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Intraperitoneal curcumin and vitamin E combination for the treatment of cisplatin-induced ototoxicity in rats

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ABSTRACT

Introduction: Cisplatin ototoxicity is characterized by irreversible, progressive, bilateral sensorineural hearing loss at high frequencies, accompanied by tinnitus. The aim of this study is to demonstrate the protective action of curcumin alone or in combination with vitamin E against cisplatin-induced ototoxicity in animal models.

Material and methods: The study included 42 rats. Experimental animals were randomized into 6 groups. In the first group, intra-peritoneal cisplatin was administered alone. In the second group, intra-peritoneal cisplatin and curcumin were administered together. In the third group, intra-peritoneal cisplatin and vitamin E were administered together. In the fourth group, intra-peritoneal cisplatin was administered together with curcumin in combination with vitamin E. In the fifth group, intra-peritoneal curcumin was administered alone. The sixth group was sacrificed directly without administration of any drugs. A distortion product otoacoustic emission (DPOAE) test was applied to both ears of all experimental animals. Curcumin was administered 1 h before cisplatin treatment continued for three successive days. Vitamin E was administered only as a single dose 30 min prior to cisplatin. All animals were sacrificed following DPOAE testing on the 5th day of cisplatin administration. Histopathological findings included a TUNEL (TdT-mediated deoxyuridine triphosphate nick end-labeling) assay, and the percentage of apoptotic cells was calculated. DPOAE values and the percentage of apoptotic cells were compared before and after treatment and between experimental groups.

Results: In Group 1, DPOAE values were significantly decreased at all frequencies (3000 Hz, 4000 Hz and 6000 Hz; $P < 0.05$). In Groups 2, 3, 4 and 5 there was no significant difference between the pre- and post-treatment DPOAE results ($p > 0.05$). Apoptotic index values were lower in all treatment groups compared to the cisplatin group, however the difference was only statistically significant in group 3 ($p = 0.009$).

Conclusion: In rats, cisplatin ototoxicity can be prevented with curcumin or curcumin-vitamin E combination.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum II) is widely used in the treatment of solid tumors (squamous head and neck carcinoma, testicular and ovarian carcinoma, lung carcinoma, advanced bladder cancer, malignant gliomas and metastatic cancers such as melanoma, mesothelioma, prostate and breast cancer) [1,2]. Known side effects of cisplatin treatment include ototoxicity, myelotoxicity, nephrotoxicity, and gastrointestinal toxicity. While nephrotoxicity can be controlled through hydration therapy, ototoxicity is a critical limitation for cisplatin chemotherapy [1]. Cisplatin ototoxicity is characterized by irreversible, bilateral, progressive sensorineural hearing loss at high frequencies accompanied by tinnitus and ototoxicity. Hearing loss can be a major dose-limiting factor in some patients. Cisplatin causes progressive damage to the outer hair cells

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of cochlea. Approximately 60–80% of patients treated with cisplatin develop permanent, bilateral, and progressive hearing loss [3]. Deafness is not a life-threatening condition and can vary widely in severity. Nevertheless, hearing loss has a significant impact on quality of life and can lead to communicative disorders. The reduction of cisplatin ototoxicity remains a major goal of anti-tumor therapy and the molecular and cellular determinants of cisplatin toxicity are not well understood. Cislatin-induced apoptosis may occur through multiple pathways, including overproduction of reactive oxygen species (ROS). Emerging evidence suggests that increases in ROS production and decreased expression of endogenous antioxidants may contribute to cisplatin ototoxicity [2,4,5]. The resulting oxidative stress in a primary driver of cochlear cell loss [4].

Amelioration of cisplatin ototoxicity using antioxidants N-acetyl cysteine, sodium thiosulfate, amifostine, D-methionine, vitamin E, B, C, L-carnitine, dexamethasone, salicylates, *Gingko biloba* extract, allopurinol and ebselen, neurotropines, flunarizine, melatonin, ringer's lactate, thymoquinone, dexamethasone, resveratrol, molsidomine, curcumin, lutein, oxytocin [2–16]. Currently there is no FDA-approved treatment with demonstrated efficacy in preventing or reducing cisplatin ototoxicity [2]. A polyphenol isolated from turmeric (*Curcuma longa*), curcumin has a variety of antioxidant, anti-inflammatory, anti-neoplastic properties. The effects of curcumin are dependent on interactions with multiple molecular targets including growth factors, kinases, transcription factors, inflammatory cytokines, apoptosis-related proteins, adhesion molecules, and enzymes associated with inflammation and cellular proliferation [3,13].

Vitamin E is the major lipid-soluble antioxidant in the body. Vitamin E maintains the integrity of membranes by inhibiting lipid peroxidation. It also protects neurological structure and function [14,15]. Serum antioxidant concentrations are decreased in cancer patients undergoing cisplatin treatment. Cycles of cisplatin chemotherapy are associated with temporary decreases in circulating vitamin C, vitamin E, ceruloplasmin, and uric acid [11]. In the present study we administered a combination of curcumin and vitamin E as an anti-oxidant supplement in rats undergoing cisplatin treatment.

The aim of this study was to evaluate damage to the inner ear in rats treated with curcumin and vitamin E during experimental cisplatin treatment using DPOAE measurements and histopathological analyses.

2. Materials and methods

The Ethical Committee on Animal Research of Tokat Gaziosmanpaşa University reviewed and approved all study procedures. The research was conducted at the experimental research laboratory of Gaziosmanpaşa University.

2.1. Animals

42 adult male Wistar albino rats weighing 240–320 g each were utilized in this study. Animals were maintained at 25 °C constant temperature in the separate cages in a temperature controlled room with a 12 h light/dark cycle and unlimited access to food and water. An operating ear microscope was used to examine the tympanic membranes and external ear canals of all rats. Cerumen was removed from the ear canal. Exclusion criteria were as follows: signs of otitis media, tympanic membrane perforation, and opacification.

2.1.1. Experimental groups

42 rats were randomized into six groups of seven animals each and treated as follows:

Group 1: one dose 16 mg/kg intra-peritoneal (i.p.) injection of cisplatin (Cisplatin DBL, 100 mg/100 ml, Australia).

Group 2: one dose 16 mg/kg i.p. injection cisplatin + i.p. curcumin 200 mg/kg (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 1 h before cisplatin administration for 4 days.

Group 3: one dose 16 mg/kg i.p. injection of cisplatin + a single dose 50 mg/kg i.p. injection of Vitamin E (Evigen 2 ml, Aksu Farma Corp., Turkey), 30 min before cisplatin administration.

Group 4: one dose 16 mg/kg i.p. injection of cisplatin + a single dose 50 mg/kg i.p. injection of Vitamin E, 30 min before cisplatin administration + i.p. curcumin 200 mg/kg, 1 h before cisplatin administration for 4 days.

Group 5: i.p. curcumin 200 mg/kg, for 4 days.

Group 6: sacrificed without any drug administration.

2.2. Preparation of curcuma extracts

2.5 g of curcumin powder was dissolved in sequential DMSO and ethanol, incubated on the shaker at room temperature for 10 min and centrifuged at 2000 rpm for 10 min before removal of the DMSO fraction. The remaining pellet was then dispersed in 96% ethanol and the procedure was repeated. The remaining pellet was diluted in 25 cc sterile saline solution, after removal of the ethanol fraction [17]. So curcumin stock solution was prepared at a 2.5 g/25 ml. Fractions were prepared fresh for each experimental treatment to minimize the damage cause by extended storage.

2.3. Study design

Intraperitoneal anesthetic of 60 mg/kg ketamine and 10 mg/kg xylazine was administered to all rats. Ear microscopic examination, pre-treatment and post-treatment distortion product otoacoustic emissions (DPOAEs) measurements were obtained after the animals were anesthetized. All drugs were injected intraperitoneally. After an observation period of 5 days, DPOAEs measurements were obtained for comparison with the pretreatment values. Euthanasia was performed under deep anesthesia. The temporal bones and cochleas were excised from all experimental animals. TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) staining of apoptotic cells was performed on the cochlear tissue.

2.4. DPOAEs measurements

Hearing was assessed by DPOAEs under deep anesthesia. DPOAEs measurements were collected in a noise-controlled environment.

Madsen Capella (Otometrics, Denmark) was used to perform the distortion otoacoustic emissions testing using an infant hearing screening probe. The sound stimulus consisted of two simultaneous permanent pure tones at different frequencies (f2/f1 ratio = 1.22) at 80 dB SPL (L1 = L2). DPOAEs were measured at four frequencies ranging from 2 kHz to 6 kHz (2,002, 3,003, 4,004, 6,006, Hz).

2.5. TUNEL assay

TUNEL staining was used to evaluate the extent of apoptosis in the organ or Corti in the cisplatin-treated animals. The TUNEL staining assay kit (In Situ Cell Death Detection Kit, AP; Roche) was used according to the manufacturer's instructions. Briefly, five-μm-thick sections from 4% neutral buffered formalin fixed and paraffin embedded cochlear tissues were deparaffinized in xylenes and rehydrated through a graded ethanol series and double distilled water. The investigators were blinded to the animal grouping during analysis of the TUNEL-stained tissue sections. The apoptotic

index of the organ of Corti was calculated by counting the number of TUNEL positive cells over the total cell number in each section using light microscopy and a 40X objective lens. (four slides was analyzed in each rat, there were three sections on each slide and average four organs of corty in each section).

2.6. Statistical analysis

The Kolmogorov- Smirnov and Shapiro Wilk tests were used to evaluate adherence to the normal distribution among continuous variables. Data are expressed as the mean ± SD for continuous variables. Observed differences of apopitotic index values were compared using the Least Significant Differences (LSD) multiple comparison test, one-way analysis of variance (ANOVA). Paired samples *t*-test was used to compare the DPOAE values before and after drug application in each group. The significance of differences between DPOAE mean values of groups was evaluated using ANOVA. Statistical Package for Social Sciences (SPSS) version 21.0 Software for Windows was used for all statistics analysis. Differences were considered statistically significant at $P < 0.05$.

3. Results

One rat from the group 4 died under anesthesia during study and 41 rats completed the study without any complication.

3.1. Auditory function evaluation

The Otoacoustic emission assessment was performed for 82 ears of 41 rats. Table 1 demonstrates the DPOAE values before and after drug administration for all groups. DPOAE values were compared before and after drug administration (Fig. 1). Pre-treatment DPOAE values did not differ significantly between the treatment groups. In Group 1, DPOAE values were significantly decreased at all frequencies (3000 Hz, 4000 Hz and 6000 Hz) after treatment ($P < 0.05$). In Groups 2,3,4,5 the post-treatment DPOAE values were not significantly different from the pre-treatment measurements ($P > 0.05$).

There was no statistically significant difference in the post-treatment DPOAE values between the curcumin and vitamin E treatment groups.

3.2. Histomorphologic results

Light microscopy analysis revealed that rats in the control group had a normal histological appearance within the organ of Corti. Profound histological damage to the organ of Corti was present in rats in all other treatment groups, particularly in the cisplatin group (Fig. 2). Table 2 demonstrates the mean apoptotic cell index values of inner-outer hair cells for all groups. Relative to controls, apoptotic cell index were markedly increased in the inner and outer hair cell regions and throughout the organ of Corti in the cisplatin treatment group ($P < 0.001$). In all treatment groups except for the vitamin E treatment group the inner hair cell apoptotic index was elevated relative to the control group. However, the apoptotic cell index was similar to the control group in the vitamin E treatment group ($P = 0.078$) and significantly decreased relative to the cisplatin treatment group ($P = 0.004$).

The outer hair cell apoptotic index was higher in all treatment groups in comparison to the control group. The outer hair cell apoptotic index of the vitamin E and curcumin groups did not differ significantly from the cisplatin group ($P > 0.05$). The total organ of

Table 1
DPOAE values in all groups.

Group	2000 Hz (mean ± SD; dB)			3000 Hz (mean ± SD; dB)			4000 Hz (mean ± SD; dB)			6000 Hz (mean ± SD; dB)		
	Pre	Post	P[a]	Pre	Post	P[a]	Pre	Post	P[a]	Pre	Post	p[a]
1 Cis	16.25 ± 4.04	8.67 ± 11.17	P > 0.05	27.64 ± 10.20	17.24 ± 10.10	P < 0.05	35.92 ± 16.47	23.54 ± 10.60	P < 0.05	48.88 ± 22.59	30.12 ± 14.13	P < 0.05
2 Cis + Cur	15.42 ± 3.03	8.18 ± 6.79	P < 0.05	25.30 ± 10.82	14.35 ± 11.69	p > 0.05	36.01 ± 6.80	26.14 ± 16.18	p > 0.05	52.68 ± 16.27	46.44 ± 16.67	p > 0.05
3 Cis + Vit E	14.64 ± 4.15	16.22 ± 10.30	p > 0.05	25.81 ± 6.61	26.45 ± 18.32	p > 0.05	37.60 ± 9.54	36.25 ± 21.82	p > 0.05	46.40 ± 19.51	56.34 ± 10.11	p > 0.05
4 Cis + Vit E + Cur	19.98 ± 4.90	12.22 ± 6.07	p > 0.05	32.30 ± 11.42	20.56 ± 13.08	p > 0.05	42.24 ± 13.88	34.68 ± 8.09	p > 0.05	50.78 ± 14.18	51.40 ± 11.77	p > 0.05
5 Cur	14.12 ± 4.36	12.11 ± 8.17	p > 0.05	23.15 ± 10.49	25.25 ± 13.57	p > 0.05	26.04 ± 12.83	30.25 ± 17.09	p > 0.05	41.74 ± 22.46	39.95 ± 20.09	p > 0.05

DPOAE = Distortion product otoacoustic emission; SD = standard deviation; cis = cisplatin; Cur = curcumin; vit = vitamin; pre = pre-treatment; post = post-treatment.
[a] Table 1 was obtained with the paired T test.

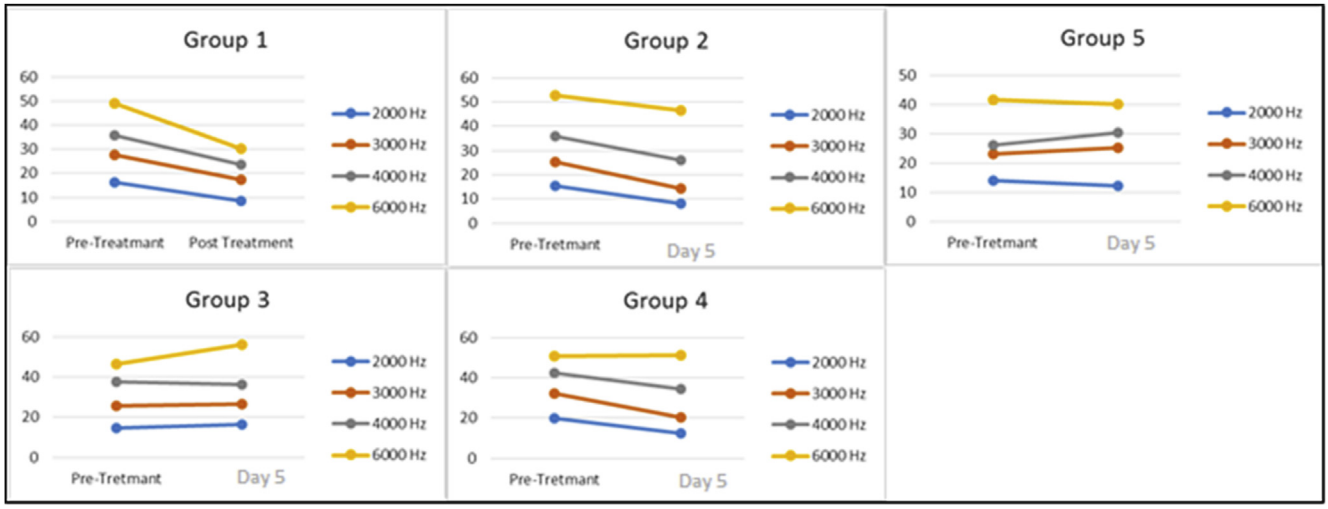

Fig. 1. Graphic shows the reduction in DPOAE amplitudes at different frequencies before and after treatment in each of the experimental groups.

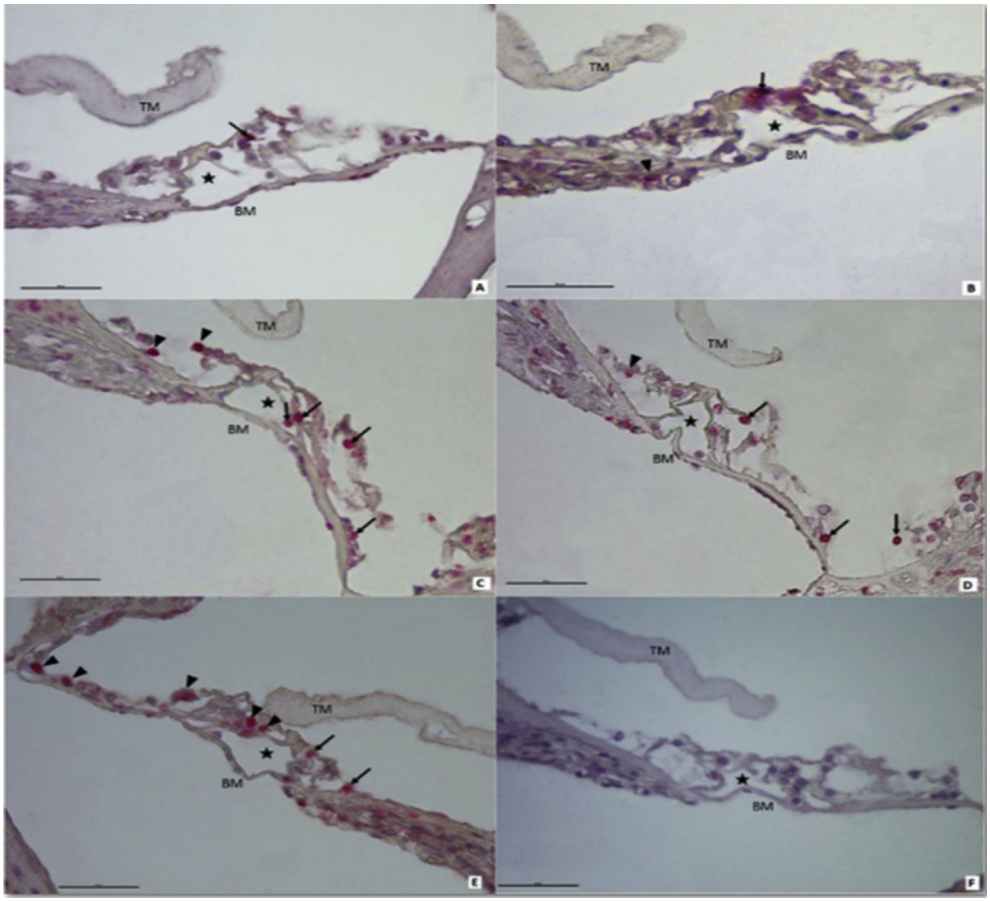

Fig. 2. Histological micro-photographs of organ of Corti in the inner ear A: Control group. B: Cisplatin- Vitamin E group. C: Cisplatin-curcumin group. D: Cisplatin-curcumin + Vitamin E group. E: Cisplatin group. F: Curcumin group. Arrows show inner hair cells; arrowheads show outer hair cells. TM: tectorial membrane, BM: Basal membrane. The border between inner and outer hair cells is marked with a star. (TUNEL staining method, Bar: 50 μm).

Table 2
Mean apoptotic cell index values of groups. (±: SEM, letters over the values indicate statistical differences and similarities). İAPİ = inner hair cell apopitotic index; OAPİ = outer hair cell apopitotic index; TAPİ = total hair cell apopitotic index.

	Group					
	Control	Cis-Cur-Vit E	Cis-Cur	Cis	Cis-Vit E	Cur
İAPi	$2,29 \pm 1,48^{a}$	$16,93 \pm 1,97^{b1}$	$18,39 \pm 4,06^{b2}$	$24,06 \pm 4,11^{b3}$	$10,80 \pm 1,87^{a,b1,b2}$	$1,85 \pm 1,85^{a}$
OAPİ	$3,59 \pm 1,81^{a}$	$18,33 \pm 1,50^{b}$	$16,41 \pm 2,93^{b}$	$20,93 \pm 1,28^{b}$	$18,51 \pm 2,58^{b}$	$1,67 \pm 1,67^{1}$
TAPİ	$3,21 \pm 1,23^{a}$	$18,40 \pm 1,02^{b}$	$17,62 \pm 3,19^{b}$	$22,92 \pm 2,07^{b}$	$14,73 \pm 1,48^{c}$	$1,92 \pm 1,22^{A}$

Corti apoptotic index was significantly increased in all treatment groups compared to the control group ($P = 0.001$). The apoptotic cell index was decreased in all treatment groups compared to the cisplatin group, however the decrease was statistically significant only in the vitamin E group ($P = 0.009$). The apoptotic cell index was notably decreased in the cisplatin + curcumin group compared to cisplatin group, however, this difference did not meet the threshold of statistical significance ($P = 0.07$). Fig. 3 shows the comparison of mean apoptotic cell index values across groups.

4. Discussion

Deafness is not a life-threatening condition and can vary widely in severity. Nevertheless, hearing loss has a significant impact on quality of life and can lead to communicative disorders. The development of effective methods for limiting cisplatin ototoxicity has the potential to protect auditory function while maintaining the potent anti-tumor activity of the chemotherapy.

The present study indicates that curcumin alone or vitamin E curcumin combination may have a protective effect against cisplatin-induced ototoxicity in male adult Wistar rats. We utilized the TUNEL method to detect fragmented DNA fragmented in cisplatin-induced apoptotic cells within the organ of corti.

Since the first report of cisplatin-induced ototoxicity by Rossof et al. in 1972, the topic has been widely studied [18]. However, the molecular and cellular mechanisms of cytotoxicity remain poorly understood. Cisplatin may destroy the outer hair cells of the cochlea, leading to excessive production of free oxygen radicals in the organ of Corti, stria vascularis, spiral ligament, and spiral ganglionic cells [2,19]. Reduced expression of endogenous antioxidants is also associated with cisplatin treatment, leading to increased oxidative damage. Cisplatin within the cochlear tissues can also lead to DNA damage and excessive free radical generation [5].

Apoptosis is a form of controlled cell death that may occur under

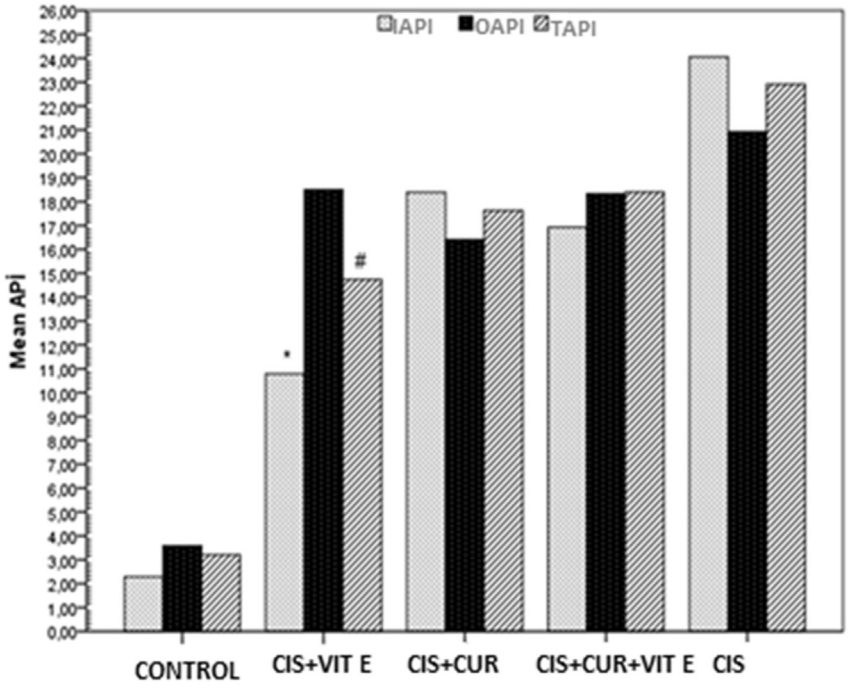

Fig. 3. Comparison of mean apoptotic cell index values across groups (compared to cisplatin; inner hair cells *: P = 0.004, total organ of Corti #: P = 0.009).

normal growth and development, or as part of the response to radiation, toxin exposure, or ischemia. Apoptosis may be the primary outcome of cisplatin-induced ototoxicity. Freitas et al. [18] demonstrated that 16 mg/kg cisplatin results in apoptosis after 3 days. Therefore, cisplatin was administered at the same dose in this experimental study.

Curcumin has multifaceted anti-inflammatory, anti-oxidant, and anti-cancer activity that may limit the side effects of some chemotherapy agents. The mechanism of curcumin-mediated cellular protection is unclear. Some evidence indicates that curcumin may act as a free radical scavenger while other studies suggest that curcumin may promote free radical generation in tumor cells, causing apoptosis [20,21]. The pro-oxidant and pro-apoptotic effects of curcumin may be highly dose-dependent in cancer cells as well as within the cochlear tissue.

Decreased lipid peroxidation following curcumin treatment may be the result of direct free radical scavenging activity [13]. Heme oxygenase-1 (HO-1) activity is modulated by curcumin and may serve as a source of cytoprotection [13]. Stress-inducible HO-1 has both anti-oxidant and anti-inflammatory activity. HO-1 is expressed in the cochlea and has been detected in cisplatin-treated organ of Corti explants [22].

Curcumin is widely considered to be well-tolerated (no hepatotoxicity is detectable in curcumin-treated animals) and may also attenuate hepatotoxicity cause by other drugs, including cisplatin [13]. Nephrotoxicity is recognized as the primary dose-limiting factor in cisplatin treatment, however hepatotoxicity may occur at high levels of cisplatin exposure. Reports in the literature suggest that mitochondrial dysfunction contributes to both hepatotoxicity and nephrotoxicity in cisplatin-treated patients [3,13].

Vitamin E contains tocopherol and tocotrienol, free radical scavengers, and limits cisplatin nephrotoxicity and endothelial cell toxicity [11]. Vitamin E acts to preserve the structure of the unsaturated fatty acids that make up the phospholipid membrane from free radical damage. Vitamin E is a potent inhibitor of lipid peroxidation reactions [11,12].

Salehi et al. administered curcumin in combination with dexamethasone, resulting in increased tissue protection. Fetoni et al. [13] showed that curcumin reduced cisplatin ototoxicity by amplifying heme oxygenase 1 enzyme expression *in vivo*. Kalkanis et al. [21] demonstrated that vitamin E supplementation can reduce cisplatin-mediated ototoxicity in rats. Intratympanic administration of vitamin E and dexamethasone is also highly effective in ameliorating the side-effects of cisplatin treatment [11].

In this study, we administered curcumin and vitamin E either alone or in combination to examine effects on cisplatin ototoxicity.

Fetoni et al. [13] applied a dose of 200 mg/kg of curcumin, increasing the toxic of cisplatin by approximately 50%. Interestingly, even higher doses of curcumin did not result in enhanced protection and may have had some detrimental effects [13]. In our study, we also applied curcumin at a dose of 200 mg/kg.

Our results demonstrate the protective effects of both curcumin and vitamin E against apoptosis using the TUNEL assay. In addition, curcumin resulted in enhanced functional hearing capacity, as indicated by the DPOAE test. However, we were unable to detect superiority of combination treatment over administration of curcumin or vitamin E alone using electrophysiological and morphological methods. In our study, the percentage of apoptotic cells were decreased in the vitamin E and curcumin treatment groups in comparison to the cisplatin group. However, this decrease was statistically significant only in the vitamin E treatment group.

To the best of our knowledge, only 2 previous experimental animal studies have examined the effects of curcumin against cisplatin ototoxicity [4,13]. However, no previous experimental animal studies have evaluated curcumin and vitamin E together in the treatment of cisplatin ototoxicity.

Our results indicate the intra-peritoneal injection of curcumin and vitamin E may be a useful adjunct therapy for the preservation of the sensory cells of the cochlea during cisplatin therapy.

5. Conclusion

Curcumin provides marked protection against cisplatin-induced hearing loss when administered in combination with vitamin E,

potentially as a result of reduced ROS damage to auditory cells.

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