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OTOLOGY

Is there a gender-related susceptibility for cisplatin ototoxicity?

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Abstract Ototoxicity is a well-known side effect of cisplatin. Some genetic and non-genetic risk factors were described for cisplatin ototoxicity. Although there are some studies which point out a sex-related difference for cisplatin nephrotoxicity and neurotoxicity, sex-related differences for cisplatin ototoxicity have not been studied. The aim of this study is to reveal whether there is any gender-related difference for susceptibility to cisplatin ototoxicity in rats. Fourteen male, 14 female Wistar albino rats were divided into four groups; a female control, a male control, a female cisplatin and a male cisplatin group. Distortion Product Otoacoustic Emission and, Auditory Brainstem Response measurements were obtained. For the cisplatin groups 16 mg/kg of cisplatin was applied. On the 4th day audiological examinations were repeated. After killing, cochleae and brainstem tissues were evaluated by light and electron microscopy. The hearing of the female rat cisplatin group was found to have deteriorated more than the hearing of the male rat cisplatin group. Histopathological evaluation revealed more serious damage in the spiral ganglion and brainstem tissues of female rats. Hearing of female rats deteriorated more than the hearing of male rats upon application of cisplatin. This difference in hearing can be attributed to the more severe damage seen in neuronal tissues such as spiral ganglion cells and brainstem neurons.

Keywords Ototoxicity · Sex · Cisplatin

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Introduction

Cisplatin (CDDP) is a widely used chemotherapeutic agent for the treatment of various malignancies. Unfortunately it has some dose limiting side effects like ototoxicity, nephrotoxicity and neurotoxicity. Incidence of cisplatin ototoxicity has been reported as 13–96 % in different studies. It usually presents as a bilateral sensorineural hearing loss which first involves high frequencies. Apoptosis due to reactive oxygen species has been shown to be the major cause of ototoxicity. Pre-existing hearing loss, radiotherapy to the head and neck region, combined therapy with other ototoxic drugs, young age (<5), have been defined as non-genetic risk factors for CDDP ototoxicity. Apart from these factors genetic risk factors like megalin and glutathione S-transferases gene polymorphism have also been found to play a role. Although there are some studies which point out a sex-related difference for cisplatin nephrotoxicity and

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neurotoxicity [1–3] currently there is no study which addresses the same differences for CDDP ototoxicity. While our team was conducting different in vitro studies on cisplatin ototoxicity [4, 5] we thought that there may be a sex-related difference among animals, however, none of these studies were planned to research this aspect. In this study we aimed to reveal whether there is any sex-related difference for susceptibility to CDDP ototoxicity in rats.

Materials and methods

The study was approved by the Animal Experiments Local Ethic Committee of Dokuz Eylul University Medical School. Fourteen female and 14 male Wistar albino rats weighting 250–300 g were randomly divided into four groups.

Study groups

Group I ($n = 7$): male control group
Group II ($n = 7$): female control group
Group III ($n = 7$): male cisplatin group
Group IV ($n = 7$): female cisplatin group

The rats were kept in ordinary cages with free access to food and water at a temperature of 23 ± 3 °C and a relative humidity of 50 ± 10 % with artificial lighting for a period of 12 h each day. All animals were anesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (5 mg/kg) injections prior to interventions.

On the first day of the study hearing thresholds were determined by distortion product otacoustic emissions (DPOAE) and auditory brainstem responses (ABR). On the 4th day of the study a single dose of 16 mg/kg CDDP was administrated intraperitoneally to group III and IV and the same volume of saline was given intraperitoneally to group I and II. On the 7th day of the study DPOAE and ABR tests were repeated. Immediately after tests all animals were killed and cochleae and brainstems were collected for light and electron microscopic examination.

Auditory assessment

The tympanic membranes and external auditory canals of all animals were inspected prior to the study. Animals with signs of any external or middle ear problems were excluded from the study. Rats were sedated with ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (5 mg/kg). All DPOAEs and ABR tests were performed in a quiet room on the first day of the study (baseline measurement) and repeated on the 7th day of the study (72 h following the intraperitoneal cisplatin administration).

To test the integrity of the outer hair cells, DPOAE recordings were elicited from the right and left ear of each rat using the ILO-96 cochlear emission analyzer apparatus (Otodynamic Ltd, London, UK). The baseline hearing thresholds of all rats were determined by DPgram, and the signal-to-noise ratio was recorded at 7 frequencies as previously described [4]. ABRs were recorded using ICS Medical Charter equipment via insert earphones as previously described [6.]. Wave II, the wave which had the largest amplitude was used to define hearing thresholds.

Killing and tissue sampling

All rats were killed under ether anesthesia after the final auditory assessments. Temporal bones and brainstem tissues were collected for histopathologic examination.

Histopathologic examinations

Apoptosis detection

In cochlea tissues cell death was assessed with TUNEL assay that can detect fragmented DNA in the nucleus (GenScript Tunel Apoptosis Detection Kit. L00300, for paraffin embedded tissue sections, FITC-labeled POD). The kit was applied on slides according to manufacturer's instruction. After deparaffinization and rehydration of sections, they were incubated with Proteinase K and blocking was done by 3 % H_2O_2, tunel reaction mix containing Equilibration Buffer, FITC-12-dUTP and TdT was applied for 60 min at 37 °C. Assay was done with Olympus fluorescence microscope using excitation wave 450–500 nm and emission wave 515–565 nm (green). 5,000 cells per condition were evaluated and scored as % of apoptosis per all cells. All cell number was assessed by DAPI counterstain. DAPI-stained photos show nucleus of all cells in bright blue by staining DNA of the nuclei. After DAPI staining apoptotic cells are labeled by FITC by tunel method TdT. Apoptotic cells are seen in bright green.

Brainstem examination

The brainstem samples were evaluated with light microscopy using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) and caspase-3 methods as previously described in our published studies [6].
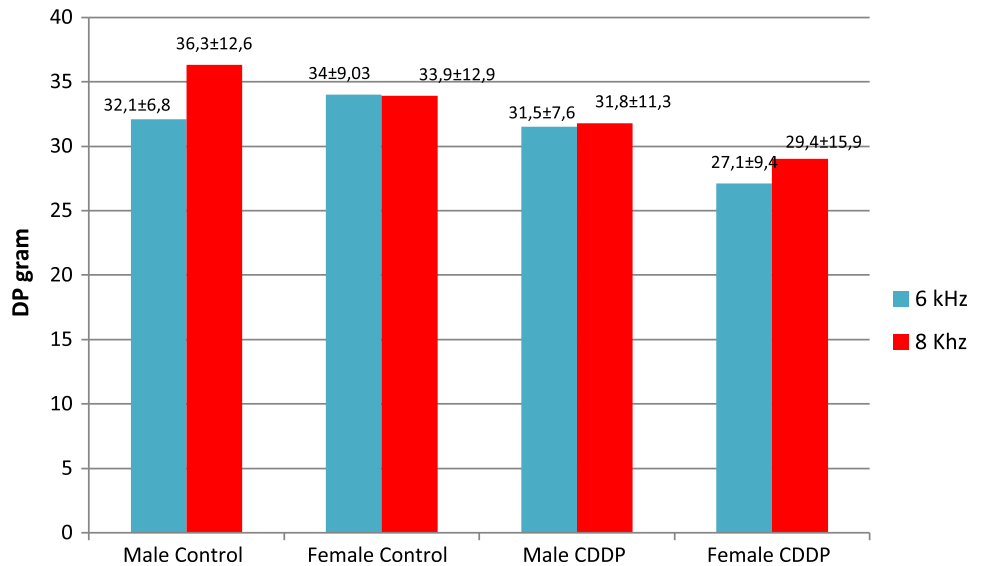
Electron microscopic examination

For electron microscopic examination of cochlear structures, temporal bones were carefully harvested and after

Fig. 1 Mean and standard deviation basal ABR values in dB SPL for each experimental groups
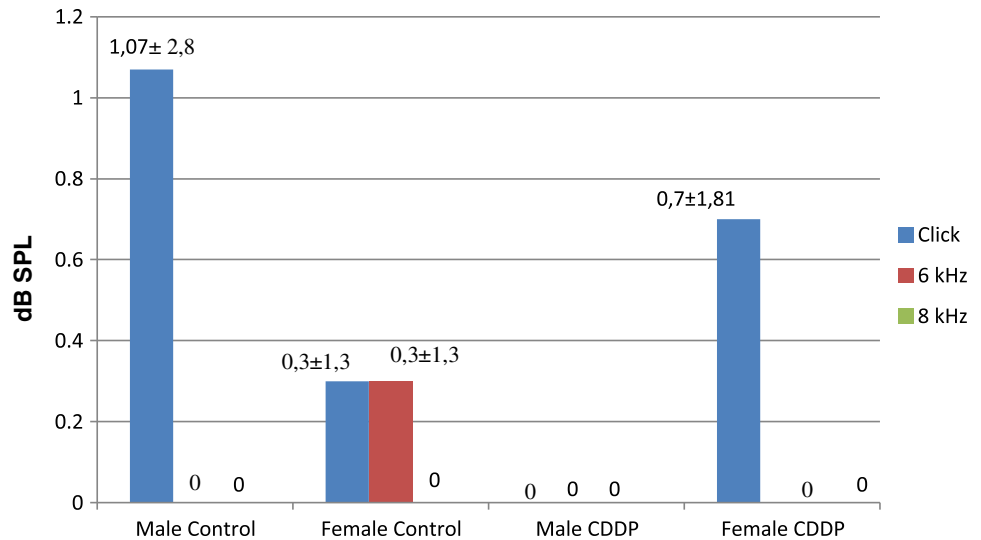
Fig. 2 Mean and standard deviation of basal DPOAE values in DP gram for each experimental groups

cochleas were opened laterally and samples were treated according to the technique described previously [6].

Statistical analyses

Statistical analyses were performed using SPSS 15.0 software package for Windows (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA) and p values less than 0.05 were considered as statistically significant. Results are presented as means $\pm$ standard deviation. One-way analysis of variance (ANOVA) post hoc Bonferroni test was used to compare the DPOAE values and ABR thresholds in each group, while Mann–Whitney U test was used to compare the apoptosis percentages of the groups. Wilcoxon test was used to test the retest variability for control groups.

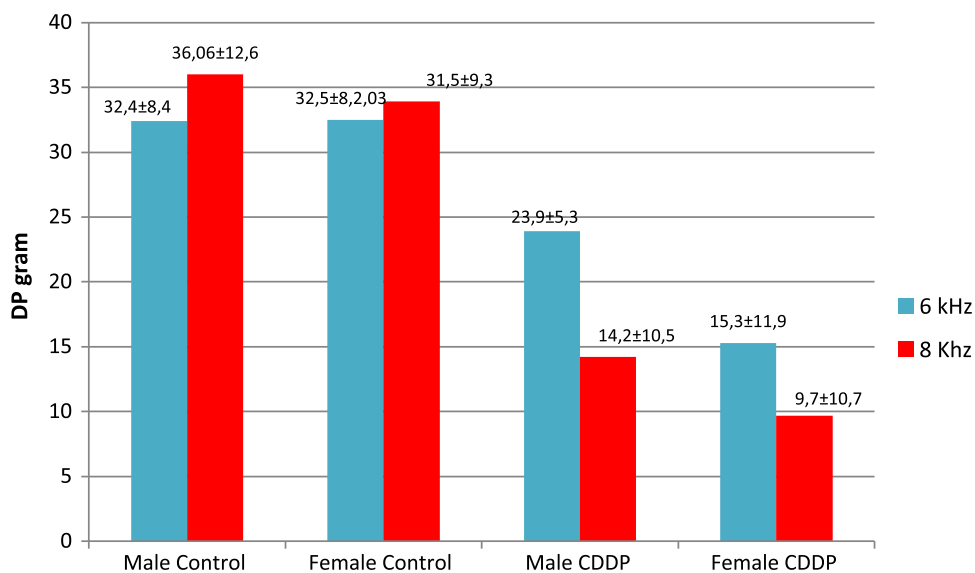## Results

Audiological results

Regarding the baseline audiological examinations no statistically significant difference was found among groups ($p > 0.05$) (Figs. 1, 2). We did not find any significancy for retest variability between control groups ($p > 0.05$). The last audiological examination revealed that CDDP administration led to ototoxicity both in male and female rats. In both male and female CDDP-administered groups; click, 6 and 8 kHz ABR and 4, 6, and 8 kHZ DPOAEs values were significantly worse than those of the control groups ($p < 0.05$). (Figs. 3, 4).

When comparing male and female CDDP-administered groups we found that 6 and 8 kHz ABR and

Fig. 3 Mean and standard deviation final ABR values in dB SPL for each experimental groups
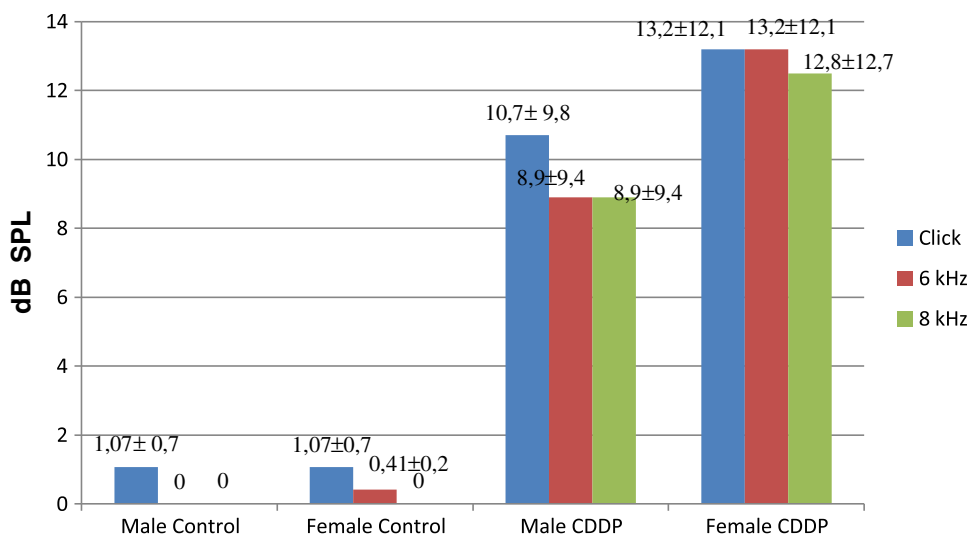
Fig. 4 Mean and standard deviation of final DPOAE values in DP gram for each experimental groups

DPOAE values of female rats were significantly worse than those of male rats ($p < 0.05$). However, click ABR values showed no significant difference between these two groups.

Histopathological results

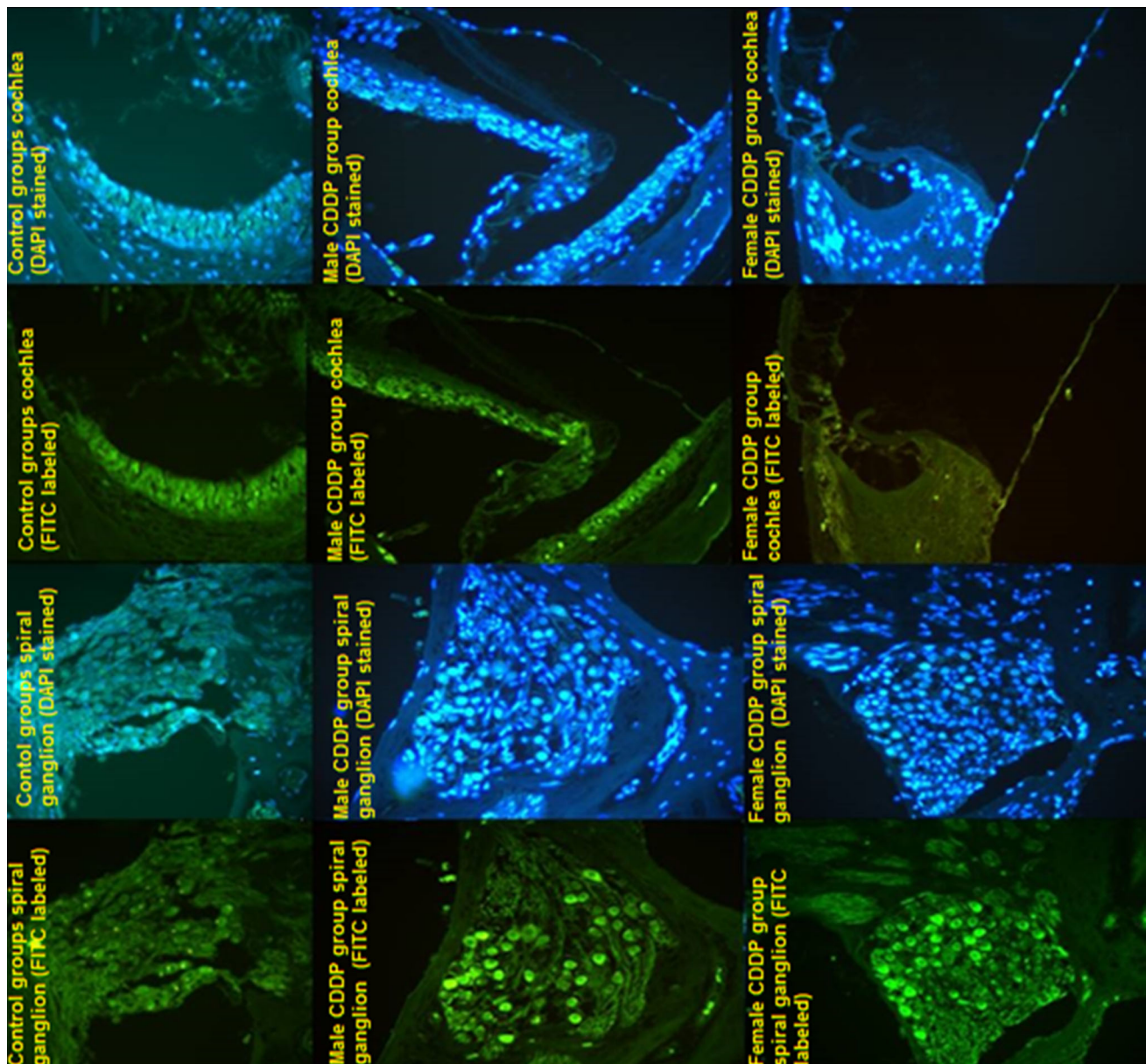*Light microscopic findings*

Inner ear histopathological examination Apoptosis: The mean percentage of apoptotic cells in the spiral ganglion were 1.1, 1.2, 10.4 and 16.7 % in Group I, Group II, Group III and Group IV, respectively. The apoptosis ratio in spiral ganglion neurons was significantly higher in Group III and IV compared with Groups I, II ($p = 0.0001$). Moreover,

when we compare both CDDP groups; we found that in the female CDDP group (group IV) there were more apoptotic spiral ganglion neurons than in the male CDDP group (group III) ($p < 0.05$). But cochlea apoptotic cell ratio between group III and IV was not statistically significant (5.8 vs 6.4 %) (Fig. 5).

Brainstem histopathological findings

In both female and male CDDP groups apoptosis was seen in brainstem tissues (Figs. 6, 7). Apoptotic caspase-3 and TUNEL + cells were statistically higher than their control groups. In the comparison of the CDDP groups in females, apoptosis was clearly more severe than males (Fig. 8).

Fig. 5 Immunofluorescence images of cochlea and spiral ganglions of rats. In DAPI-stained photos nucleus of all cells are seen bright *blue*. In FITC-labeled photos apoptotic cells are seen in bright *green*

Electron microscopic findings

In male control and female control groups, the ultrastructure of hair cells and supporting cells was normal. The boundaries of the cell membrane and nucleus were regular. Mitochondria showed normal properties. In male CDDP and female CDDP groups, intracellular degenerative areas and irregularities on the boundaries of the cell membrane were observed (Fig. 9).

In male control and female control groups, the ultrastructure of ganglion cells and satellite cells was normal. The boundaries and shapes of the cells were regular. In

male CDDP and female CDDP groups, irregularity at the cell membranes and cell nucleus was seen. There were cytoplasmic degenerative areas and loss of satellite cells in both groups (Fig. 10).

Discussion

Sex differentiation begins at embryonal life and it is under hormonal and genetic control. Sex-specific differences of effects of various molecules have not been widely studied in comparative studies [7, 8]. Apart from

Fig. 6 Brainstem TUNEL stainings. *Arrows* showing TUNEL positive (apoptotic) cells
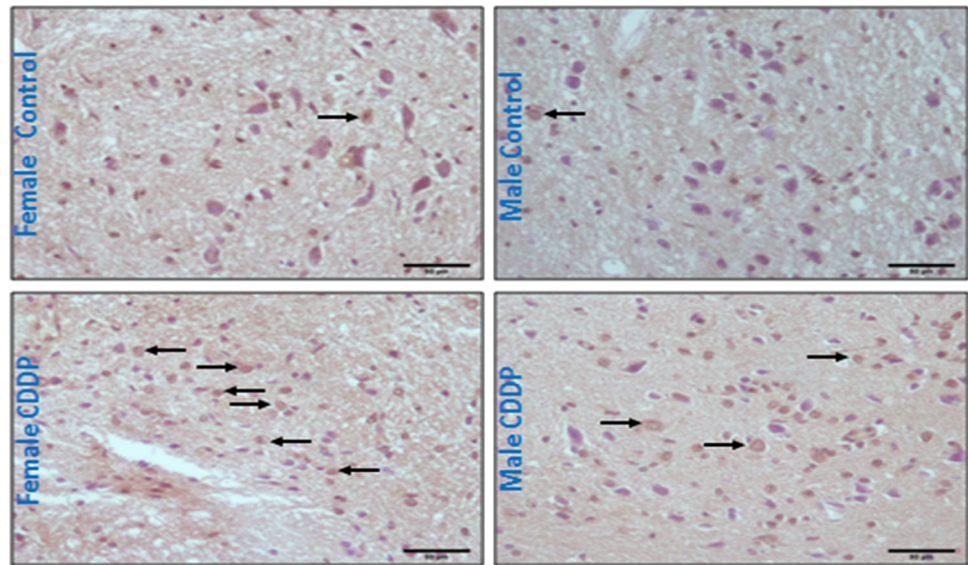
Fig. 7 Brainstem Caspase-3 stainings. *Arrows* showing Caspase-3 positive (apoptotic) cells
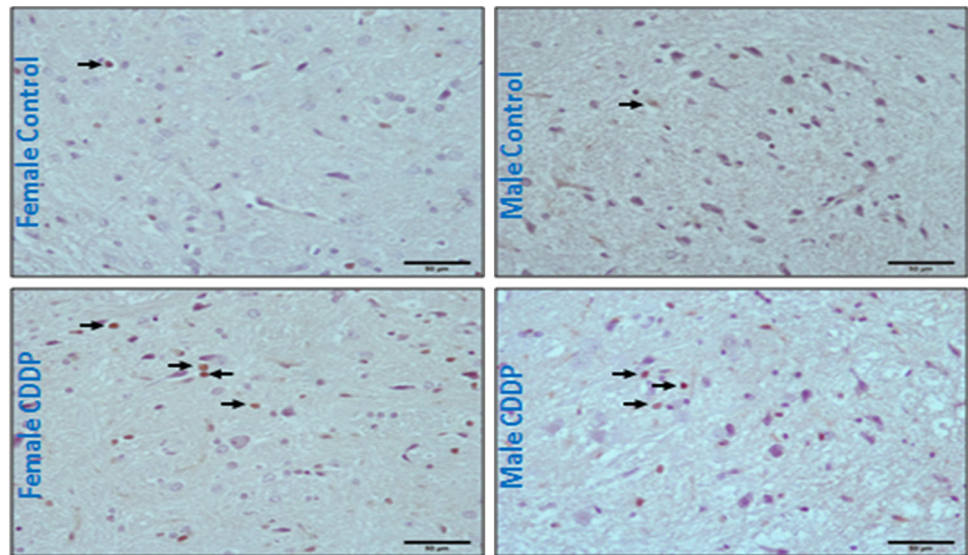

effects of sex hormones many factors can lead to different responses to toxic agents in different sexes [9]. This may be attributed to toxicogenetics-related with body size, blood volume size of the target organ, absorbtion differences and protein bindings. Toxicodynamics related with receptors, responsiveness of target organs, reached dose at the site and interactions with other molecules are also effective in different responses. For many drugs the exact mechanism of different responsiveness has not been well understood. Indeed, the effect of a given molecule may be different in various tissues of the male and female [9, 10]. Moreover, these differences may be seen before sexual maturation and may be under genetic sex control e.g. can be linked to sex chromosomes. Franconi et al. [11] concluded that

pharmacokinetics, pharmacodynamics and side effects of drugs differ between sexes.

Cisplatin is a widely used antineoplastic agent. Ototoxicity is one of the commonest side effects of CDDP which hampers application of high doses. Toxic effects of CDDP on various organs may differ among sexes. However, currently there are no studies on the sex-specific ototoxic effect of this agent.

In humans many factors such as cranial irradiation, young age, bolus and high-dose injections of cisplatin are accepted as risk factors for cisplatin ototoxicity [12–16]. But there is no consensus on whether sex is a risk factor or not for this undesirable effect. Although some studies conclude that male sex is more under threat for this side effect [13, 14] others find no relation with sex [12, 15].

Fig. 8 Apoptotic cell ratio in brainstem. *Asterisk* statistically significant compared to control. *Hash* statistically significant compared to male CDDP group
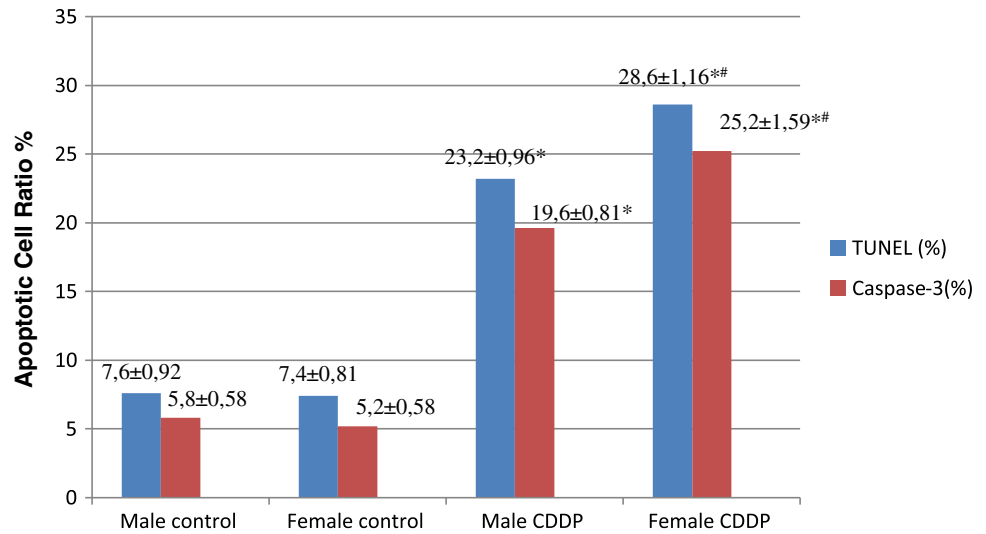
Fig. 9 Organ of Corti. In male control and female control groups, normal hair cell morphology, normal nucleus boundaries (*N*) and cilia in apical surface (*black arrows*) were seen. In male CDDP and female CDDP groups, cilia were preserved but degenerative areas and irregular cell boundaries (*red arrows*) were observed
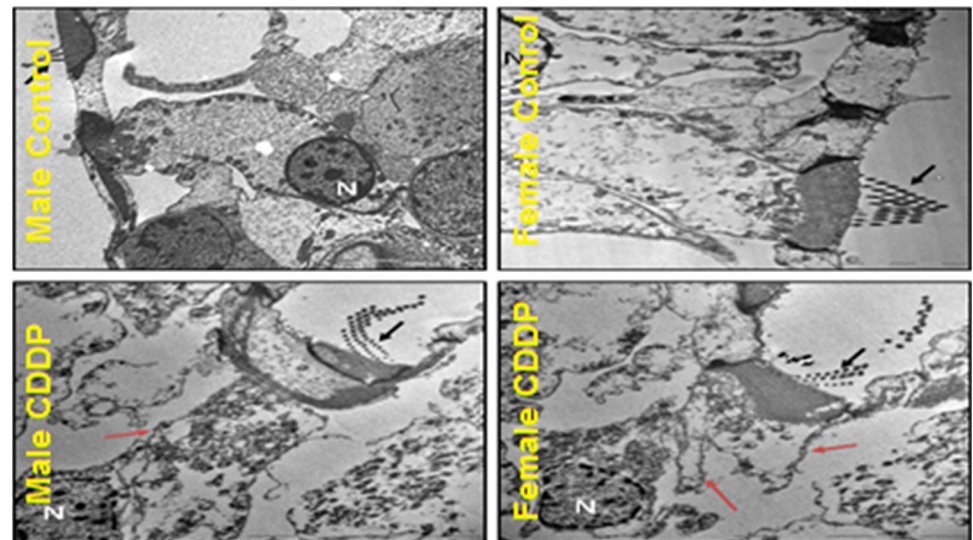
Fig. 10 Spiral ganglions: The ganglion cells in male control and female control groups showed normal morphology. The ganglion cells in male CDDP and female CDDP groups were degenerative with irregular cell membrane (*red arrows*), irregular nuclear boundaries (*white arrows*) and intracellular degenerative areas (*). Those pathological changes were more evident on female CDDP group. *N* nucleus
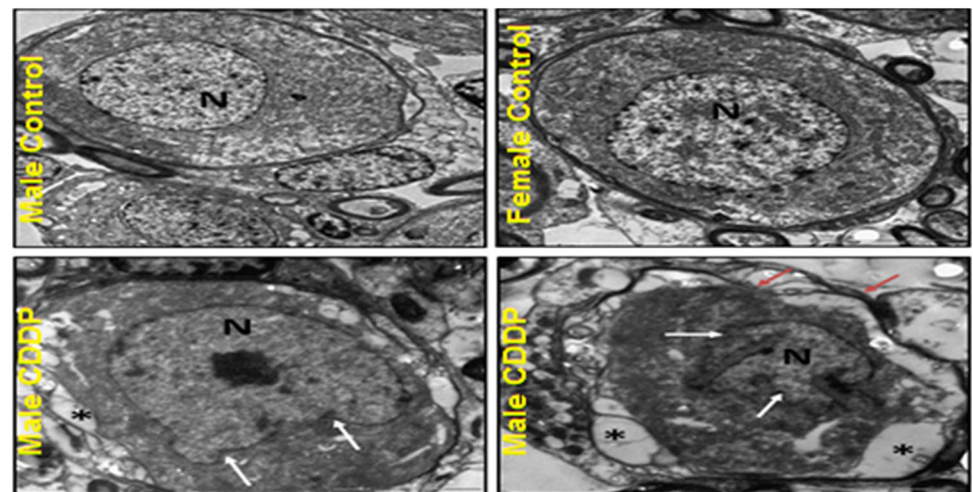
The literature on sex-related CDDP toxicity reveals that results are conflicting even on the same tissues [17–19]. Lu et al. [18] compared CDDP toxicity on renal tubular epithelial cell cultures in both sexes of monkeys and rats. Although rat cells were more sensitive than monkey cells there were not any sex-related differences in either species. In another nephrotoxicity study, female rats were found to be more susceptible to CDDP than males. These differences can be attributed to the effect of estrogen [19]. Estrogen is known to have cardioprotective and nephroprotective properties. However, in a study conducted on ovariectomised rats estrogen replacement led to increased CDDP nephrotoxicity. It is thought that high estrogen levels may cause enhanced oxidative stress by nitrous oxide (NO) production [19]. Stakisaitis et al. [20] proved that CDDP can cause more severe tubular damage and increased sodium excretion in male rats. In some studies it was concluded that estrogen may increase CDDP nephrotoxicity in rats [2, 19]. Effects of protective agents such as recombinant erythropoietin against CDDP nephrotoxicity may also differ between sexes [3].

Regarding neurotoxicity there are some confusing publications. Wongtawatchai et al. [1] proved that CDDP led to more neuronal damage in male rats. However, myelinated fiber density and myelin diameter were more severely affected in females. However, Shabani et al. [21] found that although hippocampal and cerebellum functions were severely affected upon CDDP exposure in rats there were not any differences between genders.

Recently many in vitro and in vivo studies have been conducted on the effects of different molecules for the prevention of CDDP ototoxicity [4, 5, 16, 22, 23]. However, none of them took sex differences into consideration. While our team was working on the otoprotective effect of resveratrol [4], acetyl L-carnitine [5], Korean red ginseng (unpublished data) it was observed that ototoxic effects of CDDP were different among sexes. We had used female rats for acetyl L-carnitine and male rats for other molecules. For each study the decision about which gender of animal to use was random but CDDP doses and the administration route were exactly the same. Overall evaluation of these studies showed that CDDP ototoxicity was more prominent in females. Because of this we decided to conduct this comparative study.

Our findings implied that CDDP more severely damaged hearing in female rats. Otoacoustic emissions deteriorated in both sexes at 6 and 8 kHz. However, this deterioration was more severe in females. In a similar way ABR levels were also elevated in both sexes but more in females. Histopathologic analyses showed us more severely damaged spiral ganglion cells in female rats on electron microscopic examinations. Moreover, immunofluorescence images revealed many more apoptotic spiral ganglion cells

in the female CDDP group than in the male CDDP group. In brainstem samples apoptosis was more evident for the female CDDP group but in hair cells CDDP-related damage was not obviously different between sexes. In the light of these findings we can say that the more severe hearing loss seen in female rats may mostly be attributable to neurotoxicity seen in spiral ganglion cells and brainstem. Since metabolism of various molecules which would be used against CDDP ototoxicity is also expected to differ among genders, any ototoxicity study on rats should take sex into consideration.

Conclusion

Our results implied that hearing of female rats deteriorated more than that of males upon application of Cisplatin. This difference can be attributed to the more severe damage seen in neuronal tissues such as spiral ganglion cells and brainstem neurons. To clarify the causes of these different damage patterns more studies are necessary. We think that in every cisplatin ototoxicity study sex of the animals should be taken into account.

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