

# Protective effect of ethyl pyruvate on amikacin-induced ototoxicity in rats

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**Abstract. – OBJECTIVE:** Amikacin (AMK) is a widely used antibiotic, but its ototoxic side effects limit its use. This study investigated the effects of ethyl pyruvate (EP), known for its antioxidant and anti-inflammatory effects, against AMK ototoxicity.

**MATERIALS AND METHODS:** 32 Wistar albino rats (n: 8) were used in this study. To cause ototoxicity, AMK 600 mg/kg/day dose was applied intramuscularly for 14 days. EP was administered *via ip* at a dose of 50 mg/kg/day for 14 days.

**RESULTS:** The Auditory Brainstem Responses (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) tests were performed on the study's 0, 7, and 14 days. The results have shown that the hearing functions were significantly impaired with the AMK application. A significant improvement was observed in the AMK+EP group. While total oxidant status (TOS), oxidative stress index (OSI), and malondialdehyde (MDA) levels were found to be significantly higher in the AMK group compared to the control group, total antioxidant status (TAS) level was found to be significantly lower. In the AMK+EP group, on the other hand, deterioration in TOS, OSI, and MDA levels detected in the AMK group was not observed. No elevated pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were present in the EP+AMK group, which were detected in the AMK group.

**CONCLUSIONS:** Hearing tests and biochemical results show that ethyl pyruvate has protective effects against amikacin ototoxicity due to its antioxidant and anti-inflammatory effects.

*Key Words:*

Amikacin, Ototoxicity, Ethyle pyruvate, Auditory brainstem responses (ABR), Distortion product otoacoustic emissions (DPOAE), Total oxidant status (TOS), Total antioxidant status (TAS), Pro-inflammatory cytokines.

## Introduction

Ototoxicity is a clinical condition caused by some chemicals, including drugs. Hearing and

balance function disorders may be seen in this clinical situation<sup>1</sup>. More than a hundred drugs have been reported to cause ototoxicity. Aminoglycosides (AG), loop diuretics, and antineoplastic drugs are among the drugs known to cause ototoxicity<sup>2</sup>. AGs are antibiotics that have been frequently preferred for many years to treat gram-negative bacterial infections<sup>3</sup>. Amikacin (AMK) is a semi-synthetic AG derivative produced from the naturally occurring compound kanamycin<sup>2,4</sup>. While AGs, such as streptomycin mainly damage the vestibular system, AGs such as AMK and kanamycin primarily impair hearing functions<sup>5,6</sup>. Although many years have passed since the discovery of AMK, physicians still frequently prefer it due to its cheapness and synergistic effect with penicillin. In parallel, at least 10% of AMK users have experienced ototoxic side effects<sup>5</sup>. Hearing loss caused by AMK is usually bilateral, progressive and mostly directed towards high frequency sounds<sup>5</sup>.

It has been shown that the formation of reactive oxygen species (ROS) in the ear with the use of AMK plays a critical role in studies carried out to show by which mechanism it causes ototoxicity. Increased ROS in cochlear tissue results in apoptosis and ototoxicity<sup>2,7</sup>. In many studies<sup>2,7-9</sup> based on this information, the protective effects of antioxidant active substances against AMK toxicity have been demonstrated.

Pyruvate reduces hydrogen peroxide non-enzymatically and its successful hydroxyl radical scavenging effects. However, it is not stable in solutions. Therefore, it is unsuitable for use as a therapeutic agent<sup>10</sup>. Ethyl pyruvate (EP) is a non-toxic food additive, a simple aliphatic ester derivative of pyruvic acid<sup>11</sup>. Studies<sup>11-13</sup> have shown that EP has potent antioxidant and anti-inflammatory effects. It shows its anti-inflammatory effects by inhibiting high mobility group box protein-1 (HMGB1)<sup>14</sup>. HMGB1 is a nuclear

protein found in all cells. HMGB1 is actively secreted extracellularly in stimulated cells and is involved in activating many inflammatory pathways<sup>14</sup>.

This study aimed to investigate the possible protective effects of EP with known antioxidant and anti-inflammatory effects against AMK ototoxicity and whether its anti-inflammatory properties contribute to this effect.

## Materials and Methods

### Animals

This study was carried out with Dicle University Experimental Animals Local Ethics Committee approval (2021/3). The NIH's principles regarding the "Protection of Animal Rights" concerning animal rights were meticulously followed during the study. 32 male Wistar albino rats weighing approximately 200-250-grams were used in the experimental procedures. The rats were housed in separate cages in standard laboratory conditions (12 hours of light/dark cycle, 23±2°C, and 60% humidity). The rats were fed *ad libitum*.

### Experimental Groups and Drug Administration

The study was designed over four groups (n: 8). Control group (CNT): 1 ml of saline (intramuscular, i.m.) injection was given once a day for 14 days. AMK group: To create ototoxicity, AMK (i.m.) was administered at a dose of 600 mg/kg daily for 14 days. AMK+EP group was administered 600 mg/kg daily AMK (im) + 50 mg/kg dose EP (i.p.) for 14 days. EP group: Ethyl pyruvate (i.p.) at a dose of 50 mg/kg daily was administered for 14 days. A The drug called Amikaver (Osel Pharmaceuticals, Istanbul, Marmara, Turkey) was used as AMK. EP was obtained from Sigma Chemical (Sigma Chemical Co, St. Louis, MO, USA). The doses applied for both drugs were decided after the literature review<sup>2,15</sup>. On days 0, 7, and 14 of the study, rats were anesthetized by giving ketamine+xylazine (i.m.) (ketamine: 50 mg/kg, Ketalar; Pfizer, Istanbul, Marmara, Turkey. xylazine hydrochloride: 8 mg/kg, Rompun; Bayer, Istanbul, Marmara, Turkey). First of all, the external ear canal and tympanic membranes of the rats were evaluated by otoscopy and the study was continued with the rats that were found to be normal. Auditory Brainstem Responses (ABR)

and Distortion Product Otoacoustic Emissions (DPOAE) measurements were performed in rats under anesthesia on days 0, 7, and 14. After the ABR and DPOAE measurements were made on the last day of the study, the rats were sacrificed, and their cochlea tissues were taken and kept at -80°C for biochemical analysis.

### ABR Measurement

The ABR threshold measurements were performed in anesthetized rats in a quiet environment. Measurements were made with the Interacoustics EP25 (Interacoustics, Eden Prairie, MN, USA) device using disposable surface snap electrodes. Electrodes were placed on the vertex, glabella and mastoid areas. The filter was adjusted in the band range of 30-1500 Hz, with a repetition rate of 21.1 seconds. The stimuli were started at 80 dB and decreased by 10 dB each time. At least two measurements were made to confirm the threshold value.

### DPOAE Measurement

Peripheral hearing system of rats was evaluated *via* the DPOAE test using the ILO-288 otoacoustic Emission Instrument (Otodynamics, Herts, London, UK). Measurements were made in a special quiet room while the rats were under anesthesia. Neonatal rubber probes were used for measurements. Otoacoustic emissions were measured using different frequencies and intensities. Equilevel primary tones f 1 (65 SPL) and f 2 (55 SPL) were fixed at f2/f1 = 1.22, and the DPOAE were measured at 8 different frequencies between 1000 and 8000 Hz (1000, 2000, 3000, 4000, 5000, 6000, 7000 and 8000 Hz). The DPOAEs were established in DP-grams.

### Biochemical Analyzes

Cochlear tissue total oxidant status (TOS), total antioxidant status (TAS), and malondialdehyde (MDA) levels were analyzed by the ELISA method. Tissue samples were homogenized by adding 9 times their weight of iced phosphate-buffered saline (PBS; 0.01M, pH:7.4) solution. Centrifugation was done at 5000 g for 5 minutes, and the supernatant was separated for analysis. Commercial kits were used to measure TOS, TAS (Rel Assay Diagnostic, Gaziantep, Guneydoğu Anadolu, Turkey), and MDA (Elabscience, Wuhan, Hubei, China) levels using an automated ELISA plate analyzer (Robonik readwell touch, Ambarnath, Thane, India). MDA results were expressed as ng/gram tissue, TOS

results were expressed in terms of micromolar hydrogen peroxide equivalent/L, and TAS results were expressed as mmol Trolox equivalent/L. The oxidative stress index (OSI), which is a good indicator of the degree of oxidative stress, was calculated by dividing the TOS value by the TAS value ( $OSI=TOS/TAS$ ).

Gene expression levels of pro-inflammatory cytokines interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- $\alpha$ ) were measured by real-time PCR. The ready kit (RNA Easy kit, Qiagen, Hilden, Germany) was used as described by the manufacturer for the isolation of total RNA from tissue. The extracted RNA was dissolved in nuclease-free distilled water and stored at -20°C. Real-time PCR was performed using 2  $\mu$ L template in a 20- $\mu$ L reaction containing 0.25  $\mu$ M of each primer and 12.5  $\mu$ L Sybr Green Real-time PCR MasterMix (Qiagen, Hilden, Germany). Each run consisted of 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 60 s in a real-time qPCR (Rotor Gene Q, Qiagen, Hilden, Germany).

### Statistical Analysis

The compatibility of the data obtained in the study with normal distribution was checked with the Kolmogorov-Smirnov test. Normally distributed data were expressed as arithmetic mean  $\pm$  standard deviation. One-way ANOVA and post hoc Tukey test were used to analyze these data. Repeated measures ANOVA and post-hoc Bonferroni test were used to analyze repeated measures. SPSS 21.0 program (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The significance level was taken as  $p < 0.05$ .

## Results

### ABR Results

The ABR thresholds results of the subjects and the in-group and intergroup comparison results of the data are given in Table I. While there was no significant difference between the groups in terms of Day 0 results ( $p > 0.05$ ), there was a significant difference between the group's Day 7 and Day 14 ABR thresholds results. In both measurements, the results obtained in the AMK group were significantly higher than the other three ( $p = 0$  for post-hoc Tukey test).

No significant change was observed in the CNT, AMK+EP, and EP groups in analyzing the data obtained in the repeated measurements of the subjects at 0, 7, and 14 days ( $p > 0.05$ ). Considering the repeated measurements made in the AMK group, the ABR value obtained on day 0 was  $26.5 \pm 2.26$ , while it reached  $37.3 \pm 4.06$  on day 7, and  $44.2 \pm 2.54$  on day 14. The continuous increase observed in repeated measurements in the AMK group was statistically significant ( $p = 0.004$ ).

### DPOAE Results

DPOAE measurements were made in the frequency range of 1000-8000 on days 0, 7, and 14 of the study. On days 7 and 14, DPOAE values were significantly lower in the AMK group than the day 0 ( $p < 0.05$ , Figure 1). In the CNT, AMK+EP, and EP groups, no significant difference was observed between 0-, 7-, and 14-day values ( $p > 0.05$ , Figures 2-4).

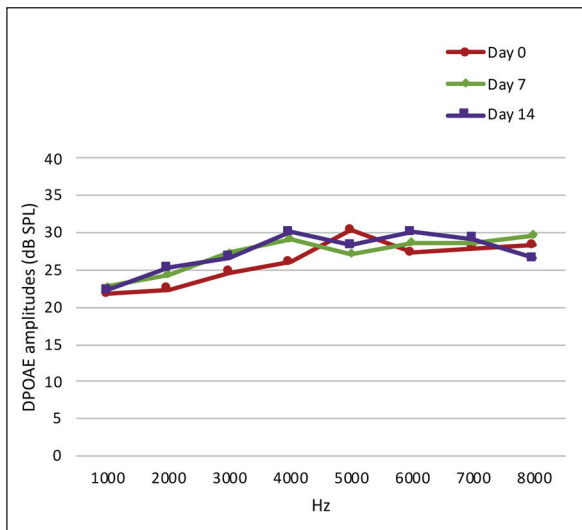
### TAS, TOS, OSI, and MDA Results

To determine the effect of EP on oxidant and antioxidant parameters, TOS, TAS, OSI, and MDA levels were analyzed in cochlear tissue samples.

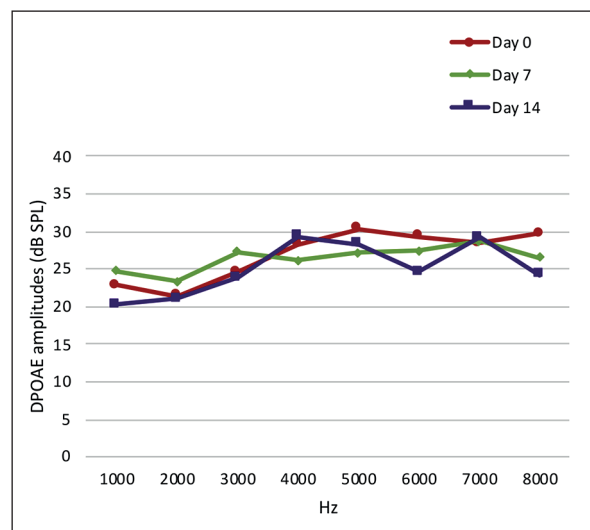
**Table I.** Comparison of ABR thresholds within groups and between groups.

Groups	Day 0	Day 7	Day 14	Repeated (ANOVA)	Bonferroni test
CNT	$26.7 \pm 2.03$	$26.1 \pm 2.72$	$25.6 \pm 2.84$	$p = 0.358$	For AMK group
AMK	$26.5 \pm 2.26$	$37.3 \pm 4.06$	$44.2 \pm 2.54$	$p = 0.004$	D0 vs. D7 ( $p = 0.003$ )
AMK+EP	$27.0 \pm 3.70$	$26.8 \pm 2.64$	$27.2 \pm 2.91$	$p = 0.687$	D1 vs. D14 ( $p = 0.001$ )
EP	$26.3 \pm 2.99$	$25.5 \pm 2.77$	$24.5 \pm 2.07$	$p = 0.590$	D7 vs. D14 ( $p = 0.009$ )
ANOVA (between groups)	$p = 0.925$	$p = 0$	$p = 0$		
Post-hoc Tukey test		For day 7 and 14			
		AMK vs. CNT ( $p = 0$ )			
		AMK vs. AMK+EP ( $p = 0$ )			
		AMK and EP ( $p = 0$ )			

Data are shown as mean  $\pm$  standard deviation. CNT: Control, AMK: Amikacin, EP: Ethyl pyruvate.



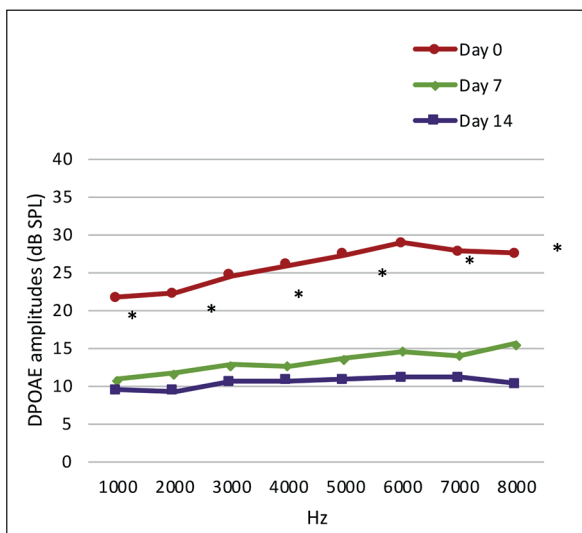
**Figure 1.** DPOAE results measured on days 0, 7 and 14 of the control group. According to the repeated measures ANOVA test performed for each frequency, no difference was found between the measurements made at 0, 7 and 14 days ( $p>0.05$ ). DPOAE: Distortion Product Otoacoustic Emissions.



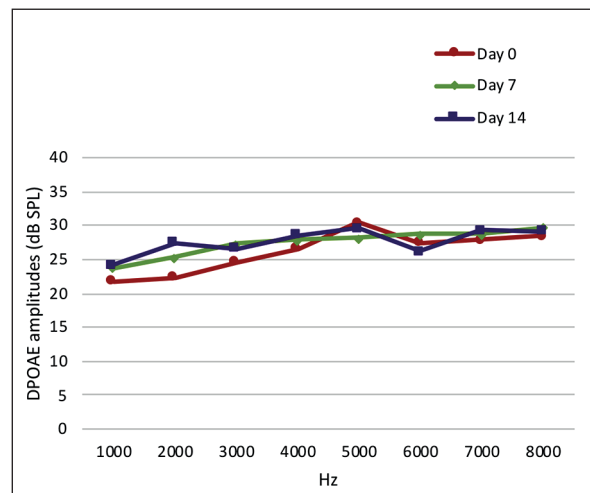
**Figure 3.** DPOAE results obtained on Day 0, 7 and 14 days in the AMK+EP group. According to the repeated measures ANOVA test performed for each frequency, no difference was found between the measurements made on 0, 7 and 14 days ( $p>0.05$ ). AMK: Amikacin, EP: Ethyl pyruvate, DPOAE: Distortion Product Otoacoustic Emissions According.

The obtained results are presented in Table II. Compared to the CNT group, TOS, OSI, and MDA levels ( $p=0.002$ ,  $p=0.001$ , and  $p=0$ , respectively) were increased in the AMK group, while the TAS level decreased significantly ( $p=0$ ). In the AMK+EP group, the deterioration in TOS,

OSI, and MDA levels observed in the AMK group improved significantly ( $p=0.021$ ,  $p=0.006$ , and  $p=0.001$ , respectively). In terms of TAS levels, a significant decrease was observed in the AMK+EP group compared to the AMK group, but this difference was not statistically significant ( $p>0.05$ ).



**Figure 2.** DPOAE results obtained on Day 0, 7 and 14 in the AMK group. AMK: Amikacin, DPOAE: Distortion Product Otoacoustic Emissions. \* $p<0.01$  for Day 0 vs. Day 7 and Day 14 (repeated measures ANOVA test).



**Figure 4.** DPOAE results obtained on Day 0, 7 and 14 in the EP group. According to the repeated measures ANOVA test performed for each frequency, no difference was found between the measurements made at 0, 7 and 14 days ( $p>0.05$ ). EP: Ethyl pyruvate, DPOAE: Distortion Product Otoacoustic Emissions.

**Table II.** TOS, TAS, OSI and MDA results of the groups.

Groups	TOS ( $\mu\text{mol H}_2\text{O}_2$ Eqv/L)	TAS (mmol Trolox Eqv/L)	OSI (TOS/TAS)	MDA (ng/g tissue)
CNT	5.96 $\pm$ 2.97	2.05 $\pm$ 0.47	3.05 $\pm$ 1.58	1.68 $\pm$ 0.69
AMK	13.5 $\pm$ 6.34	1.15 $\pm$ 0.30	13.3 $\pm$ 8.25	4.75 $\pm$ 1.74
AMK+EP	7.76 $\pm$ 2.54	1.51 $\pm$ 0.30	5.43 $\pm$ 2.33	2.42 $\pm$ 0.73
EP	4.93 $\pm$ 1.69	2.15 $\pm$ 0.52	2.29 $\pm$ 0.71	1.59 $\pm$ 0.61
ANOVA test	$p=0$	$p=0$	$p=0$	$p=0$
Post-hoc	CNT vs. AMK ( $p=0.002$ )	CNT vs. AMK ( $p=0.001$ )	CNT vs. AMK ( $p=0$ )	CNT vs. AMK ( $p=0$ )
Tukey test	AMK vs. AMK+EP ( $p=0.021$ ) AMK vs. EP ( $p=0$ )	AMK vs. EP ( $p=0$ )	AMK vs. AMK+EP ( $p=0.006$ ) AMK vs. EP ( $p=0$ )	AMK vs. AMK+EP ( $p=0.001$ ) AMK vs. EP ( $p=0$ )

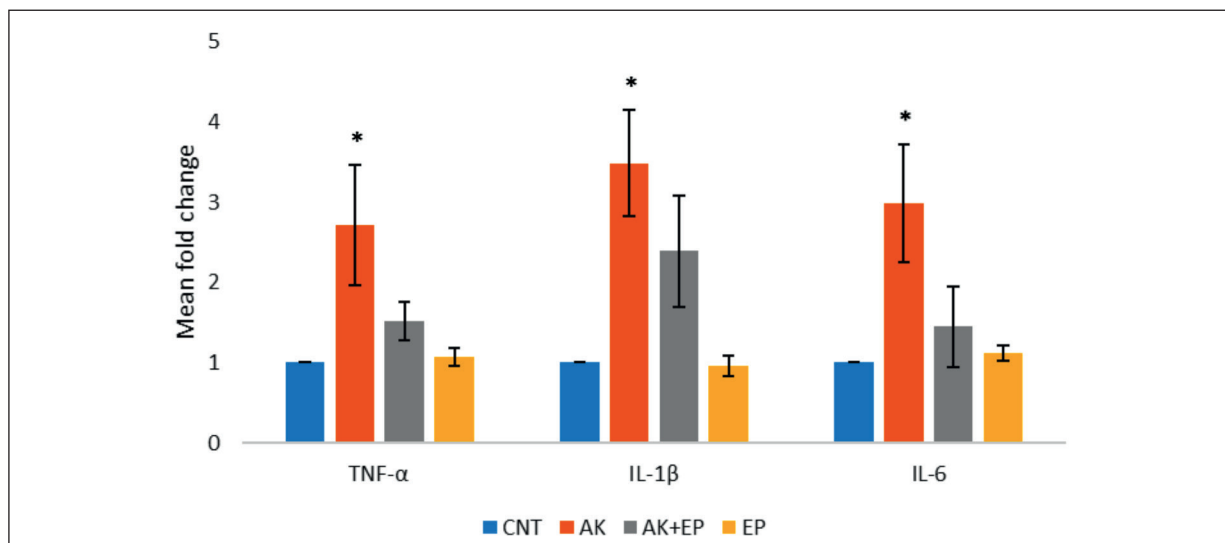
Data are presented as mean  $\pm$  standard deviation. CNT: Control, AMK: Amikacin, EP: Ethyl pyruvate, TOS: Total oxidant status, TAS: Total antioxidant status, OSI: oxidative stress index, MDA: Malondialdehyde.

### TNF- $\alpha$ , IL-1 $\beta$ and IL-6 Results

Pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in cochlear tissue were determined by Real-Time PCR method. It was observed that the levels of all three pro-inflammatory cytokines increased statistically significantly in the AMK group compared to the CNT group ( $p<0.001$ , Figure 5). In the AMK+EP group, the increase in pro-inflammatory cytokine levels observed with AMK application was not observed. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels were significantly lower in the AMK+EP group than in the AMK group ( $p<0.001$ , Figure 5).

### Discussion

Due to AMK use, the cochlea expands from the base to the apex in ototoxicity. In advanced cases, the damage may extend to the vascular structure and the 8<sup>th</sup> nerve<sup>16</sup>. Scholars<sup>17</sup> have shown that the initiating cause of AMK ototoxicity is an increase in the amount of ROS. The cochlea can secrete many ROS-scavenging antioxidant enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT). GPx converts glutathione, which is involved in H<sub>2</sub>O<sub>2</sub> detoxification, from oxidized to reduced form.



**Figure 5.** TNF- $\alpha$ , IL-1 $\beta$  and IL-6 values measured by real time PCR. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 values in the AMK group were found to be significantly higher when compared to other groups ( $*p<0.001$  vs. other groups, ANOVA and post-hoc Tukey test). CNT: Control, AMK: Amikacin, EP: Ethyl pyruvate.

Other enzymes, SOD, catalyze singlet oxygen and CAT, catalyze hydrogen peroxide. Ototoxic drugs cause an increase in ROS and a decrease in antioxidant enzymes in the cochlea<sup>2,16,17</sup>. Cellular damage begins when the amount of accumulated radicals exceeds the capacity of the ROS scavenging mechanisms. ROS primarily reacts with the phospholipid, protein, and DNA structure of the cell. As a result, irreversible damage occurs in outer hair cells, and apoptosis is triggered<sup>8,15,18</sup>.

The two most damaged structures in AMK ototoxicity are the outer hair cells and, in advanced cases, the auditory pathways extending up to the 8<sup>th</sup> nerve<sup>16,19,20</sup>. The functions of these structures can be evaluated objectively with DPOAE and ABR tests<sup>2,16,18</sup>. In this study, DPOAE values were reduced with AMK treatments on the 7<sup>th</sup> and 14<sup>th</sup> days compared to initial values. This reduction occurred at all frequencies in the 1000-8000 Hz range. These results show us that AMK causes outer hair cell damage. However, a similar decrease was not observed in the AMK+EP group. However, this reduction was prevented with EP treatments.

ABR test is utilized to evaluate hearing functions. ABR thresholds were similar between the test groups initially. The thresholds were increased on the 7<sup>th</sup> and 14<sup>th</sup>-day evaluations in the AMK group. Similar increments were not observed in EP-applied groups (including the AMK+EP group). Considering the ABR and DPOAE test results, toxic effects of AMK on the outer hair cell and auditory pathways were apparent. EP was observed to be advantageous in reducing these alterations.

EP is an antioxidant and anti-inflammatory molecule. Restorative effects of EP were shown in many pathological conditions<sup>11,12,15,21</sup>. EP non-enzymatically reduces hydrogen peroxide, and it is a successful hydroxyl radical scavenger<sup>10,22</sup>. By inhibiting the HMGB1 protein, EP possesses anti-inflammatory effects<sup>14,23</sup>. HMGB1 is a highly conserved nuclear protein present in all cell types. It is a multi-facet protein exerting functions both inside and outside of cells. Under pathological conditions, excessive extracellular release of HMGB1 increases pro-inflammatory cytokines. This increment leads to signal transducer and activator of transcription-1 (STAT-1) activation and eventually to inflammation and apoptosis<sup>12,14,23</sup>.

In this study, we measured TOS and TAS levels to determine the effects of AMK and EP on the oxidant/antioxidant balance in the cochlear tissue. We calculated the OSI value by com-

paring these two parameters to each other. We preferred to measure TAS and TOS levels practically because they allow revealing the situation with a single parameter instead of measuring individual antioxidant and oxidant parameters, respectively<sup>24,25</sup>. The OSI value (TOS/TAS) has been defined as a good indicator of oxidative stress, and the MDA level is an essential indicator of lipid peroxidation<sup>24,25</sup>. In this study, the TAS levels were lower while MDA levels, TOS and OSI levels were higher in the AMK group than in the CNT group. Concordant with the literature, these results<sup>2,8,16,18</sup> demonstrate disrupting effects of AMK in the cochlear tissue through affecting the oxidant/antioxidant balance. TOS, OSI, and MDA levels were significantly lower in the AMK+EP group than in the AMK group. No difference was observed in terms of TAS level. These results show that EP inhibits lipid peroxidation by reducing pro-oxidant molecules in cochlear tissue. The absence of difference in TAS levels may be due to the effects of EP on non-enzymatic rather than enzymatic antioxidant molecules.

This study measured the levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 with a real-time PCR. The levels of these three cytokines in the AMK group were higher than in the CNT group. This result showed that inflammation might have an essential role in AMK ototoxicity. Mentioned cytokine levels were reduced with EP treatments. Previous studies<sup>12,14</sup> have reported the anti-inflammatory effects of EP. EP shows its anti-inflammatory effect by inhibiting the extracellular secretion of a nuclear protein, HMGB1. Excessive release of HMGB1 due to ROS stimulus in cells causes an increase in the levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. These cytokines lead to STAT-1 activation and ultimately to inflammation and apoptosis<sup>12</sup>. These changes in pro-inflammatory cytokine levels observed in AMK and AMK+EP groups indicate that increased inflammation contributes significantly to AMK ototoxicity. The protective effects of EP against AMK ototoxicity are possibly due to its anti-inflammatory effects through HMGB1 inhibition and antioxidant effects.

## Conclusions

Considering the results obtained in the study, AMK increases oxidation and inflammation in the ear and impairs hearing functions. Ototoxicity caused by AMK could be prevented with EP

treatment. This effect appears to be due to EP's antioxidant and anti-inflammatory effect through inhibition of HMGB1. EP can be considered an adjunct therapeutic option while prescribing ototoxic molecules, such as amikacin.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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### Authors' Contribution

All authors contributed toward study conception and design, literature search, data analysis, drafting, and critically revising the paper, gave final approval of the version to be published.

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