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ORIGINAL ARTICLE

Medicine Science 2021;10(2):577-82

Medicine Science International Medical Journal

Protective effect of nimesulide on the external ear damage induced by staphylococcus aureus inoculation in rats

Ertugrul Erhan[1], Ismail Salcan[2], Muhammet Dilber[3], Sumeyye Akyuz[4], Bulent Dabanlioglu[5] Bahadir Suleman[6], Taha Abdulkadir Coban[7], Halis Suleyman[6], Renad Mammadov[6]

[1]Erzincan Binali Yildirim University, Faculty of Medicine, Department of Otorhinolaryngology, Erzincan, Turkey
[2]Mengucek Gazi Training and Research Hospital, Department of Otorhinolaryngology, Erzincan, Turkey
[3]ENT Special Doctor, Istanbul, Turkey
[4]Mengucek Gazi Training and Research Hospital, Department of Microbiology, Erzincan, Turkey
[5]Erzincan Binali Yildirim University, Faculty of Medicine, Department of Microbiology, Erzincan, Turkey
[6]Erzincan Binali Yildirim University, Faculty of Medicine, Department of Pharmacology, Erzincan, Turkey
[7]Erzincan Binali Yildirim University, Faculty of Medicine, Department of Biochemistry, Erzincan, Turkey

Received 23 February 2021; Accepted 02 May 2021
Available online 11.05.2021 with doi: 10.5455/medscience.2021.02.050

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Abstract

Since the external ear is covered with skin, infections that cause otitis externa are often produced by distinct skin flora in several different areas. Staphylococcus aureus (S. aureus) reproduces the most out of all bacteria isolated from external auditory canal skin culture samples. Proinflammatory cytokines are the main components responsible for tissue damage pathogenesis due to S. aureus. Nimesulide is a nonsteroidal anti-inflammatory drug that selectively inhibits cyclooxygenase-2 and also demonstrates antioxidant properties. The present study aimed to examine the antibacterial activity of nimesulide against S. aureus and to compare its effectiveness on otitis externa induced by S. aureus in male albino Wistar rats with that of cefazolin. The antimicrobial activity testing was conducted using the Kirby-Bauer disk diffusion method as described by the European Committee on Antimicrobial Susceptibility Testing. To induce otitis externa, we applied 0.5 ml of S. aureus to the ear skin using a hypodermic syringe (S. aureus strain TACK 25923 was dispersed on the 900x10-6 concentration of colony forming units per ml). Nimesulide and cefazolin were administered orally at a dose of 50 mg/kg. The antibacterial activity of cefazolin when used in equal doses (50 µg/ml) was more powerful against S. aureus than nimesulide. However, nimesulide reduced the macroscopic findings (such as oedema and redness) induced by S. aureus better than cefazolin. Additionally, nimesulide inhibited the increase of oxidant and proinflammatory indicators related to S. aureus, while cefazolin was able to inhibit only the increase in proinflammatory indicators. Our results showed that nimesulide was superior to cefazolin in terms of reducing the external ear damage caused by S. aureus. Therefore, nimesulide monotherapy or a combination of nimesulide and cefazolin therapy may be beneficial in the treatment of bacterial otitis externa.

Keywords: Nimesulide, cefazolin, staphylococcus aureus, ear damage, rat

Introduction

The external ear is a structure covered with skin. Therefore, infections responsible for otitis externa are caused by the skin flora of different areas [1]. Otitis externa is the inflammation and infection of the ear canal and the external ear and may be due to various causes [2]. Malcok HK et al. reported that Staphylococcus aureus (S. aureus) and Pseudomonas aeruginosa (P. aeruginosa) were the most common among 800 bacteria isolated from external auditory canal skin culture samples [3]. Additionally, S. aureus is the most commonly detected pathogen in acute localized otitis externa [1]. Otitis externa may occur due to various traumas or the use of nonsterile equipment [3]. The disease has different clinical forms that range from mild and local involvement to life-threatening necrotizing otitis externa [4]. Common symptoms are pain, itching, discharge, and partial hearing loss. Although rare, some serious complications may develop [5]. Therefore, it is essential to be aware of the etiopathogenesis of otitis externa to identify and successfully implement the most suitable treatment.

Cyclooxygenase (COX-2) NF-ƙB and other proinflammatory cytokines are mainly responsible for the tissue damage pathogenesis induced by S. aureus [6]. S. aureus causes inflammatory skin disorders by inducing tumour necrosis factor-alpha (TNF-α),

***Corresponding Author:** Halis Suleyman, Erzincan Binali Yildirim University, Faculty of Medicine, Department of Pharmacology, Erzincan, Turkey,
E-mail: halis.suleyman@gmail.com

another inflammatory cytokine [7]. However, reactive oxygen species (ROS), which cause oxidative damage, do not play important roles in the tissue damage pathogenesis produced by S. aureus [6]. Nevertheless, the relationship between oxidative stress related to ROS and inflammation should not be ignored [8]. An increase in proinflammatory cytokines stimulates inflammation and ROS-induced oxidative stress [9]. Additionally, activated COX-2 enables the release of -proinflammatory prostaglandins and ROS from arachidonic acids [10].

Nimesulide, whose protective effect against damage (otitis externa) induced by S. aureus inoculation of the external ear skin was examined, is a selective COX-2 inhibitor as well as an antipyretic, analgesic and anti-inflammatory non-steroidal drug (NSAID) [11]. Structural COX-2 enzyme inhibition cases the therapeutic effect of the NSAIDs, while COX-1 induces their associated side effects [12]. COX-2 is produced in reaction to agents (cytokines and growth factors, etc.) that cause inflammation [13]. According to the literature, nimesulide inhibits the reproduction of proinflammatory cytokines, such as nuclear factor kappa B (NF-kB) and TNF-α, and also has antioxidant properties [14-16]. It is reported that nimesulide displays antimicrobial activity against gram-positive organisms such as S.aureus and gram-negative organisms such as Pseudomonas aeruginosa Klebsiella pneumonia, Enterobacter cloacae, Proteus mirabilis, and Pseudomonas fluoroacetate[17]. Experimental studies have shown that selective COX-2 inhibitors display antibacterial activity [18]. Based on this information, nimesulide is useful for the treatment of external ear damage resulting from S. aureus infection. The aim of the present study was to investigate the protective effect of nimesulide on otitis externa induced by S. aureus and compare it with cefazolin.

Material and Methods

Study design

Bacteria isolation and inoculum preparation

We tested the antimicrobial effectiveness of cefazolin and nimesulide antibiotics on a standard S. aureus ATCC 29213 strain supplied from Erzincan Mengücek Gazi Training and Research Hospital Medical Microbiology Laboratory. The standard strain stored at -80ºC was cultivated in the medium with 5% sheep blood (Biomerieux, France) and incubated at 37ºC for 16–18 hours. The same process was repeated for a second time the following day. A purified passage from the colonies that had grown in sheep blood medium was dispersed onto a new liquid medium that contained 5% sheep blood (BioMérieux, France), and the density was set to 0.5 MacFarland ($1.5x10^8$ cfu/ml) using the DensiCHEK Plus (Biomerieux, France) densitometer device.

Antimicrobial Activity Testing

The antimicrobial effect of the antibiotics tested was determined with the Kirby-Bauer disk diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing. The prepared bacterial solution was spread onto the Mueller-Hinton agar medium (BioMérieux, France) with a cotton swab and was left for 10 minutes to dry. Then, 20 µl of the stock solutions of cefazolin and nimesulide in equal concentrations (50 µg/ml) were added to standard blank discs 6 mm in diameter (Bioanalyse, Turkey) and given 15 minutes to dry. After the antibiotic-saturated discs dried, they were placed on the surface of the medium with a sterile clamp, and then the plates were incubated for 16–18 hours at 37ºC. The diameter of the zone without bacterial reproduction around the discs was measured with a ruler and noted. The same processes were repeated a total of six times for both antibiotics.

Experimental Animals

Twenty-four male Wistar rats with a body weight ranging from 280–290 grams were included in the experiment. The rats were supplied by the Atatürk University Medical Experimental Practice and Research Centre. They were kept at room temperature under suitable conditions in a suitable laboratory environment and were fed. The local Animal Experimentation Ethics Committee (Date: 17.12.2020, Meeting No.:13, Decision:18) approved the study's protocols and procedures.

Chemicals

Nimesulide was provided by Deva-Turkey. Cefazolin was supplied by Mustafa Nevzat Ilac Sanayi-Turkey, while the thiopental sodium for the experiment was purchased from I.E. Ulagay-Turkey.

Experimental Groups

We divided the experimental animals into the following groups: the group inoculated on the external ear skin with S. aureus (SaG), the healthy control group (HG), the group inoculated on the external ear skin with S. aureus and treated with nimesulide (SaN) and the group inoculated on the external ear skin with S. aureus and treated with cefazolin (SaC).

Experimental Procedures

The animals in the HG (n=8), SaG (n=8), SaN (n=8) and SaC (n=8) groups were administered anaesthesia through intraperitoneal injection of 25 mg/kg thiopental sodium. The animals' immobility period in the supine state was suitable for surgical intervention [19]. The external ear skin of all groups was sterilized with povidone-iodine at this time. S. aureus (0.5 ml) was inoculated onto the external ear skin of the SaG, SaN and SaC rats (excluding the HG) using a hypodermic syringe for the otitis externa induction (the S. aureus strain TACK 25923 was dispersed at a concentration of 900 x 10-6 colony forming units per ml). The oral administration of nimesulide (50 mg/kg) in the SaN group and of cefazolin (50 mg/kg) in the SaC group was carried out by gavage 24 hours after the bacterial inoculation. The HG and SaG groups received distilled water with the same volume as a dissolvent. This procedure was implemented every day for seven days. The external ear area of the animals was macroscopically evaluated after some time had passed. Then, the animals were sacrificed under high dosage anaesthesia (50 mg/kg), and their external ear tissues were removed. The total glutathione (tGSH), malondialdehyde (MDA), NF-kB, TNF-α, interleukin a beta (IL-1β), and COX-2 levels of the extracted tissues were measured. We compared the results of the HG and SaN groups with the SaG group's results.

Biochemical Analyses

MDA analysis

Ohkawa et al. used a particular method as the basis for assessing MDA [20]. In this approach, the absorption of a pink complex created by MDA and thiobarbituric acid (TBA) at a wavelength of 532 nm at a high temperature (95°C) underwent a spectrophotometric assessment. We centrifuged the homogenates for 20 minutes at 5000 g and determined the amount of MDA using these supernatants. We vortexed 100 µl of 8% SDS, 250 µl of the homogenates, 750 µl of 0.08% TBA, 750 µl of 20% acetic acid and 150 µl distilled water into capped test tubes using a pipette. We incubated the mixture at 100°C for 60 minutes and added 2.5 ml n-butanol to the mixture and then performed the spectrophotometric analysis. Next, 3 ml cuvettes were used to measure the amounts of resultant red colour at 532 nm. We determined the samples' MDA amounts using standard graphics that were produced using the MDA stock solution before considering the coefficients of the dilution.

tGSH analysis

5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB) is a disulphide chromogen in the assessment environment that can be easily reduced by sulfhydryl group compounds. We assessed the resultant yellow colour spectrophotometrically at 412 nm [21]. We centrifuged the homogenates for 10 minutes at 12.000 g and used the supernatants to determine the MDA amount. We vortexed 250 µl measuring buffer (pH=8.2, 200 mM Tris-HCl with 0.2 mM EDTA), 100 µl DTNB, 500 µl of supernatant, and 7900 µl methanol through a pipette into capped test tubes. We incubated the mixture at 37°C for 30 minutes and then conducted the spectrophotometric analysis after then measured the resultant yellow colour amounts at 412 nm using 3-ml quartz cuvettes. We determined the samples GSH amounts using the standard graphics prepared with the GSH stock solution previously made based on the coefficients of dilution.

Measurements of TOS and TAS

A new automated measurement method and commercial kits (Rel Assay Diagnostics, Turkey) developed by Erel were used to determine the tissue homogenates' TAS and TOS levels [22,23].

In the TAS method, the characteristic colour of a stable ABTS radical cation is bleached by antioxidants and is estimated at 660 nm. We expressed the results as H_2O_2 equivalent/l. In the TOS method, the ferrous ion-o-dianisidine complex was oxidized to the ferric ion by the oxidants in the sample. The oxidation reaction was developed by the glycerol molecules in the reaction medium. A coloured complex was produced by the ferric ion in an acidic medium using xylenol orange.

The colour strength that could be spectrophotometrically measured at 530 nm was associated with the total number of oxidant molecules. We expressed the findings as the equivalent/l of µmol Trolox. The TOS-to-TAS percentage ratio was used as the OSI. The TOS was divided by 100 and then multiplied by the TAS to determine the OSI.

TNF-α, NF-ƙB and IL-1β analyses

Rat-specific sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure the tissue-homogenate NF-ƙB and TNF-α concentrations. Rat TNF-α, Rat IL-1β ELISA kits (Cat no.: YHB1098Ra, Shanghai LZ) and Rat NF-ƙB ELISA immunoassay kits (Cat.No.:201-11-0288, SunRed) were used. We performed the analyses as instructed by the manufacturers. In summary, we coated the monoclonal antibody of rat TNF-α, NF-ƙB and IL-1β onto microplate wells. We pipetted the tissue homogenate standards and biotinylated monoclonal antibody specific and streptavidin-HRP into these wells and incubated them for 60 min at 37ºC. After washing, we then added chromogen reagent A and chromogen reagent Bs, applied by the bound enzyme to yield a colour. We incubated the samples for 10 min at 37ºC. Finally, we added the stop solution. There was a direct proportion between the intensity of this coloured product and the rat TNF-α, NF-ƙB and IL-1β concentrations in the original specimen. In the end, we read the well plates at 450 nm. We calculated the samples' absorbance with formulas based on the standard graphics.

COX activity measurement

A COX activity assay kit (Cayman, Ann Arbor, MI, USA) was used to measure the COX activity in the tissue. Free ovarian membranes were collected from the tissue and washed completely with an ice-cold Tris buffer with a pH of 7.4 and also with 0.16 mg/ml of heparin (to separate all clots and red blood cells) and then maintained at -80°C until the time of the assay. We homogenized a sample of liver tissue in a 5-ml cold buffer (0.1 mol Tris-HCl with 1 mmol EDTA, pH: 7.8) per gram of tissue and centrifuged it at 4°C at 10,000 g for 15 min. For the assay measurements, we removed the supernatant and kept it on ice. The Bradford method was used to measure the concentration of protein in the supernatant [18]. The COX peroxidase activity was measured with the COX kit. We monitored the oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine at 590 nm by assaying the peroxidase activity colourimetrically. The activity of COX-2 was measured with a powerful COX-1-specific inhibitor, SC-560 (we used the reagent as supplied). We displayed the results as units per milligram of protein for both COX-2 and COX-1 activity.

Statistical Analyses

The results are shown as the mean value ± standard deviation (x±SEM). The significance of the difference was determined between the groups using a one-way analysis of variance test. We performed a post hoc Fisher's least significant difference test. All statistical procedures were conducted in the Statistical Package for the Social Sciences (SPSS) for Windows software, version 18.0, and $p < 0.05$ was regarded as significant.

Results

Antimicrobial Test Results

As shown in Table 1, nimesulide and cefazolin were found to be effective on standard bacteria. Cefazolin was more effective against S. aureus than nimesulide. Cefazolin formed a 42.3±0.8 mm±SD (standard deviation $p < 0.001$) inhibition zone on S. aureus (Figure 1A), while this same value was only 19.3±0.8 mm for nimesulide (Figure 1B).

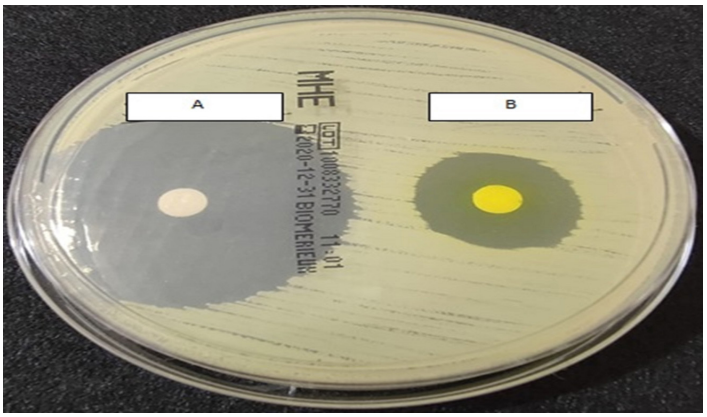

Figure 1. A; Antibacterial area of the SaC group, B; Antibacterial area of the SaN group

Macroscopic Results

As shown in Figure 2, the external ear tissue of the healthy group had no pathological findings (Figure 2A) macroscopically. However, there was severe oedema and redness of the external ear tissue of the group inoculated with S. aureus (Figure 2B). While the oedema and redness were mild in the nimesulide group (Figure 2C), they were moderately severe in the cefazolin group (Figure 2D).

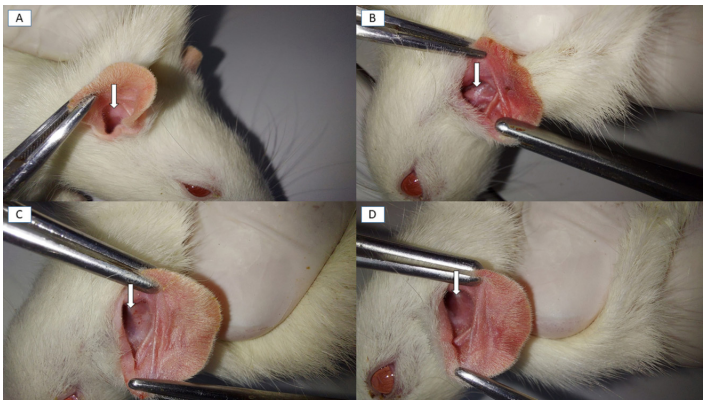

Figure 2. A; The external ear tissue of the HG group. B; The external ear tissue of the SaG group. C; The external ear tissue of the SaN group. D; The external ear tissue of the SaC group

Biochemical Results

The results of tGSH and MDA analysis: Figure 3 shows the significantly higher MDA level on the external ear tissue of the SaG group inoculated with S. aureus than HG and SaN ($p < 0.0001$). However, the SaG and SaC groups demonstrated no significant difference in the MDA amounts ($p > 0.05$), while the difference between HG and SaN was also insignificant. Similarly, the difference between the SaC and SaG groups and that between the SaN and HG based on the tGSH amounts were insignificant.

The results of the TAS and TOS analysis indicated that the S. aureus group had a significantly higher TOS amount in the external ear tissue than the HG and SaN groups ($p < 0.001$), while the amount of the TAS was lower ($p < 0.001$). However, the SaC group and the S. aureus group did not differ significantly, and the HG and SaN groups also did not differ significantly in TOS and TAS amounts ($p > 0.05$) (Figure 4).

The IL-1β, NF-κB and TNF-α analysis results revealed that the levels of these substances in the external ear tissue of the group inoculated with S. aureus were higher than those of the HG, SaN and SaC groups. The HG and SaN groups did not differ significantly in TNF-α, NF-κB and IL-1β amounts ($p < 0.001$). However, the SaN group had significantly lower proinflammatory cytokine levels than the SaC group ($p < 0.05$) (Figure 5).

COX-2 activity in the external ear tissue of the S. aureus group was significantly higher than in the HG and SaN groups ($p < 0.0001$). However, COX-2 activity in the S. aureus group was significantly lower than in the SaC group ($p < 0.01$) (Graph 6).

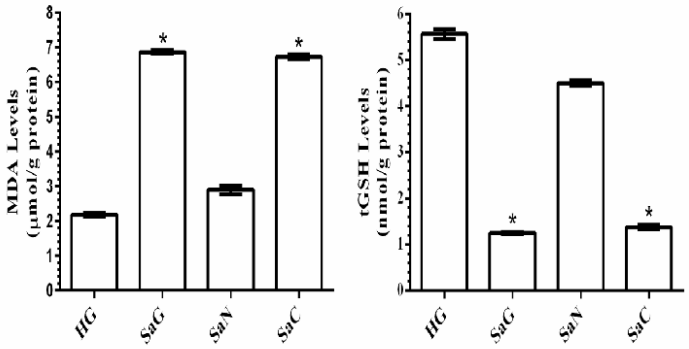

Figure 3. tGSH and MDA levels in the external ear tissues. SaG and SaC compared to HG and SaN groups. *=p<0.0001, n=8

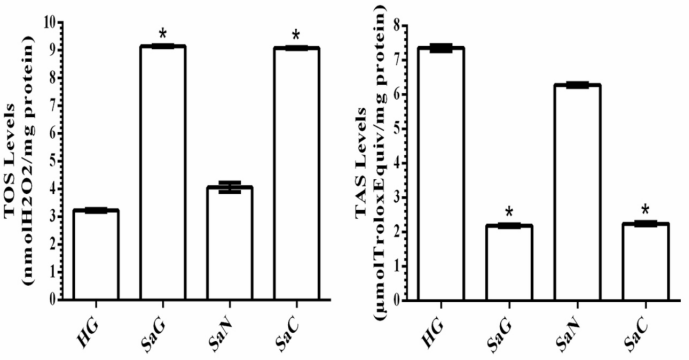

Figure 4. TAS and TOS levels in the external ear tissues. SaG and SaC compared to HG and SaN groups. *=p<0.0001, n=8

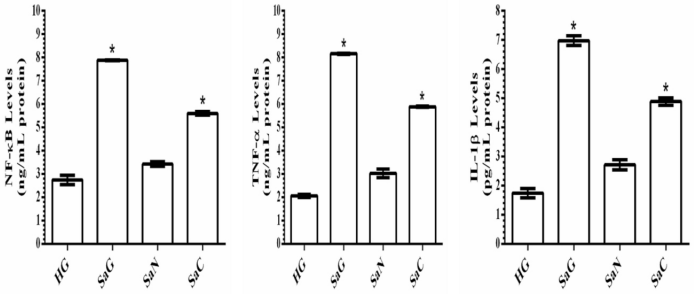

Figure 5. TNF-α , NF-κB, and IL-1β levels in the external ear tissues. SaG and SaC compared with HG and SaN groups. *=p<0.0001, n=8

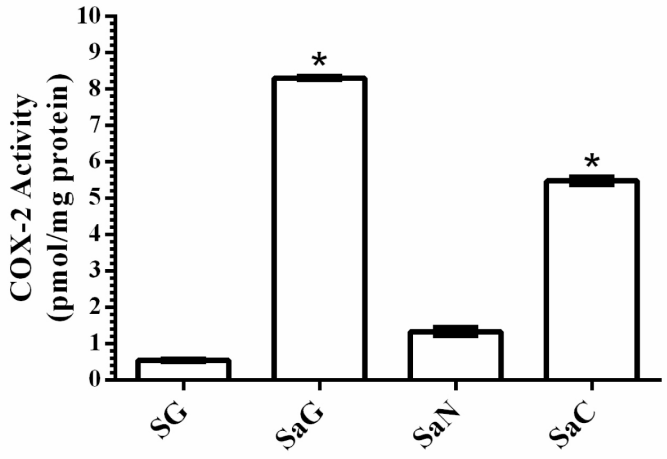

Figure 6. COX-2 level in the external ear tissues. SaG and SaC compared with HG and SaN groups. *=p<0.0001, n=8

Discussion

In this study, the antibacterial effects of cefazolin and nimesulide against S. aureus were evaluated, and the protective effect of nimesulide on otitis externa induced by inoculation of S. aureus onto the external ear skin was biochemically examined and compared with cefazolin. S. aureus is a common pathogen that can cause infection in the soft tissues, the human skin, the respiratory tract and the blood [24]. This organism is one of two pathogens that most frequently cause otitis externa [25]. The antibiogram results in this study revealed that nimesulide and cefazolin were effective against S. aureus. However, the antibacterial effect of nimesulide was weaker compared to that of cefazolin. The antibacterial activity of nimesulide against S. aureus has been documented in previous studies [10].

Macroscopically, oedema and redness of the external ear tissue were apparent in the S. aureus group. Oedema and redness are among the most important indicators of inflammation. Previous studies have stated that redness and oedema are macroscopic findings observed in otitis externa that is related to S. aureus. Nimesulide, whose antibacterial activity against S. aureus is weaker than that of cefazolin, was superior at reducing oedema and redness of the external ear tissue. As previously specified, no studies have examined the effect of nimesulide on external ear damage related to S. aureus and compared it with cefazolin. However, a clinical evaluation by Reiner M reported that nimesulide provided better recovery for signs and symptoms such as chest pain, cough and oropharyngeal hyperaemia in cases treated with antibiotics [26].

The biochemical experiment results indicated that MDA and TOS levels in the external ear tissues of the animals injected with S. aureus were higher and the tGSH and TAS levels were lower than the nimesulide and healthy groups; these results indicate that oxidative stress developed in the external ear tissue of the S. aureus group. There was no evidence that S. aureus generated oxidative damage in the external ear tissue. However, previous studies reported that oxidative stress mediates allergic and inflammatory skin disorders [27]. Chakraborty SP et al. showed that S. aureus infection caused oxidative stress in neutrophils [28]. Another study explained that S. aureus caused damage by increasing the oxidant level and decreasing the antioxidant level in liver tissue [29]. Increased MDA as a result of S. aureus inoculation of the external ear tissue in this study is one of the aldehydes that is produced when ROS oxidize cell membrane lipids. MDA induces a cytotoxic effect by causing the polymerization and cross-linking of membrane components [30].

In the nimesulide group, the MDA level was low due to its antioxidant effect. Prior studies have shown that nimesulide has antioxidant properties [16]. The antioxidant activity is a mechanism that develops to protect against the harmful effects of ROS in cells and tissues. Excessively reproduced ROS neutralize GSH and other non-enzymatic and enzymatic endogenous antioxidant defence systems [31]. The fact that tGSH in the nimesulide group was close to that of the healthy group indicates that the results of this study are in agreement with the results of previous studies. Because cefazolin was not as effective as nimesulide in reducing external ear damage macroscopically, it was unable to avoid increasing MDA and decreasing tGSH levels.

Studies in the literature have reported that tissue damage related to S. aureus is associated with increases in COX-2, NF-ƙB, TNF-α and other proinflammatory cytokines [6 7]. In this study, nimesulide demonstrated superiority to cefazolin in suppressing the S. aureus-induced increase in NF-κB, IL-1β, TNF-α and COX-2 levels. No studies have examined the effect of nimesulide and cefazolin on the external ear damage induced by S. aureus. However, nimesulide is a selective COX-2 inhibitor drug that antagonizes the NF-kB, TNF-α and IL-1β effects beforehand [11,14,15,32]. Recent studies have reported that cefazolin generated an inhibitory effect on proinflammatory cytokines [33]. However, the literature does not indicate whether cefazolin inhibits COX-2 activity. The results of this study and the existing literature indicate that there is a correlation between MDA, TOS, TNF-α, NF-ƙB, IL-1β, COX-2, tGSH and TAS expression. This association has been proven in previous studies [9,10]. In conclusion, nimesulide had a weaker antibacterial effect against S. aureus compared to cefazolin. Nimesulide significantly suppressed the oxidative and inflammatory damage induced by S. aureus. Cefazolin only reduced the amount of inflammatory damage but could not reduce oxidative damage. Additionally, nimesulide was quite superior to cefazolin at suppressing the development of oedema and redness due to the inoculation of S. aureus on the external ear skin.

Conclusion

This study indicated that nimesulide performed better than cefazolin at reducing external ear oxidative and inflammatory damage caused by S. aureus. Therefore, nimesulide monotherapy or combination nimesulide and cefazolin therapy may be beneficial in the treatment of bacterial otitis externa.

Conflict of interests
The authors have no any conflict of interest.

Financial Disclosure
All authors declare no financial support.

Ethical approval
The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 17.12.2020, Meeting no: 13, Decision: 18).

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