Objectives: The purpose of this study is to investigate the effectiveness of pomegranate extract (PE) as a protection agent against cisplatin ototoxicity.

Materials and Methods: Healthy Wistar rats (n = 18) were randomly divided into three groups. The rats in group 1 received neither cisplatin nor PE. The rats in group 2 underwent cisplatin injections for three days. PE (100 µL/day) was administered via gavage to group 3 for one day before cisplatin injections and 3 days together with cisplatin injections. Before and 3 days after injections, the ototoxic effect was measured with distortion product otoacoustic emissions (DPOAE). After DPOAE measurement, the animals were sacrificed, and the right cochlea were harvested. The level of reactive oxygen species (ROS) was measured using a chemiluminescence method.

Results: DPOAE amplitudes decreased significantly in group 2 between baseline and third day of injection at the frequencies tested (3, 4, 6 and 8 kHz) (p<0.05). Otherwise, in group 3, there were no significant differences in the DPOAE amplitudes (p>0.05). ROS levels were significantly lower in group 3 that received cisplatin plus systemic PE, compared to group 2 (p<0.01). However, the ROS levels were higher in group 3 than in the group 1.

Conclusions: Our results indicate that systemic PE decreased the ROS levels in the cochlea and reduced the DPOAE amplitudes after cisplatin injection.

Introduction
Cisplatin (cis-diamminedichloroplatinum II) is commonly used in the treatment of a variety of neoplasms, especially those of the head and neck. Its anticancer effect is obtained by several mechanisms, including formation of DNA adducts and production of reactive oxygen species (ROS) [1]. Its side effects include ototoxicity, nephrotoxicity, marrow suppression and gastrointestinal disorders [2]. Ototoxicity has been observed in up to 36% of patients receiving cisplatin [3]. Ototoxicity may occur within hours to days after treatment with cisplatin. The hearing loss appears to be dose related, cumulative, bilateral, usually permanent and occurs initially in the higher frequencies.

Although the mechanism of the antineoplastic effect of cisplatin has been well described, the cellular and molecular mechanisms of cisplatin-induced ototoxicity are not well understood. Increasing evidence indicates that the accumulation of ROS mediates cisplatin ototoxicity [4]. Cochlear damage is one of the most common reasons for interrupting chemotherapy with this drug. Cisplatin destroys the outer hair cells in the cochlea in a progressive manner, from the base to the apex. The ototoxicity of cisplatin is not limited to hair cells but also includes atrophy of the stria vascularis, collapse of Reissner’s membrane, and damage to the cells supporting the organ of Corti [5].

A detailed description of eventual dysfunction in cochlea caused by cisplatin ototoxicity can be obtained via recordings of the otoacoustic emissions [6]. In addition, otoacoustic emissions can establish not only the presence of an ototoxic effect, but also evidence regarding the progress of ototoxicity as seen from the perspective of the outer hair cells. Distortion products otoacoustic emissions (DPOAE) are otoacoustic emissions produced by the simultaneous presentation of two pure continuous tones related in the frequency. Because of the ease of measuring and interpretation, DPOAE are frequently used.
A relationship between oxygen-derived free radicals and cisplatin ototoxicity has been demonstrated in experimental models, and previous reports indicate that free radical scavengers, such as melatonin and N-acetylcysteine, can reduce ototoxicity caused by cisplatin \[7,8\]. We chose pomegranate to investigate its possible otoprotective effects because it has antioxidating properties and no side effects in human beings.

Pomegranates have been used to treat inflammatory disease for centuries and have been reported to prevent atherogenesis and urolithiasis \[9,10\]. Pomegranate juice (PE) is a rich source of potent polyphenolic, flavonoid antioxidants. The soluble polyphenol content in PE varies from 0.2-1.0%, depending on the variety, and it contains primarily anthocyanins, catechins, tannins, gallic acid, and ellagic acid \[11\].

The present study had two aims: to detect ROS in the cochlea of rats using the chemiluminescence method, and to investigate the protective effect of a potent polyphenolic-class antioxidant, PE, on cochlea as evaluated by DPOAE.

**Material and Methods**

**Experimental design**

The present study was approved by the Animal Ethics Committee of Istanbul University, Istanbul Medical Faculty (Istanbul, Turkey). Healthy Wistar rats (n = 18; 200-300 g) were used. All animals were housed in a 14-h light / 10-h dark cycle with free access to food and water. Prior to the study, the rats were anesthetized with 30 mg/kg ketamine hydrochloride and 4 mg/kg xylazine intraperitoneally assessed by otoscopic examination. Animals that showed signs of ear disease were excluded from the study.

**Exclusion criteria**

Exclusion criteria were as follows: otoscopically detectable external ear abnormalities (e.g. edema and hyperaemia of the external auditory canal, tumor growths or cerumen impaction); signs of middle-ear disease (e.g. bulging, opacification, hyperaemia or tympanic membrane perforation); and absence of DPOAE at any of the frequency ranges tested.

**Drugs**

The animals were anesthetized with 30 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi Ilac Sanayi ve Ticaret A.S, Luleburgaz, Turkey) and 4 mg/kg xylazine (Alf azyne 2%, Alfasan International B.V, Woerden, The Netherlands). The study used 8mg/kg cisplatin (Cisplatin DBL, Faulding Pharmaceuticals, Warwickshire, UK) as determined by de Freitas et al \[12\].

**Standardized Pomegranate extract processing**

Fresh pomegranates were washed, crushed, then squeezed, and treated enzymatically with pectinase to yield PE and by-products, which included the inner and outer peels and seeds. Pectinase hydrolyses α-1,4-galacturonide bonds in pectin and thus improves extraction and filtration and prevents the formation of pectin gels. The extract was filtered, pasteurized, concentrated, and stored at -180°C as described previously \[11\]. Concentrated PE was diluted in water (20 ml of concentrated extract in 500 ml of distilled water). Therefore the standardized average of 2.5 ml combination contains 100 µl PE, which are equivalent to 2.8 µmol total polyphenol per day.

**Study groups**

The rats were divided into three groups. In group one (n= 6), rats were untreated. In group two (n= 6), rats were treated with 8 mg/kg/day cisplatin intraperitoneally for three consecutive days. In group three (n= 6), rats were treated with 8 mg/kg/day cisplatin intraperitoneally for three consecutive days and received 2.5 ml PE (100 µL/day) via gavage for one day before cisplatin injection and 3 days together with cisplatin injections.

**Procedures**

Immediately before study, rats with normal otoscopic findings were anaesthetised deeply with 50 mg/kg ketamine plus 10 mg/kg xylazine and subjected to DPOAE testing. The rats were then separated into three groups and received their allocated therapy (group 1: nontreated, group 2: received cisplatin, and group 3: received cisplatin plus PE). Group 1 was sacrificed and right temporal bones and kidneys were harvested immediately after DPOAE testing. For the other groups, 24 h after their last injection, the animals were again anaesthetised and examined otoscopically to exclude new middle-ear abnormalities. Those with normal otoscopic findings were again subjected to DPOAE testing. Immediately after the second DPOAE test, the animals were then given a lethal dose of ketamine hydrochloride, administered intraperitoneally, and the right temporal bones and kidneys were immediately harvested. The temporal bone and kidney were used for luminol-enhanced chemiluminescence measurements of ROS.
Chemiluminescence detection of ROS levels
Cochleas were prepared as previously described \cite{13}. Each cochlea was dissected and placed in phosphate buffered saline (PBS). The bony lateral wall of the cochlea was thoroughly opened and removed under a dissecting microscope. The tissue remaining in the preparation included the modiolus and the membranous cochlea. The whole preparation of each cochlea was incubated in each separate experiment.

The tissues were washed with ice-cold saline solution and analyzed freshly as described later in this section. After 10 minutes, the specimens were studied to detect ROS levels with chemiluminescence as described previously \cite{7}.

Chemiluminescence measurements were made at room temperature using a Mini Lumat LB 9506 luminometer (EG&G Berthold) in the presence of 0.2 mmol/L luminol containing PBS-HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer (0.5mol/L phosphate buffered saline containing 20 mmol/L HEPES). Counts were obtained at 5-second intervals, and the results were given as the area under curve (AUC) for a luminol chemiluminescence counting period of 5 minutes. Tissues were weighed at the end of each experiment after the solution was drained. The results were expressed as AUC of CL and observed as rlu/mg tissue. (rlu: relative light unit, per milligram of tissue).

Distortion product evoked otoacoustic emission testing
DPOAE were tested with a Otodynamics Echoport USB cochlear emissions analyzer and Otodynamics ILO version 6.0 software (Otodynamics, London, UK) in a silent room. An infant hearing screening probe was attached to the external auditory canal. The stimulus consisted of two pure tones (F1 and F2; F1/F2 ratio= 1.22) at 70 dB SPL. A total of 1000 acquisitions were analysed. The resultant otoacoustic emissions were evaluated at 3, 4, 6 and 8 kHz. DPOAE testing was considered positive for signal to noise ratios of 6 dB SPL, as specified by the manufacturer.

Statistical Analysis
The data were analyzed using the Wilcoxon signed-rank test, Mann-Whitney U test and Kruskal Wallis variance analysis in NCSS 2007 and PASS 2008 statistical software (NCSS, Kaysville, Utah, USA). The statistical analysis subsection that data are expressed as mean ± SD, p values < 0.05 were deemed to indicate statistical significance.
property of PE played a protective role against cisplatin induced ototoxicity. To our knowledge, this is the first study to investigate the protective effects of PE against cisplatin induced ototoxicity.

DPOAE is a hearing assessment method which represents function of the outer hair cell, one of the target damaged by cisplatin. In terms of ototoxicity due to cisplatin, at dosages of 8 mg/kg/day, the animals showed DPOAE alterations and outer hair cells lesions in three days [12]. Giordano et al. suggested that the systemic administration of D-methionine has a potential otoprotective role [14]. Data from the DPOAE recordings of the study suggested good recovery of the posttreatment responses. Choe et al. demonstrated that animals in the untreated control group and the negative control normal saline group demonstrated consistent obliteration of DPOAE after cisplatin injection [8]. However, those receiving either intratympanic lactated Ringer’s solution or intratympanic 2% N-acetylcysteine solution showed significant preservation of DPOAE after cisplatin injection. In our study, DPOAE amplitudes did not decrease significantly in group 3 (cisplatin and PE treatment) between baseline and day three at the frequencies

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Distortion product evoked otoacoustic emission (DPOAE) average amplitudes in groups 2 and 3, showing results at baseline and after three days, for various test frequencies. (a) 3 kHz; (b) 4 kHz; (c) 6 kHz; (d) 8 kHz.

<table>
<thead>
<tr>
<th>n</th>
<th>Group 1 cochlea</th>
<th>Group 2 cochlea</th>
<th>Group 3 cochlea</th>
<th>Group 1 kidney</th>
<th>Group 2 kidney</th>
<th>Group 3 kidney</th>
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</table>

**Table 1.** Lumino-Amplified Chemiluminescence Values of cochlea and kidney (RLU/mg).

mean±SD 119.11±10.35 216.65±12.77 166.37±9.59 10.55±1.68 17.63±2.25 13.75±3.98
tested (3, 4, 6 and 8 kHz) compared with group 2 (cisplatin treatment only), suggesting that systemic PE had an otoprotective effect in subjects which were given cisplatin treatment.

Although the ototoxic effect of cisplatin has been extensively studied, the pathophysiology of cisplatin-induced ototoxicity is not completely understood. Currently, it appears that cisplatin causes ototoxicity by increasing ROS and altering the antioxidant defense system of the cochlea, other hair cell, spiral ganglia, stria vascularis and the spiral ligament [15]. Some authors suggest that the principal mechanism of ototoxicity is related to the production of NO, which is induced by the production of ROS and the over-induction of iNOS synthesis. In addition, ROS overload combined with decreased antioxidant system leads to cell injury and apoptosis [16].

Several studies have explored the possibility of administering an otoprotectant in an effort to reduce the negative impact of cisplatin on hearing. One area of focus has been on administering antioxidant compounds in an attempt to reduce the accumulation of ROS before they induce apoptosis in the inner ear. The literature shows various antioxidant agents being tested for protection against cisplatin ototoxicity, such as: vitamin B [17], melatonin [18], N-acetylcysteine [19], and ginseng [20].

Pomegranate is a fruit and is readily obtained. PE is rich in polyphenol antioxidants, including tannins and anthocyanins [21]. These antioxidants are more potent, on a molar basis, than many other antioxidants, including vitamins C and E, coenzyme Q-10, and α-lipolic acid [22]. The antioxidant levels in pomegranate are higher than in many other fruits, such as blueberry, cranberry, orange, and grapes. Anthocyanins have been shown to be effective inhibitors of lipid peroxidation, inducible nitric oxide synthetase (iNOS) and, thus, nitric oxide (NO) [23].

Recently, Im et al. showed that ginseng extract significantly attenuated cisplatin-induced increases in ROS [17]. And they believed that the antiapoptotic effect of ginseng extract is due to ROS inhibition because a major mechanism of cisplatin-induced toxicity involves ROS production. In our experiment only the right side cochlea was be used for chemiluminescence detection procedures. We removed the temporal bones of the animals just after sacrificing them to avoid affecting the tissue a leves of ROS. In our study, ROS levels were low in rats treated with cisplatin and systemic PE compared to levels in rats treated with cisplatin only. We suggest that PE has a protective effect against cisplatin-induced ototoxicity, possibly by reducing ROS levels in the cochlea. This protective effect may also be due to decreasing NO content and iNOS activity.

Several studies on cisplatin oto and nephrotoxicity point to alterations in cell antioxidant potentials. It was shown that levels of antioxidant enzymes are reduced in cochlea and kidney tissues leading to lipid peroxidation, thus, to cellular toxicity [20,21]. In our study kidney ROS levels were clearly different among the groups as well as cochlear ROS levels. The PE appears to preserve the organ function of rats exposed to cisplatin, possibly by its systemic antioxidant effects. This study tested the hypothesis that the administration of PE could prevent cisplatin-induced hearing loss. The results obtained suggest that the systemic administration of PE has a potential otoprotective role. Data from the DPOAE recordings and ROS levels of cochlea suggest good recovery of the posttreatment responses. In this context, it is feasible that PE can protect, completely and more efficiently against cisplatin ototoxicity. This can easily be monitored with DPOAE and measured with chemiluminescence detection. However, well-designed, placebo controlled human studies are needed to confirm our results and to determine the best PE regimen for preventing cisplatin-induced ototoxicity.

**Conclusion**

We studied the protective effect of systemic PE against cisplatin-induced ototoxicity in rats. Systemic PE might have a significant protective effect after cisplatin injection. However, further studies are
necessary to determine the appropriate indications and dosages of PE before clinical use is possible.

References