemical examinations. It may thus be concluded that the therapeutic effectiveness of BA increases in line with the concentration.

Previous studies have also investigated BA ototoxicity[15]. A guinea pig study reported that BA solution prepared with 70% alcohol exhibited ototoxic effects on auditory brainstem responses (ABRs)[22]. Another study involving guinea pigs also showed that BA solutions prepared with 40% and 60% alcohol exhibited ototoxic effects on ABRs[23]. In addition, a different study showed that the application of topical BA to the rat middle ear is safe for the inner ear[15]. A study involving two groups, one with BA powder application and one without, determined no statistically significant difference between the two groups in terms of distortion product otoacoustic emission (DPOAE)[13]. A study of children with suppurative OM reported that BA prepared with 70% alcohol was effective in treatment and did not impact on hearing thresholds[18]. No adverse effect of 4% or 8% BA solutions applied to healthy ears were observed at histopathological and immune histochemical examinations in the present study. It may therefore be concluded that BA solution can be safely used in patients with OM.

Due to their broad antimicrobial efficacy and ototoxic effects, quinolones began being employed in the treatment of middle ear infections. However, no consensus was achieved regarding whether topical or systemic therapies were more effective in COM[9]. One study described topical antibiotic as the first-line treatment and that due to its side-effect profile and antibiotic resistance, intravenous antibiotherapy was disadvantageous[24]. Another study reported that 88% of 25 *P. aeruginosa* strains were resistant to ciprofloxacin[5]. In other research, ciprofloxacin was applied in oral or oral + topical form. Orally applied systemic ciprofloxacin was reported to make no positive contribution to treatment, and in contrast led to systemic side-effects[9]. Other research recommended that floroquinolones should not be used as first-line treatment in COM[7]. Additional therapeutic alternatives are therefore needed, in addition to ciprofloxacin, described as the most important and effective agent in *P. aeruginosa* infections[11]. Both 4% and 8% BA were found to be effective against *P. aeruginosa* in the present study. In addition, 8% BA also exhibited similar efficacy against *P. aeruginosa* to that of ciprofloxacin. The equivalent efficacy revealed in the study suggested that topical BA application may be more advantageous then systemic ciprofloxacin therapy in terms of preventing the development of resistance.

Levels of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) have been reported to increase in both the acute and chronic periods of OM[25]. TNF-α has also been reported to increase microvascular permeability and to cause neutrophil infiltration and subepithelial edema[26]. TNF-α expression was similar in the COM groups in the present study. The decrease in TNF-α expression observed at immunohistochemical examination in our treatment groups shows that both BA and systemic ciprofloxacin suppress inflammation in COM.

The principal limitation of this study is that potential ototoxicity status in rats was not examined using tests such as ABR and DPOAE. Another limitation is the absence of a topical ciprofloxacin group.

The effectiveness of BA at high concentrations in COM has been shown in previous studies, although to the best of our knowledge no previous research has employed 8% BA. The results of this study showed the antimicrobial efficacy of 4% and 8% BA against *P. aeruginosa,* reported as the most widespread pathogen in experimentally-induced COM in rats. Eight percent BA was found to exhibit similar effects to those of systemic ciprofloxacin at clinical, histopathological and immunohistochemical examination. We therefore think that BA can be used instead of systemic ciprofloxacin, with its high side-effect and resistance profile, in COM resulting from *P. aeruginosa.* Whether or not 8% BA causes ototoxicity might usefully be investigated in future studies.

Yazar Katkıları: Çalışma konsepti/Tasarımı: AS, AK, KK, MSS, SK, SY; Veri toplama: AS, AK, KK, MSS, SK, SY; Veri analizi ve yorumlama: AS, AK, KK, MSS, SK, SY; Yazı taslağı: AS, AK, MSS; İçeriğin eleştirel incelenmesi: AS, AK, MSS, SK, SY; Son onay ve sorumluluk: AS, AK, KK, MSS, SK, SY; Teknik ve malzeme desteği: AS, AK, KK, MSS, SK, SY; Süpervizyon: AS, AK, KK, MSS, SK, SY; Fon sağlama (mevcut ise): yok.
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