Introduction

Cisplatin, a potent antitumour agent, is widely and successfully used in the treatment of many malignant tumours, including head and neck cancers. However, cisplatin has several serious side effects such as ototoxicity and nephrotoxicity. Ototoxicity, in particular, continues to be a serious problem. Bilateral, irreversible, sensorineural hearing loss, especially at high frequencies (4 – 8 kHz) and tinnitus can occur in patients receiving high doses of cisplatin. The ototoxic side effects of cisplatin have been shown to be caused by damage to the outer hair cells of the cochlea in experimental animal studies. Several agents have been used to prevent cisplatin-induced ototoxicity. One such agent is caffeic acid phenethyl ester (CAPE). CAPE is the major biologically-active component of propolis, which is present in beehives. CAPE has been demonstrated to possess numerous biological properties, including antioxidant, anti-inflammatory, antiviral, anti-invasive and anti-carcinogenic effects, for many organs in various studies. In this experimental study, we aimed to demonstrate the possible beneficial effects of CAPE against cisplatin ototoxicity in rats. To the best of our knowledge, this is the first study to evaluate the effects of CAPE on cisplatin ototoxicity by using, not only audiological assessments, but also histopathological evaluation.

Materials and methods

Study design and setting

We assert that all procedures contributing to this work complied with the ethical standards of the ‘Guide for the Care and Use of Laboratory Animals’, issued by the National Institutes of Health, Commission on Life Sciences and the National Research Council. The study was performed with the approval of the ethics committee of our institution (approval number: 2011/59).

Twenty-eight healthy male adult Wistar albino rats, weighing 240 – 345 g each, were used in this study. The rats were kept under suitable conditions in accordance with guidelines, in suitable cages that were maintained under standard environmental conditions (room temperature between 22 °C
Tissue preparation and histopathological examination

The cochleae of each rat were fixed for 24 hours in 10% formaldehyde solution and subjected to decalcification for three weeks in 10% ethylenediaminetetraacetic acid solution. After the fixation and decalcification processes, the cochleae were rinsed in tap water for 24 hours, dehydrated using a graded alcohol series, rendered transparent and embedded in paraffin. Each paraffin-embedded specimen was sliced with a microtome (Leica RM 2125, Leica Microsystems GmbH, Nussloch, Germany) at a thickness of 5 μm. Each section was stained with haematoxylin and eosin (H&E) for routine histological examination and observed with a Nikon ECLIPSE 80i (Japan) microscope. The remaining tissue sections were deparaffinized, reacted with an alcohol series, rinsed, incubated with 0.1% – 1% H2O2, washed with phosphate-buffered saline (PBS) and stained with avidin–biotin complex. The sections were conducted with 10% normal bovine serum. Caspase-3 rat polyclonal IgG primary antibody was diluted to a concentration of 1:400 in normal bovine serum. The obtained tissue sections were incubated with the caspase-3 solution overnight. Caspase 3 IgG antibody was used to show apoptotic cells. PBS was dripped onto the negative control sections. The next day, the sections were rinsed with PBS and incubated with the secondary antibody (bovine antirat biotin), followed by conjugation with horseradish peroxidase, washing with PBS and treatment with diaminobenzidine chromogen. The sections were rinsed with distilled water and stained with haematoxylin. They were then rinsed with distilled water until the blue stain disappeared and were coverslipped after being reacted with alcohol and xylene. The sections were assessed according to the intensity of the caspase-3 immunoreaction, under a light microscope (Nikon ECLIPSE 80i, Japan).

The degree of outer hair cell degeneration was histopathologically evaluated, by a single pathologist who was blinded to the study groups, as follows: 0, no degeneration (0% – 25% loss of outer hair cells); +, mild degeneration (26% – 50% loss of outer hair cells); ++, moderate degeneration (51% – 75% loss of hair cells); and +++, severe degeneration (76% – 100% loss of hair cells).
Audiological assessments

Hair cells were tested using DPOAE measurements. The DPOAE readings were recorded at the baseline before the initiation of the treatment and before the animals were killed. During DPOAE measurements, the rats were anaesthetized in a quiet room (less than 50 dB background noise). Otoacoustic emissions (OAEs) are the physiologic indicators of cochlear function. OAEs are a measure of coherent acoustic reflections and acoustic distortion products in the external auditory meatus. These acoustic emissions are triggered by stimuli in specific frequencies or bandwidths induced through a transducer, which is positioned close to the tympanic membrane and acoustically coupled to the external auditory meatus. The measurements performed through this simple, non-invasive and reliable method are independent of the state of consciousness or physical aptitude of the subject.

Before the DPOAE measurements were performed, otoscopy was carried out to verify that the ear canal and tympanic membrane were normal. Only rats with normal ear canals and tympanic membranes were included in the study. Additionally, animals with abnormal initial otoacoustic responses were excluded from the study. The DPOAE readings were obtained from the right ears of the animals with the help of a standard commercial ILO-96 OAE cochlear emission analyser (Otodynamics, London, UK). The obtained data were processed and evaluated using OAE software (EZ Screen 2 Otodynamics OAE Screening and Data Management Software, Hatfield, UK). The duration of each DPOAE test was approximately three minutes.

After the animals were anaesthetized, primary tones created by two distinct speakers were introduced into the auditory canals of the animals through an earphone and a plastic adapter that sealed the probe in the external auditory canal. Equi-level primary tones f1 (65 dB) and f2 (55 dB) were adjusted at an f1/f2 ratio of 1.22, and the DPOAEs were measured at five different frequencies between 2000 and 8008 Hz (2002, 3003, 4004, 6006 and 8008 Hz). The DPOAEs were recorded in DP-grams. The baseline hearing status of each animal was obtained through the DP-gram, and the signal-to-noise ratio (SNR) was calculated. The SNR was determined at five frequencies in each animal. The data collected from each individual rat were included in the statistical analysis.

Statistical analysis

The statistical analyses were carried out using the SPSS 15.0 software package for Windows (SPSS Inc., Chicago, IL). The Wilcoxon signed-rank test was used to determine the differences in the amplitudes of the DPOAEs and the corresponding noise floor differences and thresholds for each frequency. The histopathologic variations between the control, cisplatin, CAPE and cisplatin-CAPE groups were evaluated and compared using the Mann–Whitney U test. Statistically-significant differences were indicated by P-values of < 0.05.

Results

Audiological assessments

In the DP-grams, the amplitudes of the emissions were greater than the noise floor at all testing frequencies in all sessions. The baseline DPOAE measurements on day one (before the administration of the study drugs) were similar in all groups (P > 0.05; Figure 1). The second DPOAE measurements performed on day five revealed a distinct decrease in cochlear activity at all tested frequencies (2002 – 8008 Hz) in the cisplatin group (P < 0.05; Figure 2).

Figure 1
DP-grams of rats treated with saline, cisplatin, CAPE and cisplatin-CAPE on day one. CAPE, caffeic acid phenethyl ester; DP, distortion product; DPOAE, DP otoacoustic emission; SPL, sound pressure level.
Histopathologic evaluation

Light microscopy showed that the outer hair cells were mostly preserved in the control and CAPE groups. In the cisplatin group, there was moderate-to-severe loss of the outer hair cells, while in the cisplatin-CAPE group, there was mild-to-moderate degeneration of the outer hair cells (Figure 5). On immunohistochemical examination, caspase immunoreactivity was not observed in the control, CAPE and cisplatin-CAPE groups, whereas cytoplasmic or nuclear caspase-3 immunoreactivity was clearly observed in the hair cells, supporting cells and basilar membrane in the cisplatin group (Figure 6).

Discussion

Cisplatin, which contains the heavy metal platinum, is used for the treatment of many types of malignant tumours, including head and neck cancers. The drug has serious dose-limiting side effects, the major of which are nephrotoxicity and ototoxicity. It is possible to reduce the nephrotoxicity of cisplatin by intravenous hydration and diuresis, but its ototoxicity still remains a serious problem.19

Many experimental animal studies have shown that the main functional and morphological targets of cisplatin otoxicity are the outer hair cells of the cochlea, but the precise mechanism underlying the ototoxicity is not well understood.3,8,20 Electron mi-
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### Table 1

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SD: standard deviation; min: minimum; max: maximum

Figure 5

Histopathological appearance of the mostly-preserved outer hair cells (arrows) in the control (A) and CAPE (B) groups (H&E, ×400). Severe loss of outer hair cells (arrows) in the cisplatin group (C) (H&E, ×200). Closer view of C (D) (H&E, ×400). Approximately 50% – 90% outer hair cells are preserved (arrows) in the cisplatin-CAPE group (E) (H&E, ×200). Closer view of E (F) (H&E, ×400).
Antioxidant molecules have been claimed to be useful in reducing cisplatin ototoxicity. However, an agent that effectively prevents cisplatin ototoxicity is not yet available.

CAPE is extracted from propolis, which is found in beehives, and is the major biologically-active component of propolis. Many studies have shown that CAPE has strong antioxidant, anti-invasive, antimitotic, anti-inflammatory, immunomodulator and wound-healing properties. CAPE has been demonstrated to exert protective effects against many toxic compounds that affect the cochlea, tympanic membrane, kidney, heart, red blood cells, brain, spinal cord and nasal mucosa. Therefore, in the present study, we aimed to determine whether CAPE had any protective effects against cisplatin ototoxicity.

We employed DPOAE measurement, which has been shown to be very useful in detecting the audiological effects of cisplatin and streptomycin ototoxicity. Under this account, outer hair cells degenerate due to oxidative processes resulting in DNA damage. In experimental studies, significantly-higher ROS levels were found in animals treated with cisplatin than in control animals. Increased levels of ROS result in apoptosis and cell death. Immunostaining of the cells with caspase 3 indicates apoptosis. However, lower levels of ROS were found in animals that were administered cisplatin along with protective agents.

In recent years, several antioxidant protective agents have been used to prevent cisplatin ototoxicity in experimental studies. Some well-known agents are CAPE, resveratrol, aminoguanine, ginkgo biloba extract, methionine, sodium salicylate and erdosteine. In addition to these agents, many antioxidant molecules have been claimed to be useful in reducing cisplatin ototoxicity. However, an agent that effectively prevents cisplatin ototoxicity is not yet available.

Figure 6
Caspase-3 negativity in the control (A) and CAPE (B) groups, caspase-3 positivity in the cisplatin (C) group and disappearance of caspase-3 activity in the cisplatin-CAPE (D) group (immunoperoxidase, ×400).
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toxicity.2,4,7–9,19,23 The administration of cisplatin for more than five days can result in death in rats and, therefore, we chose to perform our experiment for only five days, similar to previous studies.2,4

In a study by Kızılay et al.,2 the DPOAE results revealed severe deterioration in hearing in the cisplatin group. They also showed that, when administered with cisplatin, CAPE significantly reduced cisplatin ototoxicity. Consistent with this, we found a significant decrease in DPOAE levels at 2002 – 8008 Hz on day five in the cisplatin group. The hearing abilities of the cisplatin-CAPE group also deteriorated. However, analysis of the DP-gram results revealed statistically-significant differences between the cisplatin and cisplatin-CAPE groups. Given these findings, we conclude that CAPE has a protective effect against cisplatin ototoxicity, aligning with the results reported by Kızılay et al.2

The present study is the first to assess outer hair cell loss histopathologically as well as via DPOAE measurements. Histological examination with H&E staining showed that the outer hair cells were mostly preserved in the control and CAPE groups. However, moderate-to-severe degeneration of the cochlear outer hair cells was observed in the cisplatin group. In the cisplatin-CAPE group, mild-to-moderate outer hair cell degeneration was observed. The demonstration of increased apoptotic cells in the cochlea of rats with caspase-3 indicates hearing impairment. On immunohistochemical examination, caspase-3 immunoreactivity was not observed in the control, CAPE and cisplatin-CAPE groups, but cytoplasmic or nuclear caspase-3 immunoreactivity was clearly observed in the hair cells, supporting cells and basilar membrane in the cisplatin group.

In the current study, we aimed to show effects of CAPE on cisplatin ototoxicity. Although there are some studies showing the effects of CAPE on cisplatin toxicity there is a lack of data regarding the effects of CAPE on cisplatin antitumoural activity.2,4,23 Further studies investigating the effects of CAPE on cisplatin antitumoural activity are needed.

Conclusion

Many agents have been used to prevent cisplatin ototoxicity. Because cisplatin causes cell death via ROS, antioxidant agents are becoming increasingly important. CAPE is one of these agents. Histopathological, audiological and immunohistochemical examinations in our study showed that CAPE is useful in preventing cisplatin ototoxicity.

Acknowledgements

An abstract of this manuscript was submitted to the 4th Congress of National Otology-Neurotology (21–24 April 2016, Antalya, Turkey) for oral presentation.

Conflict of Interest

We have no financial relationships with any organization.

References


