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Original Article

Effect of Transtympanic Injection of Melatonin on Cisplatin–Induced Ototoxicity

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OBJECTIVE: Cisplatin is a chemotherapeutic agent that is widely used in cancer treatment. Numerous side effects have been detected, one of which is ototoxicity. Melatonin, a product of the pineal gland, has a neuroendocrinoimmunological role in vertebrates. In the present study, we investigated the effects of melatonin on cisplatin-induced ototoxicity.

MATERIALS and METHODS: Twenty-four Wistar albino rats were divided into three groups. Group 1 was administered both intraperitoneal and transtympanic saline; Group 2, 12 mg/kg of intraperitoneal single-dose cisplatin and transtympanic saline; and Group 3, 12 mg/kg of intraperitoneal single-dose cisplatin and 0.1 mg/mL of transtympanic melatonin for 5 days. Before and after the procedure, distortion product otoacoustic emissions and auditory brainstem responses of all the rats were measured. At the end of the procedure, the cochleas of the rats were investigated at the microscopic level.

RESULTS: Group 3 had lesser threshold shift in otoacoustic emissions and auditory brainstem responses at all frequencies than Group 2 ($p<0.005$). The difference was not significant between Group 1 and Group 3. On the microscopic level, more epithelial loss and less TNF staining were detected in Group 2 than in Group 3.

CONCLUSION: As an antioxidant and immune modulator, melatonin is effective against cisplatin ototoxicity. Both hearing thresholds and tissue investigations supported this conclusion. Melatonin can also be used to treat cisplatin ototoxicity using transtympanic local application in lower doses.

KEYWORDS: Melatonin, cisplatin, transtympanic, ototoxicity, cochlea

INTRODUCTION

Cisplatin is a well-known chemotherapeutic substance that is commonly used in head and neck cancer treatment. As is the nature of various cancer drugs, cisplatin has many side effects, including bone marrow toxicity, renal toxicity, gastrointestinal toxicity, peripheral neuropathy, and ototoxicity. These clinical side effects are mostly dose-dependent. Formation of ototoxicity depends on the outer cells of the cochlea indirectly and damage of the auditory neurons directly [1]. The decrease in the antioxidant protection of the organ of corti and the increase in the synthesis of reactive oxygen species (ROS) level [2-4] play roles in cisplatin toxicity. In the cochlea, the levels of glutathione, glutathione peroxidase, and reductase decrease, and the levels of superoxide dismutase, catalase, and malondialdehyde increase [4]. However, the cochlea's protective upregulation mechanism mitigates the ototoxic effects of cisplatin [5]. Many antioxidants prevented the ototoxic effects of cisplatin such as amifostine, D-methionine, 4-methylthiobenzoic acid, sodium thiosulfate, and superoxide dismutase [2, 6, 7]. Cochlear destruction by ROS has been found on the outer hair cells and at the intraperilymphatic level [7, 8].

Melatonin is a secreted pineal gland hormone synthesized in the circadian rhythm, mainly in the dark. It has a neuroendocrinoimmunological role in the human body. The melatonin cycle affects the central nervous system, retina, cochlea, immune system, and Harderian gland through the melatonin receptors of these organ systems [9-12]. In addition, the antioxidant role of melatonin is well known [13].

The objective of our study is to scrutinize the antioxidant effect of intratympanic melatonin on cisplatin-induced ototoxicity.

MATERIALS and METHODS

Animals

Healthy adult male Wistar albino rats weighing up to 180–320 g were obtained from Marmara University Research Center for the study. The rats were preserved under ideal laboratory conditions [temperature (21±1°C), humidity (70±10%), and 12 h light–dark cy-

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cle). In addition, all rats were adjusted to the laboratory for one month before the experiment and fed freely *ad libitum*. Ethical approval was obtained from the University's ethical committee for animal studies, and all guidelines were strictly observed during the study.

Experimental Protocol

Ototoxicity was achieved by a unique dose of intraperitoneal injection of cisplatin (12 mg/kg) (Cisplatin Ebewe 100 mg/100 ml flacon; Liba Lab) at 30 min before the administration of melatonin. Twenty-four male Wistar rats aged 3 months were separated into three groups. Group 1 was the control group; the rats in this group were injected with a single dose of intraperitoneal saline at a volume equivalent to the volume of intraperitoneal cisplatin and with 0.1 cc transtympanic saline for 5 days. Group 2 rats were injected with a single dose of intraperitoneal cisplatin (12 mg/kg b.w., i.p.) and 0.1 cc transtympanic saline for 5 days. Group 3 rats were injected with both transtympanic melatonin (0.1 mg/mL IT for 5 days) (Sigma-Aldrich; St Louis, MO, USA) and a single dose of intraperitoneal cisplatin (12 mg/kg b.w., i.p.).

Melatonin application commenced on the day of cisplatin injection and lasted for 5 days.

Transtympanic injections were conducted using a 26-gauge needle through the anterosuperior quadrant of the tympanic membrane under an operational microscope. The measurements of the transtympanic injections, distortion product otoacoustic emissions (DPOAE), and auditory brainstem responses (ABR) were performed under general anesthesia with 90 mg/kg intramuscular ketamine hydrochloride (Ketalar; 36 Eczacıbaşı, İstanbul, Turkey) and with 10 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany).

Audiologic Assessment

Before and after the procedure, while the rats were under general anesthesia, both DPOAE and ABR were calculated. DPOAE was calculated on the first day of the study and 10 days after the last dose of cisplatin administration with a Bio-Logic Navigator PRO Scout Diagnostic OAE (Natus Medical; CA, USA). The initial tones were directed into the external auditory canal of the rat with an insert earphone using a plastic adapter that covered the probe in the external auditory canal. DPOAEs were recorded at 2f1–f2 frequency with a constant frequency ratio (f2/f1=1.22). The intensities of the initial stimuli were held constant and set as equilevel (L1 = L2) at 65 dB SPL (decibel sound pressure level). DPOAE data were recorded for the following frequencies: 3825, 4549, 5434, 6367, 7604, and 9071 Hertz (Hz), and were plotted as a function of f2.

The ABR values were evaluated 1 day prior to the study and recalculated in 10 days after cisplatin injection. These values were measured with Biologic sys. Corp. (version 2.3.0) and Navigator Pro electrophysiological instruments (Natus Medical Inc.; San Carlos, CA, USA) in the right ear of the rats. The ABR measurements were calculated by using needle electrodes (Technomede Europe, subdermal needle electrode). Auditory stimuli were directed with a Bio-Logic insert earphone (21/s stimuli ratio and alternating polarity). Auditory brainstem response calculation was recorded at the following frequencies: click, 4000, 6000, and 8000 Hz.

Cochlear Section

At the end of the ABR and DPOAE tests, all rats were deeply anesthetized with ketamine and decapitated. All the cochleae of the rats from all groups were harvested, and pathological and microscopic examination was conducted via light microscopy (Olympus BH-2; Tokyo, Japan). All the cochleae were studied at the microscopic level with hematoxylin and eosin and Tumor Necrosis Factor Alpha (TNF-α) dyes.

Statistical Analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) 15.0 software (IBM SPSS Statistics, IBM Corporation; Chicago, IL, USA). The groups were compared using ANOVA, post-ANOVA Tukey's B test, and Pearson's correlation analysis; $p<0.05$ was considered statistically significant.

RESULTS

The pre-treatment and post-treatment DPOAE values and threshold shifts at DPOAE of the groups were calculated (Figures 1, 2). Group 1 had significantly better thresholds in all frequencies than Group 2 ($p<0.01$). In addition, Group 1 and Group 3 did not have any significant differences in otoacoustic values. Group 3 had significantly

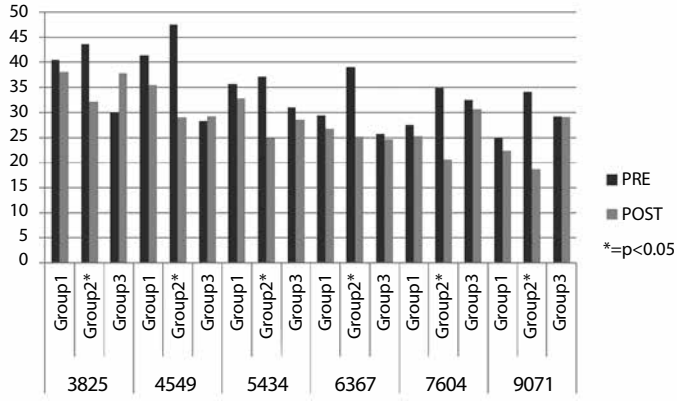

Figure 1. Distortion product otoacoustic emission (DPOAE) mean values of the study groups before and after treatment. Group 1 has statistically similar pre- and post-treatment frequencies ($p>0.05$) except 4549 Hz ($p<0.05$). Group 2 has statistically significant worse post-treatment DPOAE results in all frequencies compared to pre-treatment ($p<0.05$). In addition, Group 3 has statistically similar pre- and post-treatment frequencies ($p>0.05$)

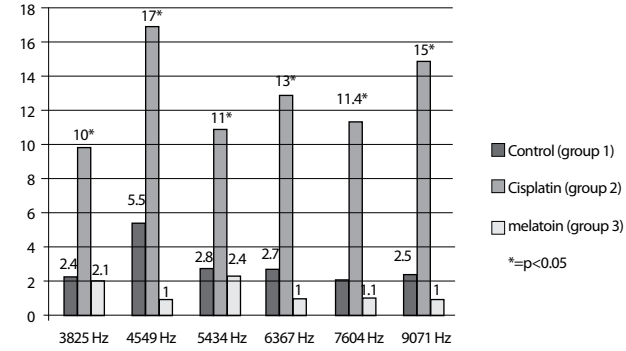

Figure 2. Threshold shifts of the DPOAE between the groups. Group 1 has significantly lower thresholds in all frequencies than Group 2 ($p<0.01$). In addition, Group 1 and Group 3 do not have any significant difference in otoacoustic values. Group 3 has significantly lower decreases at 9704, 7604, 5434, and 4549 Hz than Group 2 ($p<0.05$)

smaller decreases at 9704, 7604, 5434, and 4549 Hz than Group 2 ($p<0.05$).

In addition, the ABR values were calculated and compared with the change in the thresholds before and after treatment among the groups (Figures 3, 4). Group 3 had better ABR click at 4000, 6000, and 8000 Hz than Group 2 ($p<0.05$). Group 1 had better hearing levels than Group 2 ($p<0.01$); however, this difference was not detected between Group 1 and Group 3 ($p>0.05$).

Pathologic examination was performed subsequent to the removal of the cochlea. The analysis was conducted semi-quantitatively through the classification of the changes taking place at the tissue level. Edema, vascularity, and inflammation were classified and were scored as 0: none, 1: mild, 2: moderate, and 3: severe. Cilia loss and epithelial loss were scored as 0: normal, 1: mild loss, 2: moderate loss, and 3: severe loss.

According to the pathologic results, there was no significant difference observed between the groups. However, when comparing the means, we detected less cilia loss and epithelial loss in Group 3 than in Group 2 (Figures 5, 6). Similarly, less TNF-α staining was detected in Group 2 than in Group 3 on the microscopic level (Figures 7, 8).

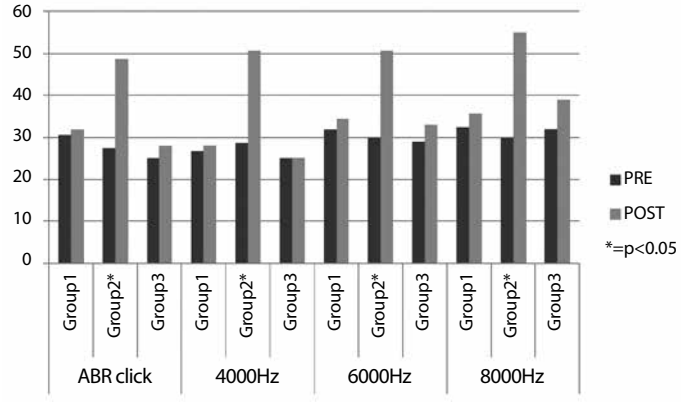

Figure 3. Pre- and post-treatment ABR mean values of the study groups. Group 1 and Group 3 have statistically similar pre- and post-treatment frequencies ($p>0.05$). Nevertheless, Group 2 has statistically significant worse post-treatment ABR results in all frequencies than pre-treatment values ($p<0.05$)

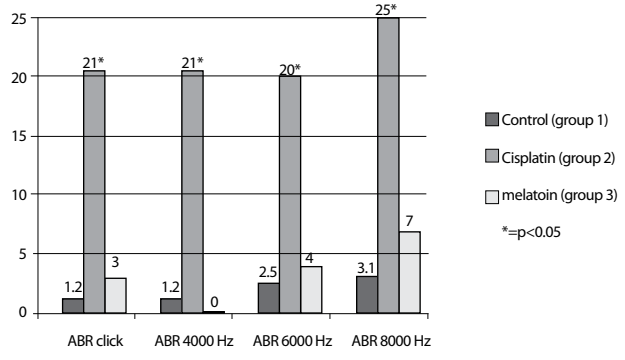

Figure 4. Threshold shifts of the ABR values between groups. Group 1 has better thresholds than Group 2 ($p<0.01$); however, this difference is not detected in Group 3 ($p>0.05$)

DISCUSSION

Cisplatin is a well-known chemotherapeutic agent used in the treatment of many types of cancer, such as head and neck cancers. The ototoxic effects as well as many other toxic impacts of cisplatin, such as renal toxicity, neural toxicity, gastrointestinal toxicity, and bone marrow toxicity, are well known and have been detected at a dose of 5 mg/kg (i.p.) in rats [2, 14]. In our study, we applied 12 mg/kg cisplatin to induce cisplatin ototoxicity. If we observe the DPOAE and ABR values of Group 2 in all frequencies, decreases in hearing can be detected. Cisplatin causes ototoxicity by increasing the concentration of reactive oxygen radicals (ROS) [2].

Melatonin, a pineal secretory product of vertebrates, is a tryptophan derivative that can be generated in numerous tissues and cells such as the cochlea [10, 13]. A melatonin receptor is also present on the cochlea [12]. Melatonin has, by nature, a neuroendocrinoimmunological role at the tissue level. It has both indirect antioxidant and direct free radical scavenger activity [13]. Melatonin provides these effects by means of transforming into its metabolites, such as cy-

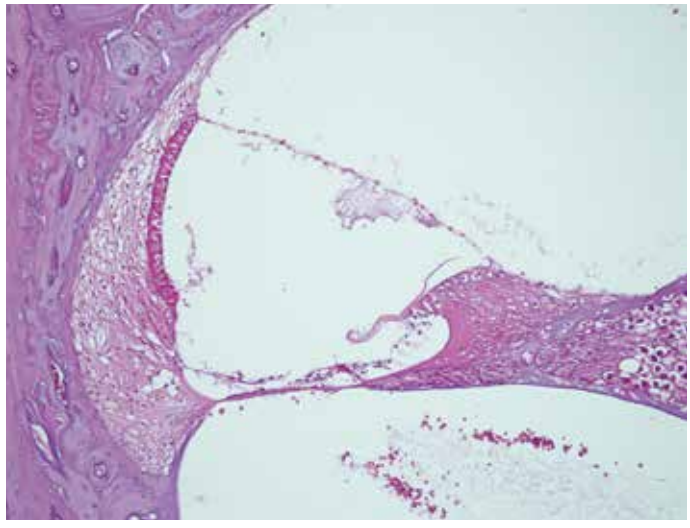

Figure 5. Epithelial loss in the cisplatin group (Group 2) (hematoxylin and eosin, 200×)

Figure 6. Reduced loss in the cochlear epithelium in the melatonin treated group (Group 3) (hematoxylin and eosin, 200×)

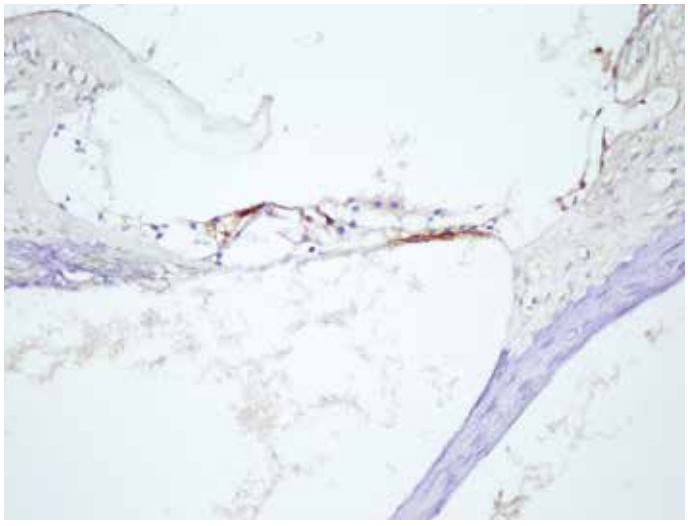

Figure 7. Reduced amount of TNF-α dye in the cochlear epithelium in Group 2 (TNF-α dye, 400×)

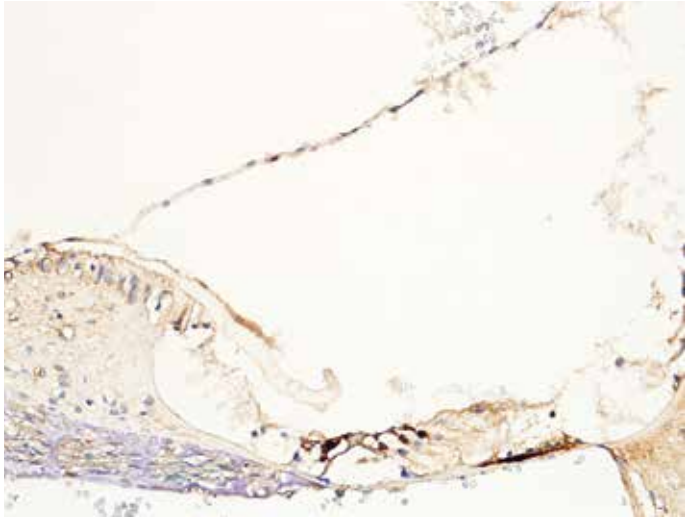

Figure 8. Relatively higher staining with TNF-α dye of Group 3 (TNF-α dye, 400×)

clic N-1-acetyl-5-methoxykynuramine, 3-hydroxymelatonin, and N-1-acetyl-N2-formyl-5-methoxykynuramine [15-17]. All these metabolites neutralize free radicals. Moreover, melatonin triggers the antioxidant system and induces the secretion of antioxidants such as glutathione [18]. In addition, melatonin, in higher concentrations, has a vasodilatory effect in the cerebral arteries [19]. According to previous studies, this protective effect has been demonstrated in cisplatin ototoxicity. Oral and intraperitoneal routes have been sought; however, the systemic side effects of melatonin were deemed to pose an obstacle for the treatment [20, 21]. In addition, it was observed that high concentrations of melatonin potentiated the ototoxicity of the amicacin-treated rats, in contrast to expectations [21]. Local therapies are important at this point. The transtympanic route is defined as a safe and usable method in patients with ototoxicity. This treatment method is very effective because electrolytes, steroids, and various antibiotics, such as gentamicin, neomycin, and streptomycin, pass through the round window and become concentrated in the perilymph [22]. Hence, we preferred the transtympanic route to alleviate the ototoxic effects of cisplatin. In our study, Group 3 had a smaller decrease in the DPOAE and OAE frequencies than Group 2. Based on this data, we determined that transtympanic melatonin had a protective effect in cisplatin ototoxicity. High frequencies in DPOAE and ABR were statistically protected with the help of melatonin. We applied a low dose of melatonin in our study; however, some studies assert that the application of relatively high doses of oral or intraperitoneal melatonin is effective against ototoxicity. As discussed earlier, it is known that the systemic therapies have some side effects, such as potentiation of the ototoxic effects of cisplatin by means of vasoconstriction of the vessels [23]. We eliminated this effect via direct penetration into the perilymph via the round window, so that the drug is concentrated at a high level in the cochlea. The protective effect of melatonin can also be seen at the tissue level. The cochleae were investigated by microscopy. We detected a loss in the epithelial tissue due to cisplatin. We also observed that TNF-α staining was reduced in the cisplatin group, which exhibited damage at the tissue level. Melatonin has an additional curative effect on cisplatin ototoxicity. Although this impact was not statistically significant, the remarkable curative process was observed in our study. Similarly, Ye et al. [24] found that the loss of epithelial hair cells induced by gentamicin toxicity is reduced by melatonin at the tissue level, and this change was statistically significant.

All these findings support the protective effect of transtympanic melatonin on cisplatin ototoxicity. We recommend melatonin treatment of cisplatin-induced ototoxicity; however, further controlled studies with patient groups should be conducted.

As a well-known antioxidant agent, melatonin can be also synthesized in the cochlea. Its protective effect via local methods, such as the transtympanic route, is demonstrated in this study. According to this data, patients with ototoxicity or sudden hearing loss can be treated with melatonin by the transtympanic route effectively.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Marmara University./61.2013.mar.

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Conflict of Interest: No conflict of interest was declared by the authors.

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