



Original Article

A Prospective Experimental Study on the Protective Effect of Resveratrol against Amikacin-Induced Ototoxicity in Rats

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OBJECTIVE: The purpose of this study was to evaluate the protective effect of resveratrol against amikacin-induced ototoxicity in rats by otoacoustic emission and histopathology of the cochlea.

MATERIALS and METHODS: This study was conducted with 31 Sprague Dawley adult female rats that were 20–21 weeks old and 190–245 g in weight. Before the drug administration, distortion product otoacoustic emission (DPOAE) tests were performed in both ears of each rat. The rats were divided into four groups. Group 1 (n=7) received ethanol 1cc 4%, Group 2 (n=8) received 600 mg/kg amikacin, Group 3 (n=8) received 10 mg/kg resveratrol and 600 mg/kg amikacin, and Group 4 (n=8) received 1cc resveratrol at 10 mg/kg. The drugs were administered once a day for 21 consecutive days. Control DPOAE tests were performed at the 7th, 14th, and 21st days after the administration of drugs. At the end of the study, the rats were sacrificed and their cochleae were dissected. The cochleae were evaluated for histopathologic changes.

RESULTS: There was no statistically significant difference in the DPOAE measurements before the procedure between groups. The DPOAE measurements significantly decreased after the procedure in the amikacin group. There was no statistically significant difference in DPOAE measurements after the procedure in the amikacin + resveratrol, resveratrol, and ethanol groups. The histopathologic findings supported these results.

CONCLUSION: We found that if resveratrol is administered with amikacin, the severity of amikacin-induced hearing loss is decreased. These findings suggest that resveratrol, a strong antioxidant, has a protective effect in amikacin ototoxicity.

KEYWORDS: Amikacin, antioxidants, otoacoustic emission, ototoxicity, resveratrol

INTRODUCTION

Ototoxicity is a general term used for damage of the cochlear and/or vestibular organs; it results from exposure to several therapeutic agents and chemical substances ^[1]. Tinnitus is usually the first symptom of ototoxicity. Other symptoms include imbalance, hearing loss, and vertigo ^[2].

Aminoglycoside antibiotics, a drug group to which the inner ear is known to be vulnerable, are widely administered for various conditions, including tuberculosis and gram-negative bacterial infections. However, the toxic potential of these drugs limits their clinical use. The most prominent toxic effects of aminoglycosides are nephrotoxicity, neurotoxicity, and ototoxicity ^[3]. Neither duration of treatment nor plasma drug concentration correlates with the ototoxic effects of aminoglycosides. Amikacin may lead to irreversible and bilaterally progressive sensory-neural hearing loss, particularly affecting the higher frequencies ^[2]. The average incidence rates for amikacin ototoxicity are between 5 and 10% ^[4].

Aminoglycosides damage the membranes of hair cells in the inner ear via increasing the levels of reactive oxygen and nitrogen species ^[5]. Therefore, patients treated with this group of drugs should be closely monitored for hearing functions; if prolonged medication is required, a revision of the treatment plan is advised.

The otoacoustic emission (OAE) test, which determines the status of outer hair cells (OHC), is an objective, non-invasive, and specific test for ototoxicity monitoring. OAEs are invaluable in the early diagnosis and prevention of ototoxicity ^[5].

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In a number of clinical and experimental studies, several agents were demonstrated to have protective effects against the ototoxic effects of aminoglycosides. These agents include iron chelators, glutathione, alpha-tocopherol, alpha lipoic acid, D-methionine, dexamethasone, trimetazidine, ebselen, N-acetylcysteine, thymoquinone, and steroids [6].

Resveratrol (3, 5, 4'-trihydroxystilbene), which is known to be a potent antioxidant, is derived from fruits, particularly black grape seeds, peanuts, and mulberries. Several studies have focused on the biological and pharmacological roles of resveratrol. These include antioxidant, anti-inflammatory, antimicrobial, antiviral, anti-aging, vasodilatory, anti-lipid peroxidation, hepatoprotective, cardioprotective, gastroprotective, anticancer, anti-aggregant, and estrogenic effects [7]. To our knowledge, there is an absence of evidence in the literature as to whether resveratrol has any protective effects against amikacin ototoxicity.

In this study, we aimed to investigate the possible protective effects of a potent antioxidant, resveratrol, against amikacin ototoxicity, which occurs through free radicals. We also aimed to determine, through otoacoustic emissions and histopathological examinations, whether resveratrol administration exerts any ototoxicity on the inner ear.

MATERIALS and METHODS

The study was approved by the Local Ethics Committee for Animal Experiments. Experiments were performed in an experimental research and application laboratory. Because of experimental animal study, informed consent is not required.

A total of 31 female Sprague Dawley 5-month-old rats with an average weight of 210 g (190–245 g), which were raised under the same environmental conditions and given a standard laboratory diet, were used in the experiments. All rats were kept in cages in the same room under the same environmental conditions, namely in a room that was illuminated and darkened for 12/12 hour cycles at a temperature of $21^{\circ}\text{C}\pm 1$ with a background noise level of under 50 dB, and the rats were fed ad libitum. All procedures were performed in compliance with the Helsinki Declaration and the International Guiding Principles for Biomedical Research Involving Animals.

Initially, each rat was anesthetized with intraperitoneal (i.p.) ketamine (40 mg/kg) (Ketalar flacon; Pfizer, New York, USA) and xylazine (5 mg/kg) (Ksilazol flacon; Provet Veterinary Products JSC, Brisbane, Australia). Following anesthesia, the ear canals and tympanic membranes of each rat were examined by otomicroscopic inspection (Opmi 1, Zeiss, Germany), which revealed no pathological findings. The distortion product OAE (DPOAE) test was performed for both ears of each animal for baseline hearing threshold evaluation, and 62 functionally normal ears of 31 rats were included in the study. On the last day of the experiment, one rat belonging to the control group did not recover from anesthesia and was excluded from the study; therefore, the control group included 7 subjects instead of 8. No other animal loss occurred during the experiments.

Formation of Groups and Experimental Studies

The subjects were randomized into 4 distinct groups as follows:

1. **Control Group (n=7):** Each of the 7 rats included in this group was given 4% ethanol (1 cc, i.p.).
2. **Amikacin Group (n=8):** Intraperitoneal amikacin (600 mg/kg) (Amikozit 500 mg flacon, Zentiva) was administered to each of the 8 rats in this group.
3. **Amikacin + Resveratrol Group (n=8):** Initially, the rats were given 10 mg/kg resveratrol (500 mg, Sigma Chemical Co.; St. Louis, MO, USA) in 4% ethanol solution with a volume of 1 cc intraperitoneally. Two hours later, intraperitoneal amikacin (600 mg/kg) (Amikozit 500 mg flacon, Zentiva) was administered.
4. **Resveratrol Group (n=8):** 10 mg/kg intraperitoneal resveratrol (500 mg, Sigma Chemical Co.; St. Louis, MO, USA) in 4% ethanol solution with a volume of 1 cc was administered to the rats in this group.

All of the injections were performed once daily for 21 days. At days 0, 7, 14, and 21, the subjects underwent anesthesia followed by DPOAE evaluation for hearing functions, and the results were recorded. Injections on the DPOAE days were given 2 hours after the rats had awakened from anesthesia.

After all injections and measurements were accomplished, the rats were sacrificed following high-dose anesthetic administration, and the cochleae were harvested and fixed in formol solution for histopathological studies.

Resveratrol was prepared by dissolving 50 mg resveratrol powder in 1 mL 100% ethanol, and the product was further diluted in 24 mL saline to obtain a final resveratrol solution in 4% ethanol. The resveratrol was maintained at -20°C and the solutions were freshly prepared every day.

Implementation of DPOAE Tests

The amplitude (L) and signal-to-noise ratio (SNR) values, which were calculated by subtracting the background noise level from the DPOAE measurements in dB, were used to interpret the test results. A Madsen (Capella; Taastrup, Denmark) OAE system and neonatal probes were used for DPOAE screening. The f_2/f_1 ratio was fixed to 1.22, and the L1-L2 difference was adjusted to 10 dB SPL (L1=70 dB SPL; L2=60 dB SPL). The DPOAEs were measured at tones equal to $2f_1-f_2$ and generated at the frequencies corresponding to the geometric mean of f_1 and f_2 . SNR and L values were recorded for both ears on days 0, 7, 14, and 21 and at 2000, 3000, 4000, 6000, and 8000 Hz.

Histopathological Studies

The cochleae were fixed in 4% formaldehyde solution for 15 days and decalcified in 10% formic acid for another 15 days. Following the decalcification step, the tissues were regularly processed and embedded in paraffin. Serial 5 μm cross-sections both parallel and vertical to the cochleae with a maximum width of 50 μm were obtained, until the whole cochlear area became visible. The slides were stained with hematoxylin and eosin for histopathological investigations, and TUNEL staining was performed for apoptosis detection.

TUNEL Method

TUNEL staining was performed to demonstrate apoptosis in the inner ear tissue. For the TUNEL staining, the In Situ Cell Death Detection

Table 1. Pretreatment and post treatment Distortion Product Otoacoustic Emission (DPOAE) responses in all frequencies (mean L, SD, minimum and maximum values) (Variables are given as mean)

	Ethanol				Amikacin				Amikacin + Resveratrol				Resveratrol			
	L	Min	Max	SD	L	Min	Max	SD	L	Min	Max	SD	L	Min	Max	SD
Pre2000	-6.39	-21	6.3	7.34	7.75	-10	25.2	10.80	5.86	-13	20.7	10.49	-4.40	-21	20.3	10.25
Post2000	-5.48	-15	3.8	5.83	-12.22	-26	-0.6	6.45	-0.33	-13	14.6	8.25	-7.51	-14	5.6	6.59
Pre3000	4.52	-13	15.8	11.23	16.43	-12	29.9	10.20	13.23	-6	24	8.92	6.47	-14	26.3	11.63
Post3000	-0.79	-14	15.4	10.61	-12.28	-24	-1.1	5.97	7.88	-12	21.3	9.46	1.95	-17	14	11.59
Pre4000	3.92	-26	21	18.86	23.51	-6	35.9	11.09	17.09	-30	31.3	15.67	7.09	-28	29.9	21.81
Post4000	-3.56	-24	20	14.17	-22.38	-34	-2.5	11.23	13.92	-11	31.9	12.07	4.91	-16	24	13.57
Pre6000	10.57	-26	27.7	20.23	25.40	-4	39.7	10.68	24.87	-3.8	37.5	11.77	12.71	-29	29.7	20.46
Post6000	1.60	-25	31.6	19.49	-22.52	-36	-15	5.18	17.11	3.6	29.7	6.51	10.06	-21	33.1	19.37
Pre8000	18.82	-15	37.8	21.93	29.33	9.6	39.5	8.93	31.73	-11	44.7	16.72	19.07	-18	41.8	21.30
Post8000	12.25	-14	39.6	21.33	-20.75	-29	-14	3.74	23.95	-2	34	11.28	16.34	-18	39.3	21.97

Amikacin treatment markedly decreased amplitude responses in all frequencies. The DPOAE changes in the other three groups were not marked. L: amplitude; SD: standard deviation

Table 2. Pretreatment and post treatment Distortion Product Otoacoustic Emission (DPOAE) responses in all frequencies (mean SNR, SD, minimum and maximum values) (Variables are given as mean)

	Ethanol				Amikacin				Amikacin + Resveratrol				Resveratrol			
	L	Min	Max	SD	L	Min	Max	SD	L	Min	Max	SD	L	Min	Max	SD
	SNR	Min	Max	SD	SNR	Min	Max	SD	SNR	Min	Max	SD	SNR	Min	Max	SD
Pre2000	7.78	1.1	11.5	2.71	15.85	6.4	30	7.88	12.84	6.2	25.9	7.10	7.72	-2.5	24.6	5.42
Post2000	8.27	3.7	13.5	2.45	7.61	0.8	14.3	3.09	12.88	6.8	24.1	6.17	7.60	6.2	10.8	1.37
Pre3000	16.26	6.2	28.2	7.33	25.73	7.3	42.9	10.09	23.29	7.2	39.4	8.41	17.48	6.1	40.2	8.96
Post3000	14.40	6.3	28.4	7.46	7.17	4.6	9.8	1.30	20.55	8.6	36.2	8.24	13.57	1.4	21.9	6.91
Pre4000	17.30	-0.8	38	12.34	32.98	6.2	48.3	11.01	27.70	-0.2	43.3	11.79	19.85	-4.7	38.3	14.98
Post4000	13.55	0.2	30.9	8.53	2.48	-5.6	11.4	4.93	23.71	6.9	45.2	9.65	20.56	6.4	35.6	10.71
Pre6000	19.76	-11	38.7	16.87	30.67	7.3	44.3	10.80	28.63	7.2	48.6	12.30	19.51	-15	35.6	16.36
Post6000	12.75	-8.4	38.1	15.39	-4.8	-17	2.7	5.38	25.15	10	37.2	6.51	17.91	-5	42.5	16.18
Pre8000	23.41	3.4	40.2	13.90	26.03	8.5	37.6	9.85	31.78	6.3	44.9	12.12	22.05	-3.7	39.7	14.04
Post8000	18.15	-1.3	38.8	15.40	-3.45	-16	6.4	4.89	24.35	7.3	34.9	10.26	24.35	-5.1	39.9	15.65

Amikacin treatment markedly decreased amplitude responses in all frequencies. The DPOAE changes in the other three groups were not marked. SNR: signal-to-noise ratio; SD: standard deviation

Kit, Fluorescein (Roche) was used according to the manufacturer's recommendations. Tissue sections with thicknesses of 5–6 µm were deparaffinized, rehydrated, and washed with phosphate-buffered saline (PBS) solution. The specimens were then placed in sodium-citrate buffer and heated in a microwave oven at 350 watts for 5 min for antigen retrieval, followed by cooling at room temperature for 20 min. After washing with PBS three times for 5 min each, the tissues were incubated with the TUNEL reaction mixture at 37°C for 60 min in a dark and humid environment. Following another washing procedure with PBS (three times for 5 min), the tissues were counter-stained with DAPI. The specimens were mounted with glycerol solution and visualized under a fluorescence microscope (Olympus BX-51) in the wavelength range of 450–500 nm.

Statistical Analysis

For statistical analysis, the variables were expressed and used as number (n), percentage (%), and mean±standard deviation. The Shapiro–Wilk

test, Q-Q, and histograms were used for assessment of the normality of the data. Comparisons were made using two-way repeated measures analysis of variance. The Bonferroni test was performed for multiple comparisons. Values of p<0.05 were considered to be statistically significant.

RESULTS

Evaluation of DPOAE Responses

The pre-experiment DPOAE measurements of the subjects revealed no statistically significant difference when intragroup and intergroup comparisons were made. Also, the group averages and the right and left ear values were statistically similar (p>0.05).

1st Group (Control Group): After treatment of the subjects in this group with 4% ethanol, the L and SNR values obtained at all tones on day 0 (pre-treatment) and day 21 were compared, and the results were statistically similar (p>0.05) (Table 1, 2).

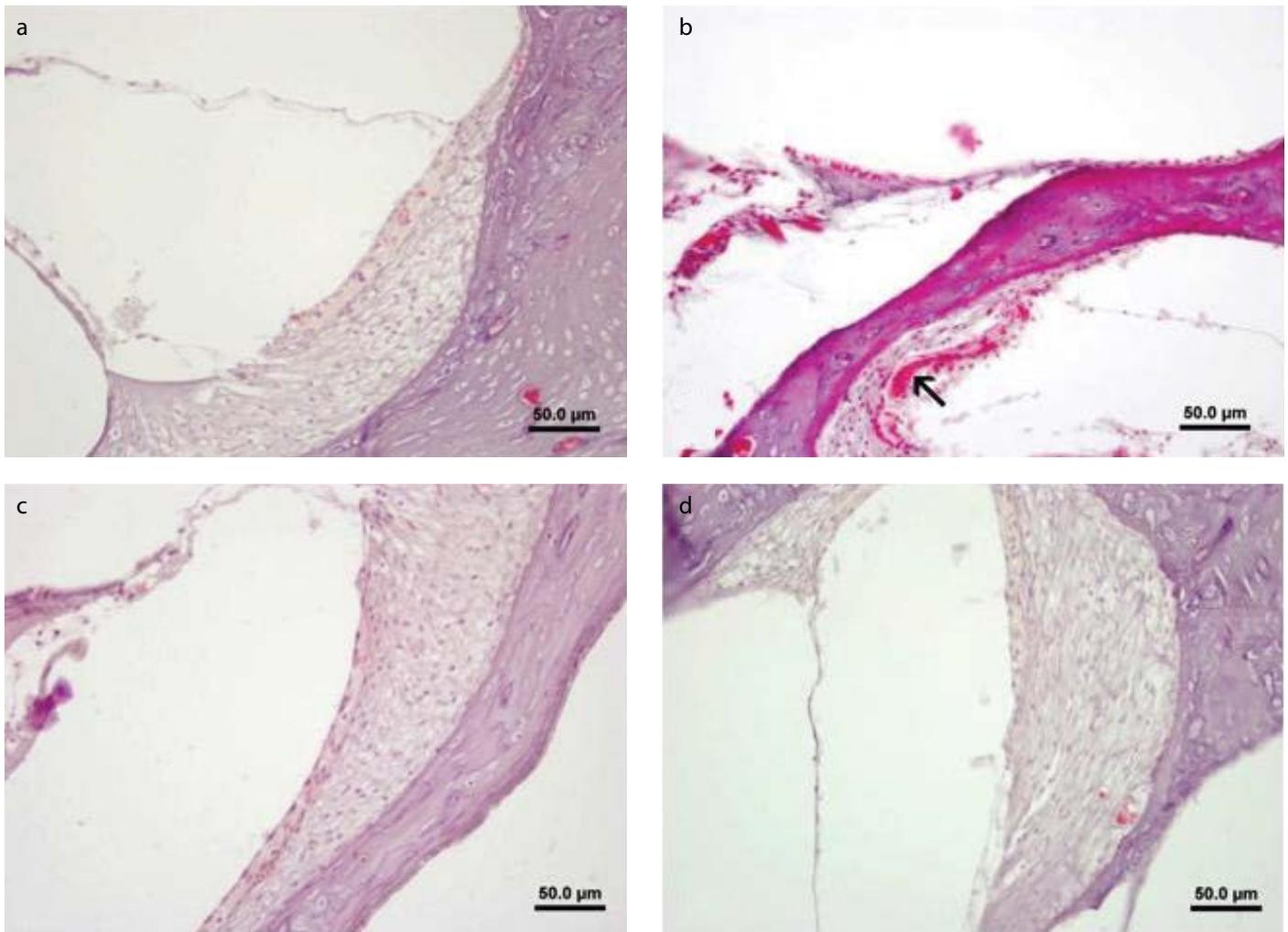


Figure 1. a-d. Microscopic images of stria vascularis. (a) Control group, (b) amikacin group; congested and dilated vascular structures are shown (arrow), (c) amikacin + resveratrol group, (d) resveratrol only group. (Hematoxylin and eosin staining. Horizontal scale bar represents 50 µm.)

2nd Group (Amikacin Group): The mean L and SNR values obtained at each tone in the amikacin group on days 0 and 21 showed significant differences ($p < 0.05$) (Table 1, 2).

3rd Group (Amikacin + Resveratrol Group): In this group, in which the subjects were administered amikacin along with resveratrol, the L and SNR values obtained at all frequencies on day 0 were compared with the corresponding values recorded on day 21, revealing no statistical difference ($p > 0.05$) (Table 1, 2).

4th Group (Resveratrol Group): After treatment of the subjects in this group with resveratrol, the L and SNR values obtained at all tones on day 0 and day 21 were compared, and the results were not statistically different ($p > 0.05$) (Table 1, 2).

Histopathological Findings

The stria vascularis, organ of Corti, and spiral ganglion were examined under a light microscope. The samples obtained from the amikacin group showed surface irregularities, vascular dilatation, and congestion within the stria vascularis. The changes were milder in the amikacin + resveratrol group. Both the control and resveratrol only groups demonstrated normal stria vascularis structures (Figure 1).

In the amikacin group, examination of the organ of Corti showed a prominent loss of outer hair cells, whereas the loss was less prominent in the amikacin + resveratrol group. The findings were similar between specimens from the control and resveratrol only groups (Figure 2).

Eosinophilic degenerated cells were detected in the spiral ganglions of the rats belonging to the amikacin group. Degenerated ganglion cells were also present in the amikacin + resveratrol group, but to a lesser extent. Tissue examinations from the resveratrol only and control groups demonstrated similar findings (Figure 3).

TUNEL staining was performed to demonstrate apoptosis in the inner ear. The organs of Corti obtained from the animals in the control and resveratrol groups did not contain any apoptotic cells. The amikacin group showed an increased number of apoptotic cells within the organ of Corti and stria vascularis, while apoptosis was absent in the amikacin + resveratrol group (Figure 4).

DISCUSSION

In the present study, the possible protective effects of resveratrol against amikacin ototoxicity were evaluated. The signal-to-noise ratios and amplitude levels obtained in response to otoacoustic emissions, along with the histomorphological changes in the cochlea

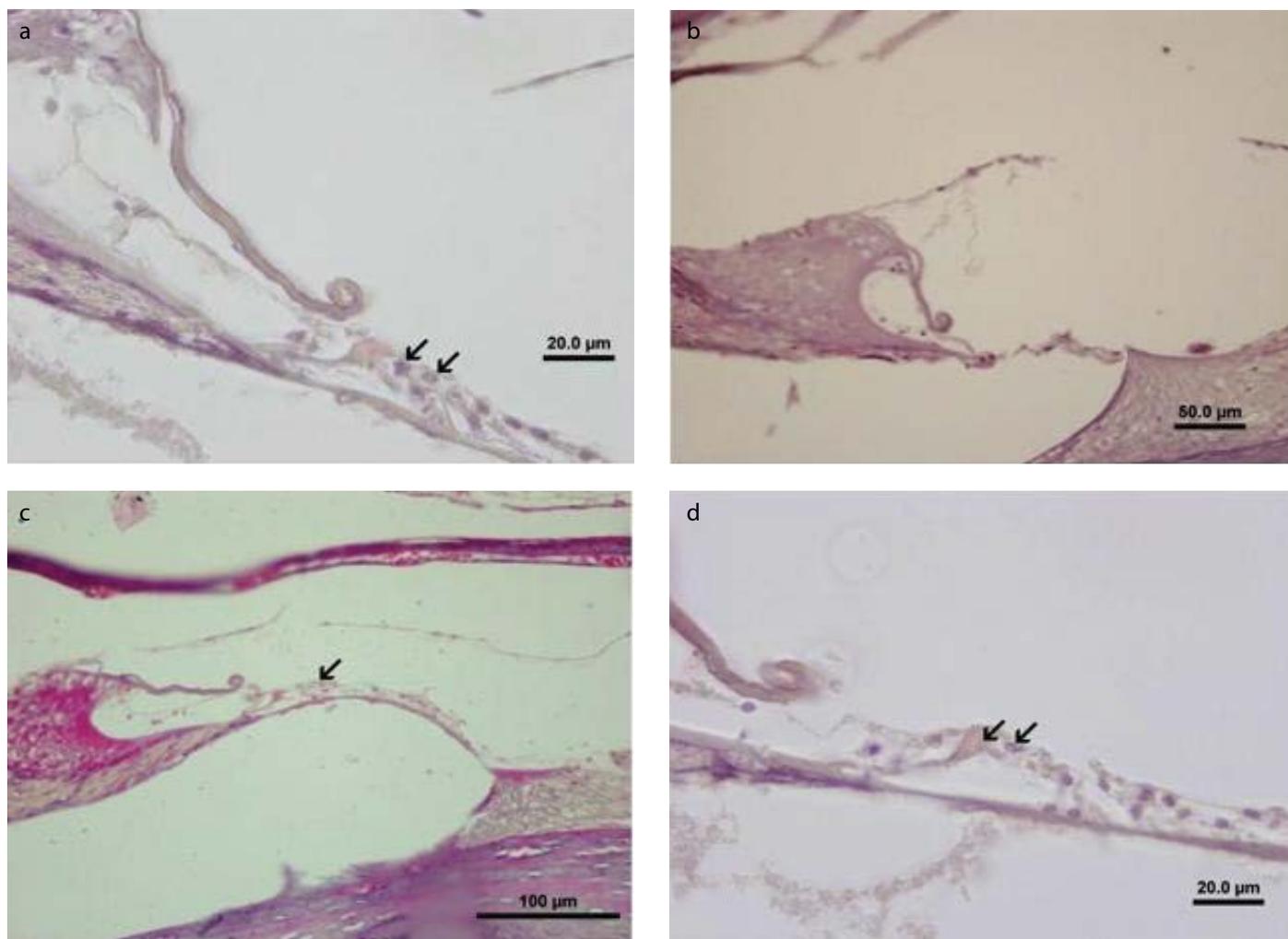


Figure 2. a-d. Microscopic images of organ of Corti. (a) Control group; outer hair cells are distinguished (arrows). (b) Amikacin group; outer hair cells are lost due to damage. (c) Amikacin + resveratrol group; only one outer hair cell is visible (arrow). (d) Resveratrol only group; outer hair cells are clearly visible (arrow). (Hematoxylin and eosin staining.)

in the amikacin group, confirmed ototoxicity. Both the otoacoustic emission results and the cochlear histopathology suggested that resveratrol had protective roles. There were more TUNEL-positive cells in the amikacin only group than in the amikacin + resveratrol group.

The ototoxicity that develops after amikacin treatment not only deteriorates quality of life, but also necessitates a change in the treatment regimen.

Studies of the ototoxic mechanism of aminoglycoside antibiotics showed that the death of OHCs occurs through apoptotic pathways^[8].

Aminoglycosides behave as free radicals and cause cell death through distinct mechanisms. Positively charged aminoglycoside molecules can easily adhere electrostatically on the negatively charged cellular and mitochondrial membranes, leading to an increase in membrane permeability by lipid peroxidation. Eventually, the cellular structures leak out, and more drug influx occurs through the membranes, which ultimately results in apoptosis^[9].

The free radicals, which are thought to play a role in ototoxicity, are byproducts of normal metabolism; also, they may arise from endog-

enous or exogenous sources such as radiation, drugs, or harmful chemicals. Drugs and radiation are the most important exogenous causes^[10]. Under normal circumstances, a baseline level of free radicals is constantly present throughout the body; the harmful effects of these free radicals are mitigated by the action of several antioxidant mechanisms^[11].

Antioxidants protect cells against the unwanted effects of drugs, carcinogens, and toxic radical reactions through several direct or indirect mechanisms. However, overproduction of free radicals or weakening of antioxidant defense mechanisms will render the occurrence of the toxic effects of these free radicals inevitable. The oxidative stress created by these free radicals is thought to play a role in the pathogenesis of ototoxicity or nephrotoxicity in diabetes, cancer, atherosclerosis, and of some drugs^[11].

The main histopathological event in amikacin ototoxicity is damage of the organ of Corti, starting from the first line OHCs residing at the basal turn (Type I cells), progressing towards the apical region, and further involving the inner hair cells. In addition to the organ of Corti, amikacin also damages the spiral ganglion and stria vascularis^[12]. In our study, OHC damage was more prominent in the amikacin group

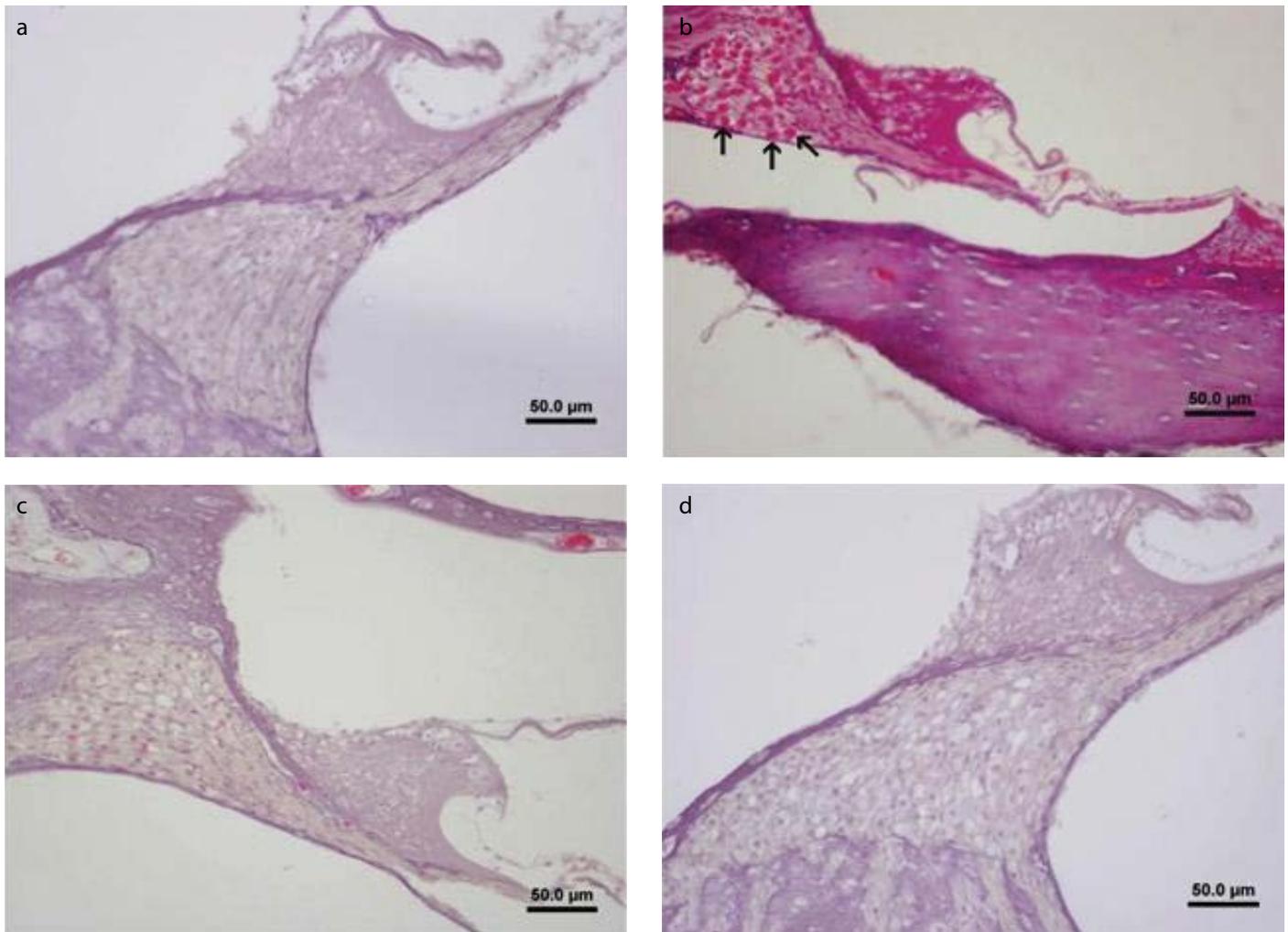


Figure 3. a-d. Microscopic images of spiral ganglion. (a) Control group, (b) amikacin group; highly eosinophilic stained degenerated neurons are depicted (arrows), (c) amikacin + resveratrol group, (d) resveratrol only group. (Hematoxylin and eosin staining. Horizontal scale bar represents 50 µm.)

than in the amikacin + resveratrol group, which may be the underlying cause of the changes in SNR and L values observed in the amikacin group.

An amikacin dose of 600 mg/kg, the dose that we used in our study, is usually sufficient to promptly observe ototoxic effects [13]. In amikacin ototoxicity, several antioxidants and other substances such as magnesium, pentoxifylline, iron chelators, glutathione, alpha-tocopherol, alpha lipoic-acid, D-methionine, dexamethasone, trimetazidine, ebselen, N-acetylcysteine, thymoquinone, and steroids have been widely used to overcome the toxic effects of the reactive oxygen species [14].

Bayındır et al. [13] showed that beta glucan had protective effects against amikacin induced cochlear damage in rats, and might be a treatment option. In the study by Bulut et al. [15] on adult Guinea pigs, magnesium was reported to have protective roles against cochlear damage caused by amikacin. Berkiten et al. [16] used Wistar albino rats as a model and found similar results for pentoxifylline, which is a derivative of methylxanthine.

Aminoglycosides are thought to exert their ototoxic effects by chelating iron molecules and behaving as free radicals. Desferrioxamine, an iron chelating agent, was shown to be partially effective in genta-

micin induced ototoxicity, through audiological and histopathological studies, by disrupting aminoglycoside-iron complexes [17]. Other iron chelating substances, such as dihydrochlorobenzoate and salicylates, also have antioxidant properties and have also been reported to be protective against amikacin toxicity [18].

In gentamicin and amikacin studies, D-methionine was demonstrated to have protective effects through several antioxidant mechanisms. This agent was proved to effect better results than a number of other antioxidants, such as glutathione, histidine, and ebselen [19]. Potent free radical scavengers such as alpha-tocopherol (Vitamin E) and alpha-lipoic acid were also found to play inhibitory roles against gentamicin ototoxicity [20,21].

Freeman et al. [22] evaluated amikacin induced cochleotoxicity and vestibulotoxicity by measuring auditory brainstem responses (ABR) and vestibular evoked potentials. They found that cochleotoxic and vestibulotoxic effects emerged on days 7 and 17, respectively. In that study, histopathological evaluation was carried out long after the amikacin treatment had terminated; this evaluation revealed serious cochlear damage and a protected utriculus and sacculus. The findings suggested that the cochlear toxicity of amikacin was greater than its vestibular toxicity.

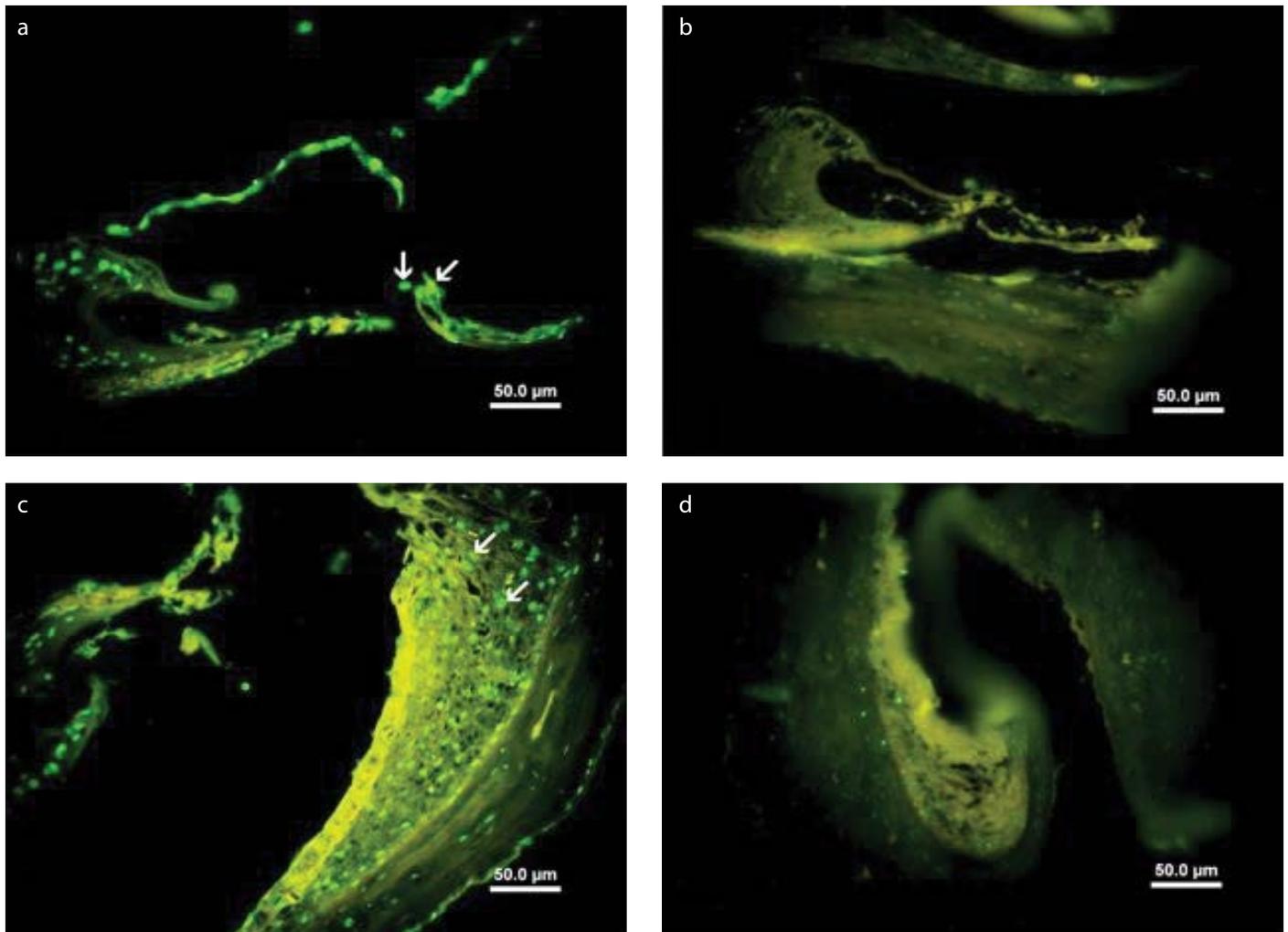


Figure 4. a-d. Fluorescent microscopic images of TUNEL stained specimens. (a) Amikacin group; apoptotic cells in the organ of Corti (arrows), (b) amikacin + resveratrol group; no apoptotic cells detected in the organ of Corti, (c) amikacin group; apoptotic cells in the stria vascularis (arrows), (d) amikacin + resveratrol group; no apoptotic cells detected in the stria vascularis.

Resveratrol (3, 5, 4'-trihydroxystilbene) is a phenolic compound found in several fruits and vegetables, especially in the skins and seed of black grapes. Plants use resveratrol as a phytoalexin for protection against a number of fungal infections (*Botrytis cinera*, in particular) and ultraviolet radiation [7].

The biological roles of resveratrol include scavenging of free radicals, inhibition of lipid peroxidation, anti-inflammatory effects, copper chelation, modification of eicosanoid synthesis, inhibition of platelet aggregation, vasodilatation, modulation of lipid metabolism, anti-cancer effects, and estrogenic activities [7].

Bonabi et al. [23] studied the protective effects of resveratrol on hair cells against gentamicin ototoxicity. They suggested that the free radical scavenging action of resveratrol may be associated with nuclear factor kappa B (NFkB) activity, which is essential for the survival of immature hair cells. They prepared cell cultures from the organ of Corti of a newborn rat and established experimental groups that were exposed to varying doses of resveratrol + gentamicin or gentamicin only. The results showed that resveratrol administered at doses of not only 10 mg/kg but also 100 mg/kg protected cells from ototoxicity.

Yumuşakhuylu et al. [24] examined the possible protective effects of resveratrol against cisplatin ototoxicity; they found a statistically significant difference between the resveratrol + cisplatin and cisplatin only groups.

Considering its effective dose, timing, and method of administration, resveratrol may serve as a valuable antioxidant agent for minimizing the ototoxicity of not only amikacin, but also other substances.

Resveratrol does not exert any adverse effects on the inner ear when used alone. In our experimental model, amikacin successfully triggered ototoxicity, which is evident from the decreasing DPOAE results and morphological findings; furthermore, resveratrol showed a significant protection from this toxicity.

Ethics Committee Approval: Ethics committee approval was received for this study from the Experimental Animals Ethics Committee of Erciyes University (No: 14/026).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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

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