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Possible Ototoxic Effects of Topical Rifamycin Application: An Electrophysiological and Ultrastructural Study

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Original Investigation

Abstract

Objective: This study aimed to investigate possible ototoxicity associated with topical rifamycin application via electrophysiological tests and ultrastructural examinations.

Methods: Electrophysiological assessment was performed with tympanometry, auditory brainstem response (ABR), and distortion product otoacoustic emission (DPOAE) measurements. This study was conducted on 40 ears of 20 guinea pigs that were detected to have normal hearing thresholds. The animals were randomly assigned to three groups: Group 1 (n=12) received 0.1 mL rifamycin, Group 2 (n=8) received 0.1 ml gentamycin, and Group 3 (n=20) received 0.1 mL physiological saline. The antibiotics and saline solutions were administered via intratympanic injections. After five injections every other day, electrophysiological tests were performed again on the 15th day. After electrophysiological measurements, the temporal bones of all guinea pigs were prepared for ultrastructural examinations and the cochlear surface morphology was examined by scanning electron microscopy (SEM).

Results: The animals in group 3 did not show a statistically significant change in their DPOAE signal/noise ratio (SNR) or ABR thresholds ($p>0.05$). In groups 1 and 2, the reduction in the DPOAE SNR and the increase in the ABR threshold were statistically significant ($p<0.05$). Regarding SEM examination results, the animals in groups 1 and 2 showed statistically significant outer hair cell damage and cochlear degeneration due to the ototoxic effect of the drugs ($p<0.05$), whereas the animals in group 3 showed no significant damage ($p>0.05$).

Conclusion: The results indicate that rifamycin application to the middle ears of guinea pigs has mild ototoxic effects on their inner ears.

Keywords: Rifamycin, ototoxicity, otoacoustic emission, auditory brainstem response, scanning electron microscopy, guinea pig

Introduction

Rifamycins have been obtained from *Streptomyces mediterranei*, and five antibiotics which are termed as rifamycin A, B, C, D, and E have been produced from them. Three semi-synthetic antibiotics have been derived from rifamycin B (1). These antibiotics, termed as rifamid, rifamycin sodium, and rifampicin, block DNA-controlled mRNA synthesis in susceptible bacteria by inhibiting the enzyme RNA polymerase and have bactericidal action (2). Rifamid is an antibiotic that is currently not used; rifamycin sodium is a drug that is rarely used parenterally or topically. Rifampicin is an important medication currently used as an antistaphylococcal drug, particularly in the treatment of tuberculosis and some other indications (3, 4). Rifamycin acts against gram-positive cocci (pneumococci, streptococci, and particularly *Staphylococcus aureus*); gram-negative cocci (particularly *Neisseria meningitidis*); gram-negative bacilli; and acid-resistant bacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* (1). Rifamycin has frequently been used since 1963 for the irrigation of open and closed wounds and for the treatment of infected surgical or cutaneous wounds. The first local use of rifamycin is to clear the lung cavities of patients with tuberculosis (5). Wound healing with rifamycin is better than that with other local antibiotics; therefore, rifamycin is frequently used for the treatment of infected skin wounds (6). Rifamycin is also used topically by otolaryngologists for the treatment of cholesteatoma-free chronic otitis media and external otitis. Although potential ototoxic effects of many topical agents have been investigated, the possible ototoxic effect of topical rifamycin application has not been investigated in

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the literature. Chronic otitis media and external otitis are very common diseases in the society, and topical rifamycin is one of the treatment options for these diseases. We aimed to investigate the possible ototoxic effect of topical rifamycin application in our study with regard to the electrophysiological and ultrastructural assessments in guinea pigs.

Methods

After obtaining approval from the local ethics committee for the 2014/10 protocol, dated March 28, 2014, and numbered 2014.04.01, 30 4-8 month-old pigmented Hartley adult guinea pigs weighing between 400 and 800 g and having auropalpebral reflexes were used in our study. Regarding the animal care and use, the rules in the regulation for the welfare and protection of animals used for experimental and other scientific purposes (dated December 13, 2011 and numbered 28141), which were declared in the International Helsinki Declaration and the Ministry of Food, Agriculture and Livestock, have been used. Intratympanic (IT) injections and electrophysiological measurements were performed in the guinea pigs under general anesthesia. General anesthesia was provided with 40-mg/kg intramuscular (i.m) ketamine hydrochloride (Ketalar ampoule; Pfizer, İstanbul, Turkey) and xylazine hydrochloride (Rhompun ampoule; Bayer, Istanbul, Turkey). One third of ketamine hydrochloride was intraperitoneally administered when repeated anesthetic dose was needed. Before the electrophysiological evaluation, all guinea pigs were oto-microscopically examined, and the external auditory canal was evaluated. An immittance metric study was performed for evaluating middle ear functions. Distortion-product otoacoustic emission (DPOAE) recordings were obtained to demonstrate the normal function of the outer hair cell, and auditory brainstem responses (ABR) were recorded for evaluating central auditory pathways and the presence of normal hearing. Although 20 guinea pigs with normal outer hair cell function and normal hearing threshold were included in the study, 10 guinea pigs with no emission response and no normal hearing threshold were excluded. Forty ears of the 20 guinea pigs were randomly divided into three groups. Group 1 was the rifamycin group, and 0.1 mL of rifamycin [rifamycin SV (Rifocin 100 mg 10 mL ear drop; Sanofi Aventis, Istanbul, Turkey)] was intratympanically administered to the right ears (n=12) of the guinea pigs. Group 2 was the gentamicin group, and 0.1 mL (containing 0.3 mg gentamicin) of gentamicin (Genta eye ear drop, İ.E. Ulagay, İstanbul, Turkey) was intratympanically administered to the right ears (n=8) of the guinea pigs. Group 3 was the physiological saline group and it was intratympanically administered to the left ears (n=20) of all guinea pigs as 0.1 mL of 0.9% NaCl. In all groups, IT injections were administered five times every other day, and the injections were administered at the same time each day. During all injections and measurements, the anesthetized guinea pigs were covered with heated pads to protect their body temperature. The procedures were performed in an environment with a 50% humidity rate and a temperature of 16°C-21°C to reduce fluid loss in the guinea pigs. DPOAE and ABR test measurements were repeated five days after the last drug application. After the measurements, decapitation was performed in the guinea pigs after high-dose ketamine hydrochloride and xylazine hydrochloride injection. Temporal bones of the guinea pigs in all groups were dissected for ultrastructural evaluations.

Electrophysiological evaluations

Immittancemetric examination

Immittancemetric examination was performed using an otoacoustic emission apparatus by Capella-Madsen (GN Otometrics A/S, Taastrup, Denmark) in the tympanometry mode. To adapt to the external auditory canal of the guinea pigs, the OAE probe tip (Capella-Madsen) was placed on the tip of tympanometry with a number 1-2 plastic probe, which was used for newborns on the 1-cm tip of plastic tube adapters. The probe tone was set to 1 kHz at 75 dB SPL. The speed of the pump was determined to be 100 daPa/s. During the measurements, the pressure range was set between +200 and –300 daPa providing a pump direction from positive to negative. The measurement process was started when the probe position was appropriate. Those in whom a compliance peak curve between +100 and –100 daPa was obtained during the measurements were evaluated as a "A"-type (normal type) tympanogram.

Distortion-product otoacoustic emission measurement

Otoacoustic emissions (2f1–f2 cubic distortion-product components) were measured in the DPOAE mode using the Capella-Madsen (GN Otometrics A/S, Taastrup, Denmark) device. The OAE probe tip (Capella-Madsen) was placed on the tip of a number 1-2 tympanometry plastic probe used for newborns on the 1-cm tip of plastic tube adapters according to the external auditory canals of the guinea pigs. The ratio between the frequencies f2 and f1 (f2/f1) was set to 1.22; the stimulus intensity was taken as L1 (L1=65 dB SPL) for frequency f1 and as L2 (L2=55 dB SPL) for frequency f2, and distortion-product emissions were measured in the 2f1–f2 mode. Otoacoustic emissions were recorded at geometric averages of frequencies f1 and f2 at 0.75, 1, 1.5, 2, 3, 4, 6, and 8 kHz. Each recording was approximately 60 s long. Recordings were taken in an environment where the noise intensity was <50 dB. When DPOAE results were evaluated based on Signal-to-Noise ratio (SNR) in the frequency bands at 0.75, 1, 1.5, 2, 3, 4, 6, and 8 kHz for each frequency, the results >6 dB were considered to be significant (7). When DPOAE responses were evaluated, SNR was found to be more reliable than DPOAE amplitudes (8, 9). In our study, percentage changes (taking the difference between the first and last measurements) of SNR responses specific to the frequency were evaluated for each guinea pig, and percentage change graphs were generated for SNR frequency bands.

Auditory brainstem responses

The Medelec Synergy ABR (Oxford Instruments, Oxford, UK) brand brainstem analyzer was used for measurement. Plastic tube adapters (1 cm in size) were connected to the E-A-R Tone 3A insert headphones according to the outer ear canal of the guinea pigs. The negative silver needle was placed in the mastoid of the tested ear, the positive needle was placed in the forehead, and the ground was placed in the contralateral side of the foot.

The electrode test on the device was used to check whether the electrodes were connected properly. Special attention was paid so that electrode impedances were <5 kOhm. Stimuli were provided as click stimulus. Eleven click stimuli were provided in a second, and an average of 300 responses was determined. The stimulation was started from 80 dB normalized hearing level (nHL) and reduced by 10 dB at each time, and the hearing level at which at least three wave forms were observed was determined as the threshold. Hearing was evaluated to be normal when ABR wave configuration was detected at 20 dB HL.

Ultrastructural evaluations

Before temporal bones were dissected, 2.5% glutaraldehyde was injected into the middle ear cavity or tympanic cavity. After temporal bone dissection, tympanic bullae were opened and the cochlear structure was reached, and then it was placed in a 2.5% glutaraldehyde-phosphate buffer (7.3 pH) for 12 h. After these prefixation procedures, the bone tissue was washed by placing in phosphate buffer (PBS) for 24 h. Afterward, the tissues were decalcified at room temperature in a solution prepared from 0.1 M Na-EDTA (Sigma, Darmstadt, Germany) at pH 7.3 for 2 weeks. After the temporal bones were decalcified, the otic capsule surrounding the cochlea was asymmetrically dissected from the base to the apex using a stereo microscope (Olympus 1×71 S8-F3, Tokyo, Japan). The dissected cochlea were kept at +4°C in PBS for 3 days and were followed up using routine scanning electron microscopy (SEM). Primarily, the corti organ surface was anatomically examined during SEM evaluation. General cell and stereocilium morphology in outer hair cells were evaluated in superficial anatomy (Table 1) (10). Among evaluated parameters, the morphological arrangement of the outer hair cell stereocilia, particularly in the cochlear frequency bands, was studied from the base to the apex.

Statistical analysis

The results are expressed as mean±standard deviation. Wilcoxon's signed-rank test was used to compare the first and last measures of ABR and DPOAE responses in the groups. The Kruskal–Wallis test was used for intergroup comparison by calculating differences between the first and last measurements of ABR and DPOAE responses; when a difference was found, the Bonferroni post-hoc test was used to determine which groups differed. Statistical analyses were performed using the Statistical Package for the Social Sciences 20.0 (IBM Corp.; Armonk, NY, USA). $p<0.05$ was considered to be the limit value of statistical significance.

Results

In our study, electrophysiological test results of 40 ears of the 20 guinea pigs in the three groups were evaluated. None of the 20 guinea pigs in the study were excluded for any reason. ABR threshold values and DPOAE SNRs were compared in the presence of click stimulus on electrophysiological measurements. On IT applications after the first and last measurements, ABR threshold responses were firstly compared within each group and then among the groups (Figure 1). While the first measurement ABR threshold value was 10.8±2.8 dB on an average before IT application for the rifamycin group (group 1), the mean ABR threshold value was 18.8±8.3 dB after the last application, and it was found to be statistically significant ($p=0.007$ and $p<0.05$, respectively). In the gentamicin group (group 2), while the ABR threshold value before IT application was 11.2±3.5, it was 45±9.2 dB after the last application, and it was considered to be statistically significant ($p=0.01$ and $p<0.05$, respectively). In the physiological saline group (group 3), the mean ABR threshold value was 10.5±2.2 dB before IT application and the mean ABR threshold value after drug application was 12±4.1 dB; the difference between these values was not statistically significant ($p=0.083$ and $p>0.05$, respectively). When the groups were compared among each other with respect to ABR threshold values, a statistically significant difference was found among all three groups ($p=0.0001$). When DPOAE SNRs were compared within the group before and after IT administration in groups 1 and 2, while a statistically significant decrease was found ($p=0.012$) at all frequencies, there was no

Table 1. Parameters for evaluating the cochlear surface morphology

Parameters	Results	Degree of degeneration	Score
General cell morphology (superficial and lateral connections)	Normal cell view	Normal	0
	Collapse, regular separation in lateral ligaments	Mild degeneration	1
	Collapse, irregular separation in lateral ligaments	Moderate degeneration	2
	Necrosis	Severe degeneration	3
Outer hair cells (EHC)	Normal stereocilia view	Normal	0
	Irregularity in the stereocilia	Mild degeneration	1
	Cohesion and partial loss in the stereocilia	Moderate degeneration	2
	Total stereocilia loss	Severe degeneration	3
Place of degeneration	No degeneration	Normal	0
	Hairy cell degeneration in 1/3 of the cochlea	Mild degeneration	1
	Hairy cell degeneration in 2/3 of the cochlea	Moderate degeneration	2
	Hairy cell degeneration in 3/3 of the cochlea	Severe degeneration	3

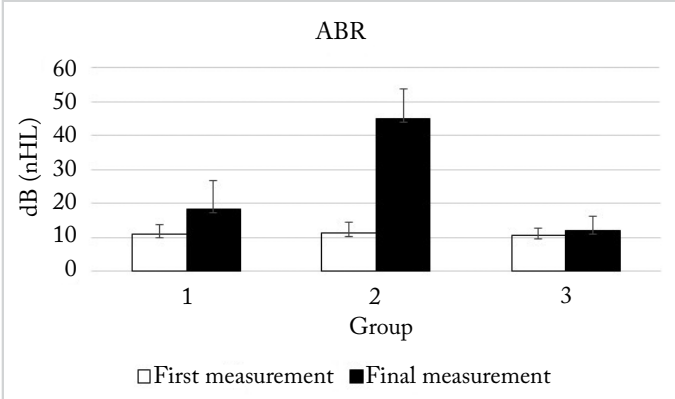

Figure 1. Auditory brainstem response threshold values before and after drug administration within the groups and among the groups (dB nHL)
Group 1: rifamycin group (n=12), group 2: gentamicin group (n=8), and group 3: physiological saline group (n=20)

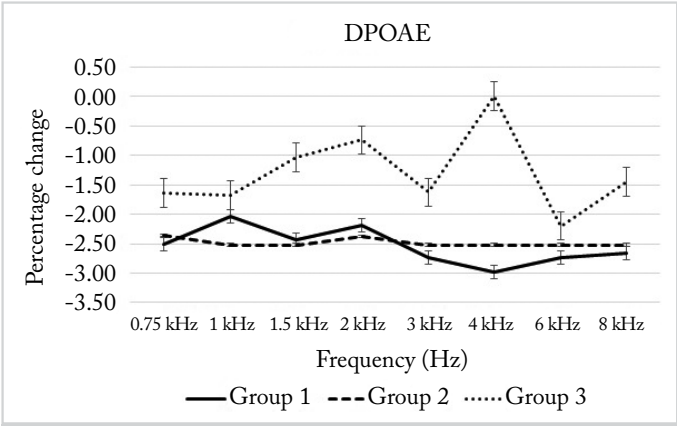

Figure 2. Comparison of percentage changes in SNRs according to frequencies before and after drug administration within groups and among groups

Group 1: rifamycin group (n=12), group 2: gentamicin group (n=8), and group 3: physiological saline group (n=20)

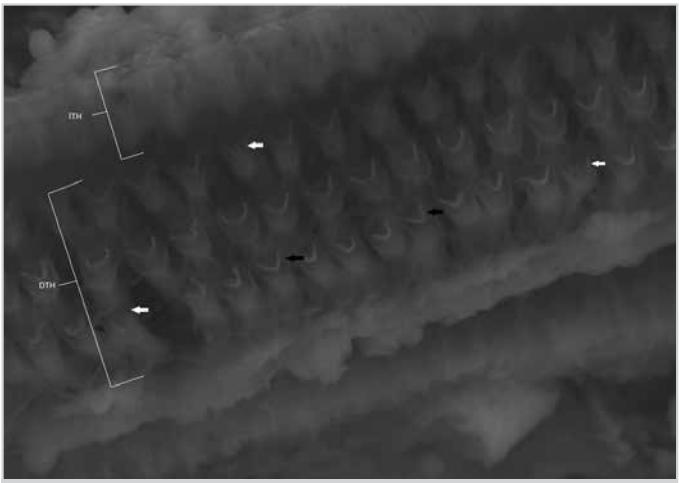

Figure 3. Irregularities are viewed in the stereocilia of the outer hair cells on the corti organ surface of a guinea pig in the rifamycin group, ×3000

OHC: outer hair cell; IHC: inner hair cell; black arrow: normal outer hair cell; white arrow: partially damaged outer hair cell

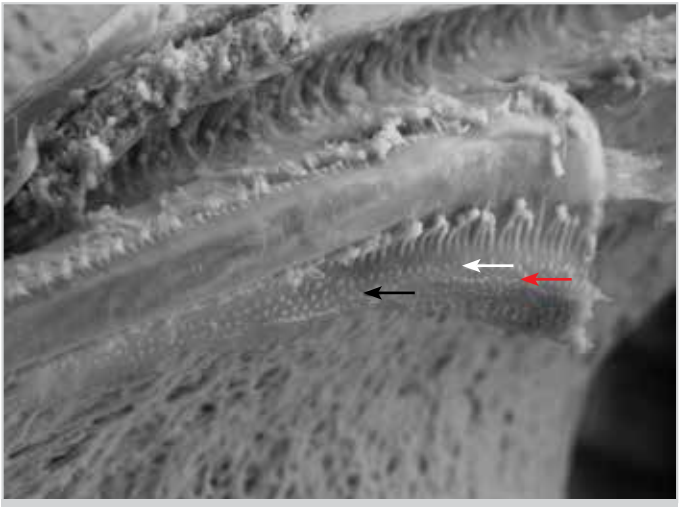

Figure 4. Stereocilia of the outer hair cells on the corti organ surface of a guinea pig in the gentamicin group are viewed to completely disappear from place to place, ×1000

Black arrow: normal outer hair cell; white arrow: total loss; and red arrow: partially damaged outer hair cell

decrease in DPOAE SNRs in group 3 (p=0.67). Percentage changes in SNRs in all three groups before and after IT administration are shown (Figure 2). General cell morphology, corti organ surface anatomy, and outer hair cell stereocilium structure were evaluated through SEM. On the examination of the corti organ surface in group 1, there were irregularities and partial losses in stereocilia of the outer hair cells of six cochleas (Figure 3). In two guinea pigs, there was an irregularity in the stereocilia of the outer hair cells in 1/3 of the cochlea. No irregularity was detected in the cochleas of four guinea pigs. Thus, four cochleas were evaluated with 3 points and four cochleas were evaluated with 2 points (mean, 1.66 points). When we compared these results with those of group 3, there was a statistical significant difference (p=0.01 and p<0.05, respectively). There was a statistically significant difference (p=0.001 and p<0.05, respectively) when group 1 was compared with group 2, though there were mild degenerations. On surface examination of the corti organ in group 2, in four cochleas, 2/3 cochleas showed irregularity in the stereocilia of the outer hair cells and a regular separation was found at the side surface connections. In the other four cochleas, there were partial or complete losses in the stereocilia (Figure 4). Thus, four cochleas were evaluated with 5 points, two cochleas with 6 points, and two cochleas were evaluated with 8 points (mean, 6 points). There was a statistically significant difference when we compared these results with those of group 3 (p=0.0001 and p<0.05, respectively). In group 3, the corti organ surface anatomy was examined; cells throughout the cochlea were evaluated as normal in 20 cochleas. There were irregularities in the stereocilia of the outer hair cells of four cochleas. On histological scoring, 16 cochleas were scored with 0 points and four cochleas were scored with 1 point (mean, 0.2 points). No degeneration was detected on evaluating the morphology and sequence of the outer hair cell stereocilia in group 3 (Figure 5).

Discussion

Ototoxicity continues to be one of the major causes disrupting hearing and balance. Major complaints emerging because of ototoxic substances are hearing loss, tinnitus, imbalance, and vertigo (11). In our study, we experimentally investigated hearing loss for assessing ototoxicity.

Topical ear drops have many advantages over systemic treatments (12). Topical application may be performed at high concentrations in the relevant area in addition to the fact that it does not cause side effects such as diarrhea, nausea, vomiting, and rash. However, the transition from the round window into the inner ear at high concentration reveals the ototoxic potential of topical applications. A wide variety of animal model studies have been used for investigating possible ototoxic effects of topical ear drops in the presence of perforation (13). In rodent models, the effects of many topical antibiotics have been investigated; in particular, gentamicin has been found to be consistently and significantly ototoxic (12-14). Therefore, in our study, we used gentamicin as the positive control group.

The most important discussion topic in electrophysiological responses for monitoring ototoxicity is the selection of the

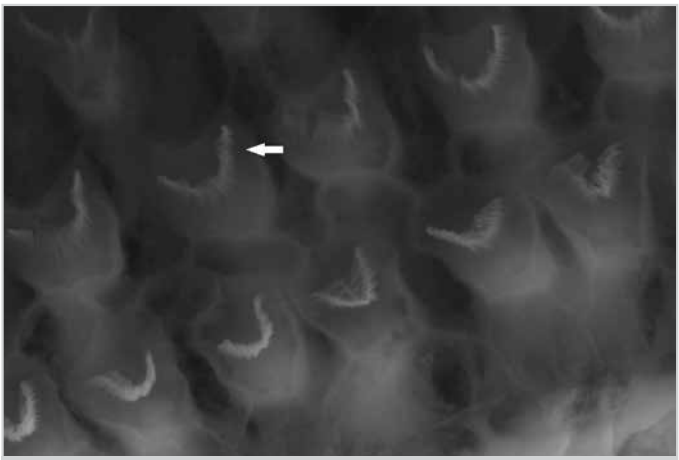

Figure 5. Stereocilia view of the outer hair cells on the corti organ surface of a guinea pig in the physiological saline group, ×10000
White arrow: normal outer hair cell

significant change criterion. Brown et al. (15) detected changes in DPOAE SNR responses after prolonged administration of gentamicin on guinea pigs. More importantly, they detected changes in DPOAE responses before the changes in the surface morphology of outer hair cells of the corti organ were observed. Therefore, they argued that the measurement of otoacoustic emissions is highly valuable for monitoring ototoxicity. DPOAE SNRs were used to assess the ototoxic effect in our study. In DPOAE measurements after the administration of rifamycin, gentamicin, and physiological saline solution, the greatest decrease in SNR was observed in the gentamicin group. In the rifamycin group, the decrease in SNR was found to be statistically significant, though less in comparison to the gentamicin group ($p<0.007$ and $p<0.05$, respectively).

De Lauretis et al. (16) showed with clinical audiological evidence that the value of ABR was significantly successful in early detection of ototoxicity of cisplatin. Campbell and Durrant (17) argued in their first study on ototoxicity that threshold differences of >15 dB in ≥1 frequencies or >20 dB in a single frequency were considered as a significant indication of change; however, in later studies, these criteria were found to exceed even in those who did not use ototoxic drug. In a study on 44 subjects using tobramycin sulfate or vancomycin, when they maintained a threshold change of at least 15 dB at a single frequency, Meyerhoff et al. (18) found that a 15 dB change criterion at a single frequency was not reliable in terms of ototoxicity. In the frequencies that were tested, they also found an average of 5 dB threshold changes in both directions of increase and decrease. We did not base our study on a change value; we compared the values in terms of significance before and after the drug administration. In our study, ABR measurements before and after IT administration most commonly revealed elevation in hearing thresholds in the gentamicin group. We also observed a similar increase in ABR threshold in the rifamycin group.

In previous studies, various methods, such as light microscopy, fluorescence microscopy, or electron microscopy, have been used to histopathologically reveal ototoxicity. Oshima et al. (19) evaluated morphological changes on the surface of corti organ through fluorescence microscopy in their study in which they examined the ototoxicity of daptomycin in guinea pigs. Olgun et al. (20) evaluated the spiral ganglion and corti organ surface morphology using electron microscopy in their study in which they detected the protective effects of red ginseng against the ototoxicity of cisplatin. In our study, we used SEM to show ototoxicity-related outer hair cell damage. For this purpose, we assessed and scored the cochlear surface in terms of cell morphology from the baseline to apex. We observed hairy cell damage in the cochlea and cochlear degeneration mostly in the gentamicin group. This damage and degeneration were also found in the rifamycin group, though to a lesser extent than the gentamicin group.

Although we have enough knowledge about the location of damage caused by aminoglycosides-induced ototoxicity in the inner ear and the clinical and pathological changes that occur because of this, there is still no common opinion on the molecular mechanism of ototoxicity. One of the causes of ototoxicity of aminoglycosides is thought to be free oxygen radicals. The formation of free oxygen radicals in the cell by aminoglycosides requires a polyunsaturated fat molecule as the iron ion and electron donor. Gentamicin and iron together bind to phosphatidylinositole, which is a membrane lipid, and lead to free oxygen radical formation (21). In the literature, we did not come across another original publication investigating the ototoxicity of rifamycin. Although we have shown outer hair cell damage in our study through SEM, the causes of ototoxicity need to be explained at the molecular level in future studies.

Conclusion

In the literature, we did not find a similar study investigating the ototoxicity of topical rifamycin. In this study, direct application of rifamycin to the middle ear in guinea pigs in the light of electrophysiological and ultrastructural evaluations was found to cause slight degeneration in the cochlear structures of the inner ear and to increase hearing threshold responses. Therefore, the ototoxic effect of rifamycin should be evaluated in similar experimental and clinical studies. In addition, rifamycin should not be preferred for the treatment of external otitis in patients with chronic otitis media and tympanic membrane perforation or in patients with ventilation tube.

Ethics Committee Approval: Ethics committee approval was received for this study from Trakya University Animal Experiments Local Ethics Committee (TÜHDYEK-2014.04.01).

Informed Consent: Not required in this study.

Peer-review: Externally peer-reviewed.

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