Int. Adv. Otol. 2010; 6:(3) 380-385

ORIGINAL ARTICLE

Protective Effect of Pomegranate Extract on Cisplatin-induced Ototoxicity

Aysenur Meric Teker, Volkan Kahya, Zahide Mine Yazici, Meral Yuksel, Orhan Gedikli, Gunter Hafiz

Dept. of Otorhinolaryngology, Vakif Gureba Training and Research Hospital, Istanbul , (AT, VK, OG)
Dept. of Otorhinolaryngology, Bakirkoy Training and Research Hospital, Istanbul , (ZY)
Dept. of Biochemistry, Vocational School of Health Related Professions, Istanbul , (MY)
Dept. of Otorhinolaryngology, Istanbul University Istanbul Medical Faculty, Istanbul, (GH)

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.

Submitted : 23 March 2010 *Revised : 01 August 2010* *Accepted : 05 September 2010*

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.

Corresponding address:
Aysenur Meric Teker, MD
Atakoy 9. Kisim A3A Blok D:36 Bakirkoy/Istanbul/Turkey
Phone number: +905325493949, Fax: +902126217580, E-mail: aysenurmeric@yahooo.com

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 chosed pomegranate to investigate its possible otoprotective effects because it has antioxydating 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 30mg/kg ketamine hydrochloride and 4 mg/kg xylazine intraperiteonally 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 (Alfazyne 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 -180C 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 seperated 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 sacrified 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 anesthetised 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 [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 [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.

Results

Functional hearing evaluation

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 value calculated for each frequency is less than 0.05). In group 3, there were no significant differences in the DPOAE amplitudes between baseline and third day of intraperitoneal cisplatin injection at the frequencies tested (3, 4, 6 and 8 kHz) (p value calculated for each frequency is more than 0.05). Figure 1 shows the comparison of DPOAE amplitudes before and after injection for groups 2 and 3.

Chemiluminescence Measurements

A statistically significant increase in cochlear ROS levels was observed following cisplatin injection. The luminol chemiluminescence levels of the rats which underwent cisplatin injection only (group 2; 216.65±12.77 RLU/mg) were significantly higher than those of the control group (group 1; 119.11±10.35 RLU/mg; $p < 0.01$). ROS levels were significantly lower in the PE treated group (group 3; 166.37±9.59 RLU/mg) than those in group 2 ($p < 0.01$); however, group 3 ROS levels were significantly higher than those in the control group. Figure 2 shows the levels of cochlear ROS before and after cisplatin injection for all groups.

At the same time, kidney ROS level was significantly higher in group 2 than group 1 (group 2;17.63±2.25 RLU/mg; $P < 0.01$). Kidney ROS levels were significantly lower in the PE treated group (group 3; 13.75±3.98 RLU/mg; $P < 0.01$) than those in group 2; however, group 3 ROS levels were significantly higher than those in the control group (group 1; 10.55±1.68 RLU/mg; $p < 0.01$). This data suggested that oral administration of PE had a systemic effect in rats. Table 1 shows the levels of cochlear and kidney ROS before and after cisplatin injection for all groups.

Discussion

Life-threatening medical conditions may require treatment with cisplatin, and the risk of cisplatin may be unavoidable. Side effects of cisplatin such as nausea, vomiting, myelosupression, and renal toxicity could be controlled by several agent such as seretonin antagonists, granulocyte stimulating factors, and hydration. Unfortunately ototoxic side effects of cisplatin can not be decreased by traditional medical treatments. Our results show that the antioxidant

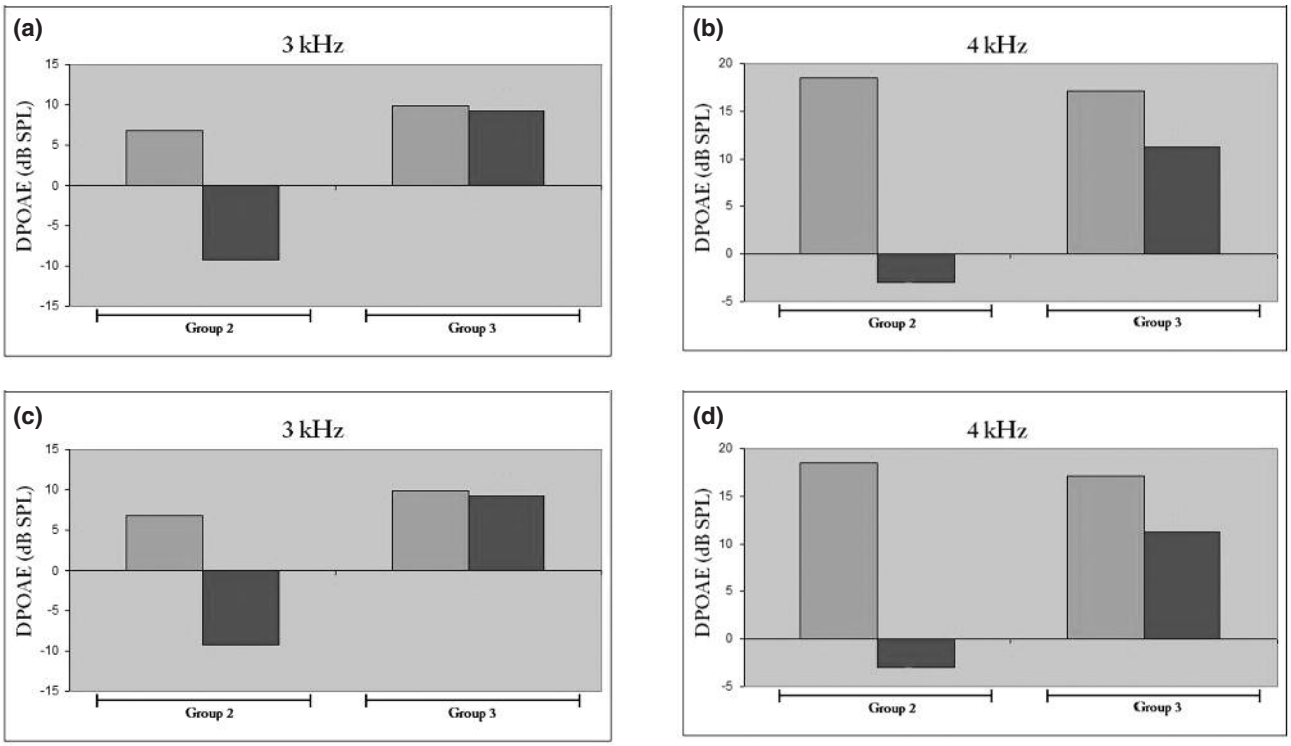

Figure 1. Distortion product evoked otoacoustic emission (DPOAE) average amplitudes in groups 2 and 3, showing results at baseline and after three days, for various test frequencies. (a) 3 kHz; (b) 4 kHz; (c) 6 kHz; (d) 8 kHz.

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

n	Group 1 cochlea	Group 2 cochlea	Group 3 cochlea	Group 1 kidney	Group 2 kidney	Group 3 kidney
1	113.5	210.0	169.3	13.3	19.6	12.7
2	102.2	205.8	152.7	10.8	16.9	6.6
3	123.3	207.5	162.2	9.7	13.9	17.0
4	123.0	224.3	175.3	8.4	17.1	15.3
5	120.0	239.0	160.7	9.8	20.2	13.4
6	132.7	213.3	178.0	11.3	18.1	17.5
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

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 show 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

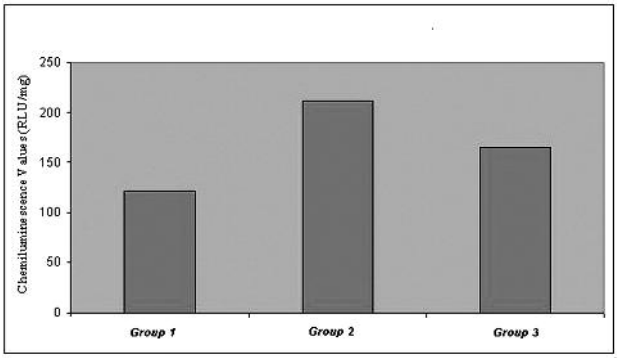

Figure 2. Comparison of Lumino-Amplified Chemiluminescence Values for cochlear ROS.

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, outher 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 [2], melatonin [7], N-acetylcysteine [8], and ginseng [17].

Pomegranate is a fruit and is readily obtained. PE is rich in polyphenol antioxidants, including tannins and anthocyanins [11]. 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 [18]. 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) [19].

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

1. De Pauw-Gillet MC, Houssier C, Fredericq E. Interaction of DNA and purine nucleosides with cis-dichlorodiammineplatinum (II) and antimitotic activity of the complexes on meristematic root cells. Chem Biol Interact 1979; 25:87-102.

2. Güneri EA, Serbetçiogllu B, Ikiz AO, Güneri A, Ceryan K. TEOAE monitoring of cisplatin induced ototoxicity in guinea pigs: the protective effect of vitamin B treatment. Auris Nasus Larynx 2001; 28:9–14.

3. Nagy JL, Adelstein DJ, Newman CW, Rybicki LA, Rice TW, Lavertu P. Cisplatin ototoxicity: the importance of baseline audiometry. Am J Clin Oncol 1999; 28:305–08.

4. Rybak LP. Mechanisms of cisplatin ototoxicity and progress in otoprotection. Curr Opin Otolaryngol Head Neck Surg 2007; 15: 364–69.

5. Laurell G, Bagger-Sjoback D. Dose-dependent inner ear changes after i.v. administration of cisplatin. J Otolaryngol 1991; 20:158–67.

6. Hatzopoulos S, Di Stefano M, Albertin A, Martini A. Evaluation of cisplatin ototoxicity in a rat animal model. Ann NY Acad Sci 1999; 884:211-25.

7. Lopez-Gonzalez MA, Guerrero JM, Rojas F, Delgado F. Ototoxicity caused by cisplatin is ameliorated by melatonin and other antioxidants. J Pineal Res 2000; 28:73-80.

8. Choe WT, Chinosornvatana N, Chang KW. Prevention of cisplatin ototoxicity using transtympanic N-acetylcysteine and lactate. Otol Neurotol 2004; 25:910-15.

9. de Nigris F, Williams-Ignarro S, Sica V, Lerman LO, D'Armiento FP, Byrns RE, et al. Effects of a pomegranate fruit extract rich in punicalagin on oxidation-sensitive genes and eNOS activity at sites of perturbed shear stress and atherogenesis. Cardiovasc Res 2007; 73:414-23.

10. Tugcu V, Kemahli E, Ozbek E, Arinci YV, Uhri M, Erturkuner P, et al. Protective effect of a potent antioxidant, pomegranate juice, in the kidney of rats with nephrolithiasis induced by ethylene glycol. J Endourol 2008; 22:2723-31.

11. Aviram M, Dornfeld L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis. 2001; 158:195-98.

12. de Freitas MR, de Castro Brito GA, de Carvalho JV Jr, Gomes RM Jr, Barreto Martins MJ, de Albuquerque Ribeiro R. Light microscopy study of cisplatin-induced ototoxicity in rats. J Laryngol Otol 2009; 123:590-97.

13. Lopez-Gonzalez MA, Lucas M, Delgado F, Diaz P. The production of free oxygen radicals and nitric oxide in the rat cochlea. Neurochem Int 1998; 33:55-9.

14. Giordano P, Lorito G, Ciorba A, Martini A, Hatzopoulos S. Protection against cisplatin ototoxicity in a Sprague-Dawley rat animal model. Acta Otorhinolaryngol Ital 2006;26:198-207.

15. Clerici WJ, DiMartino DL, Prasad MR. Direct effects of reactive oxygen species on cochlear outer hair cell shape in vitro. Hear Res 1995;84:30-40.

16. Rybak LP, Husain K, Morris C, Whitworth C, Somani S. Effect of protective agents against cisplatin ototoxicity. Am J Otol 2000;21:513-20.

17. Im GJ, Chang JW, Choi J, Chae SW, Ko EJ, Jung HH. Protective effect of Korean red ginseng extract on cisplatin ototoxicity in HEI-OC1 auditory cells. Phytother Res 2009 Dec 17.

18. Aviram M, Dornfeld L, Kaplan M, Coleman R, Gaitini D, Nitecki S, et al. Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans. Drugs Exp Clin Res 2002;28:49-62.

19. Tsuda T, Horio F, Osawa T. Cyanidin 3-O-beta-D-glucoside suppresses nitric oxide production during a zymosan treatment in rats. J Nutr Sci Vitaminol (Tokyo) 2002;48:305-10.

20. Cardinaal RM, Groot JCMJ, Huizing EH, Veldman JE, Smmorenbrg GF. Dose- depend effect of 8-day ciplatin administration upon the morphology of the albino guinea pig cochlea. Hear Res 2000;144:135-46.

21. Rybak LP, Whitworth MA, Somani S. Application of Antioxidants and Other Agents to Prevent Ciplatin Ototoxicity. Laryngoscope 1999; 109:1740-44.