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RESEARCH ARTICLE

The Potential Therapeutic and/or Protective Effects of Steroid, PRP and Melatonin on Nise-Induced Hearing Loss In Rats

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Abstract

Introduction: To investigate the potential therapeutic and protective effects of steroid, platelet-rich plasma (PRP), and melatonin treatment on noise-induced hearing loss (NIHL).

Methods: A total of 42 rats were divided into five groups: Group 1 did not receive any drug, Group 2 received methylprednisolone via the intratympanic route at 24 hours after acoustic trauma (AT), Group 3 received PRP via the intratympanic route at 24 hours after AT, Group 4 received intraperitoneal melatonin at 24 hours before AT, and Group 5 received intraperitoneal melatonin at 24 hours after AT. Two of the 42 rats were sacrificed and used for blood source material to prepare PRP. Each group was exposed to noise at the 105 dB sound pressure level for 12 hours to induce AT. Auditory brainstem responses (ABRs) were determined before AT and on days 1 and 28 after AT, and then histomorphological assessment was performed to identify cellular changes.

Results: In the ABR test performed on day 28 after AT, a statistically significant improvement was found in the hearing thresholds of all the four treatment groups compared to the control group ($p < 0.05$). The improvement in the melatonin groups (Groups 4 and 5) was statistically significantly better than in the steroid and PRP groups ($p < 0.05$). Although the hearing thresholds of the steroid group were better than those of the PRP group, this difference was not statistically significant ($p > 0.05$). As a result of the histopathological examination performed on day 28 after AT, cell loss after AT was statistically significantly reduced in all the experimental groups compared to the control group ($p < 0.05$).

Conclusion: Based on ABR testing and histopathological findings in a rat model, we conclude that melatonin may be effective in reducing NIHL and noise-induced cochlear damage.

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Introduction

Noise is defined as unpleasant and unwanted sound, and the noise-induced hearing loss (NIHL) is considered a serious occupational and environmental health problem.[1] NIHL is one of the most prominent reasons for sensorineural hearing loss (SNHL) in adults, while it also causes labor and economic loss.[2-3] According to the World Health Organization (WHO) review in 2015, it is estimated that almost 1.1 billion young people worldwide could be at risk of NIHL.[4]

The traumatizing noise may be repetitive, continuous, or pure impulsive. Continuous exposure to intense noise causes temporary threshold shifts (TTSs) and/or permanent threshold shifts (PTSs). In addition, acoustic trauma (AT), injury or damage to the inner ear caused by exposure to excessively loud noise over a short time can result in irreversible auditory damage due to the loss of hair cells in the organ of Corti and impaired cochlear microcirculation.[5] TTSs are not accompanied by cell death but mostly lead to stereocilia dysfunction and resolve within 24-48 hours. However, if the noise and/or exposure to AT persists, PTSs with cell death may develop and sensorineural hearing loss becomes irremediable.[6] In cases where auditory hair cells cannot be replaced, preventing hair cell death or a therapeutic intervention in the first 24 hours after AT is critical to maintain hearing ability.[7] The main goal in this treatment is to reduce oxidative stress and inflammation on the cochlea and to reestablish the cochlear microcirculation. For this purpose, a variety of pharmacological agents, such as corticosteroids, H1 antagonists, melatonin, thymoquinone, pentoxifylline, vasodilator agents, and volume expanders are used.[7-10]

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone secreted from the pineal gland and is particularly involved in the regulation of circadian rhythms. It is also a powerful antioxidant that stimulates antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase (GSH-Px), and glutathione reductase.[11-13] Melatonin neutralizes toxic reactants and inhibits the production of reactive oxygen species (ROS); thus, it can make cell membranes more resistant to an oxidative attack and protect DNA damage resulting from oxidizing agents.[14,15] Ischemia-reperfusion injury that develops after AT and free oxygen radicals (FORs) formed in response to this injury has an important place in cochlear sensorial epithelial damage, resulting in NIHL.[16-18] The use of melatonin, an important FOR scavenger, can also prevent and/or correct noise-related cochlear damage and related hearing loss.[8-19] Glucocorticoid has been reported to be another pharmacological agent with protective and therapeutic effects after noise exposure.[8-20] Platelet-rich plasma (PRP) is also used for the same purpose.[21] In this study, we aimed to investigate the potential therapeutic and/or protective effects of melatonin, PRP, and steroid treatment on NIHL in rats through histopathological and electrophysiological evaluations.

Material and Methods

This study was approved by the Animal Experiments Ethics Committee of Ankara Training and Research Hospital and conducted in accordance with the ethical regulations of the Declaration of Helsinki.

Animals

Forty-two healthy mature female Wistar albino rats weighing between 220 and 270 g were obtained from the Animal Experiments Laboratory of Ankara Training and Research Hospital Training and Research Hospital. Forty rats were housed in separate cages located in a temperature-controlled room (20-22 °C) under a 12-hour light/dark cycle. The ambient noise level was kept at <50 decibel (dB) at all times. All the rats were provided with free access to food and water. The remaining two rats were sacrificed and used for blood source material to prepare PRP.

Experimental protocol

An otoscopic examination was performed, and the ear wax in the external ear canal was removed under a surgical microscope (Carl Zeiss, Oberkochen, Germany). The ears with normal tympanic membranes were included in the evaluation. Hearing levels were assessed using auditory brainstem responses (ABRs) in both ears of all the animals on day 0 (baseline) under general anesthesia. The ears with a hearing loss of >20 dB and those with otitis media were excluded. The experimental design of the study is given in Table 1. Using random number tables, the 40 rats were randomized into the following five groups:

Group 1 (control; n = 8): no drug given.
Group 2 (steroid group; n = 8): Methylprednisolone was given via the intratympanic (ITM) route at 24 hours after AT.
Group 3 (PRP group; n = 8): PRP was given via the ITM route at 24 hours after AT.
Group 4 (melatonin group; n = 8): Intraperitoneal melatonin was given at 24 hours before AT.
Group 5 (melatonin group; n = 8): Intraperitoneal melatonin was given at 24 hours after AT. To induce AT, each group was exposed to noise at the 105 dB sound pressure level (SPL) for 12
To induce AT, each group was exposed to noise at the 105 dB sound pressure level (SPL) for 12 hours. At 24-four hours after AT, hearing levels were measured and compared to the baseline levels using ABRs in both ears of all the animals. On day 28, the ABR assessment was repeated for all the animals, and hearing results were recorded. Then, the animals were sacrificed under general anesthesia, and their cochlear tissues harvested for histopathological assessment.

Table 1. Experimental design of the study

Groups	Basal	Acoustic trauma	Day 1	Day 28	ITM	ITM PRP	M1	M2
Group 1	+	+	+	+				
Group 2	+	+	+	+	+			
Group 3	+	+	+	+		+		
Group 4	+	+	+	+			+	
Group 5	+	+	+	+				+

Abbreviation; ITM: Intratympanic; PRP: Platelet-rich Plasma; Group 1: Controls; Group 2: ITM Methylprednisolone; Group 3: ITM PRP; Group 4: Intraperitoneal Melatonin at 24 Hours Before Acoustic Trauma; Group 5: Intraperitoneal Melatonin at 24 Hours After Acoustic Trauma.

Two rats outside the study group were sacrificed and used for blood source material to prepare PRP. Blood was obtained by cardiac puncture (3 mL) and treated with 10% sodium citrate in a tube. Then, a two-step centrifugation process (1,000 rpm for 15 minutes and 3,000 rpm for 10 minutes) was followed. Finally, the PRP concentrate was dissolved in phosphate buffered saline (1:1). The PRP concentration was administered into both ears of the animals using a dental injector at 24 hours after AT. All the rats in PRP group received a single dose of PRP via the ITM route until the middle ear was filled (approximately 0.1 mL).
Methylprednisolone preparation and treatment

A 40 mg/mL concentration of methylprednisolone (250 mg/4mL, prednol-L, Mustafa Nevzat, Istanbul, Turkey) was administered into both ears of the animals using a dental injector at 24 hours after AT. All the rats in steroid group received a single dose of methylprednisolone via the ITM route until the middle ear was filled (approximately 0.1 mL).

Melatonin preparation and treatment

This treatment consisted of 5 mg/kg of melatonin [Melatonin Powder (CAS 73-31-4), Hangzhou Hyper Chemicals Limited, Hangzhou, China] and was intraperitoneally administered to Group 4 on the day before AT and Group 5 on the day after AT.

Auditory brainstem response measurement

The ABR test (AC 40 Interacoustics, Denmark) was performed under general anesthesia induced using 6 mg/kg intramuscular xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) and 60 mg/kg intramuscular ketamine hydrochloride (Ketalar, Pfizer Co., Istanbul, Turkey). Needle electrodes were used to collect ipsilateral auditory evoked potentials. The reference and ground electrodes were placed onto the vertex in the midline and the active electrodes on the mastoid bilaterally. The electrode-skin impedance was kept below 5 kV. Beginning at a SPL of 90 dB, the SPL of the stimuli was decreased in 10 dB increments until the ABR wave III response was no longer observable. A total of 500 clicks were applied at a rate of 11.4/s through insert earphones (ER-3A; Etymotic Research, Inc., IL, USA). Five waves were identified in waveforms.

Noise exposure

A total of 40 rats were exposed to white noise at 105 dB SPL for 15 hours. The white noise was generated using an IAC AC40 model audiometer and applied via two speakers located at a distance of 50 cm from the cage on both sides. All the rats were awake while being exposed to the noise. Sound intensity was monitored with a sound-level meter (Tromer, P.R.C.) positioned near the external auditory canal.

Histopathological assessment

After the rats were sacrificed under general anesthesia, the temporal bones were dissected macroscopically and preserved in formalin fixative. Then, the tissues were placed in 10% formic acid for decalcification at room temperature for 24 hours. The formalin-fixed paraffin-embedded blocks of cochlear samples were cut using a Leica RM2255 rotary microtome, and serial sections of 5 mm thickness were obtained. Finally, the sections were dewaxed, rehydrated, and stained with hematoxylin-eosin (H&E). For the qualitative and quantitative analysis of the outer hair cells (OHCs) and spiral ganglion cells (SGCs), images were taken using a light microscope under 40x magnification (Leica Aperio CS2, Leica Biosystems Ltd, UK). For each animal, three mid-modiolar sections were selected, including the basal, middle, and apical turns of the cochlea. The outline of Rosenthal's canal profile was then traced, and SGCs were counted from the base to the apex of the cochlea within each profile. The density of SGCs was expressed in an area of 10,000 μm2.Cell counting in SGCs was performed using the Virapath-3.0.14 cell nucleus artificial intelligence analysis program (Figure 1) (Virasoft, ARI 1 Teknokent, Istanbul, Türkiye).

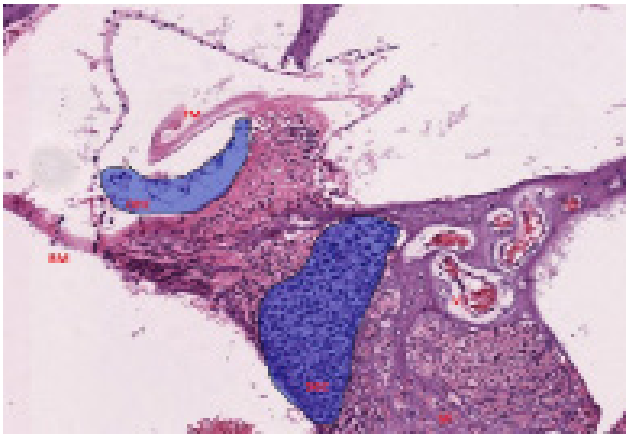

Fig. 1. Cell counting processes in spiral ganglion cells using the cell nucleus artificial intelligence analysis of Virapath software

Statistical analysis

SPSS statistical software program (SPSS, version 13.0 for windows; SPSS Inc, Chicago, Illinois, USA) was used to perform statistical calculations. Audiological results were compared with non-parametric two related (Wilcoxon) and two independent samples (Mann-Whitney U) tests. The density of SGCs (number of cell/10.000 μm2) calculated for each group was compared between the groups using one-way analysis of variance. Differences were accepted statistically significant at a p value of <0.05.

Results

Auditory brainstem responses

There was no significant difference between the groups in terms of the basal hearing thresholds before AT. Table 2 presents the me an ABR hearing thresholds for each group before AT and on days 1 and 28 after AT. No statistically significant difference was found in the three hearing threshold values between the right and left ears in each group. Therefore, the averages of the right and left ears were taken, and comparisons were made according to these values.

Statistically significant hearing loss was observed in all the groups based on the hearing thresholds obtained on day 1 after AT. According to the results of the hearing thresholds obtained on day 28 after trauma, there was a statistically significant improvement in the hearing thresholds of all the four treatment groups compared to the control group ($p < 0.05$).

The improvement in the melatonin groups (Groups 4 and 5) was statistically significantly better than in the steroid and PRP groups, while there was no significant difference between the two melatonin groups (Table 2). Although the hearing thresholds of the steroid group were better than those of the PRP group, the difference was not statistically significant ($p > 0.05$).

Histopathological findings at the end of week 4

As a result of the histopathological examination performed on day 28 day AT, the organ of Corti and spiral ganglion revealed a normal morphology in the steroid, PRP, and both melatonin groups. There was a significant decrease in the number of OHCs determined in the organ of Corti in the control group compared to the remaining groups (Figure 2, Figure 3).

The mean SGC density values determined separately for each group are given in Figure 4. Accordingly, the mean density of SGCs in all the four experimental groups was statistically significantly higher than in the control group ($p < 0.05$). There was no statistically significant difference between the remaining groups in terms of SGC density ($p > 0.05$).

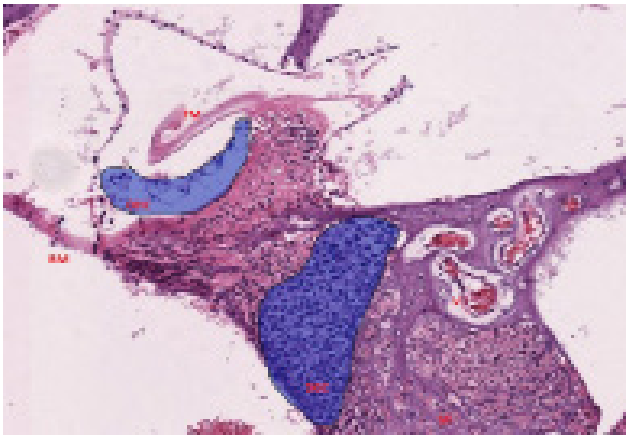

Fig. 2. Photomicrography of the organ of Corti and spiral ganglion in the control group. Note the decrease in the density of OHCs and SGCs. OHC: outer hair cell; SGC: spiral ganglion cell; BM: basilar membrane; TM: tectorial membrane

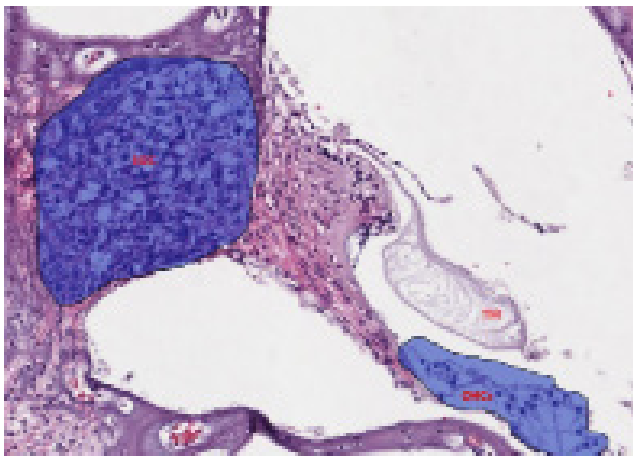

Fig. 3. Photomicrography of the organ of Corti and spiral ganglion in the group in which melatonin was administered before acoustic trauma. In this group, the organ of Corti and spiral ganglion have a normal morphology, and the density of OHCs and SGCs is higher compared to the controls. OHC: outer hair cell; SGC: spiral ganglion cell; BM: basilar membrane; TM: tectorial membrane; M: modiolus

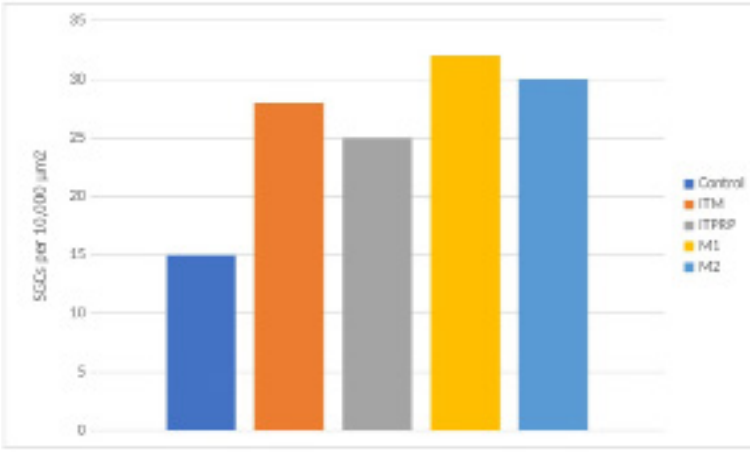

Fig. 4. Mean density of SGCs in each group

the remaining groups (Figure 2, Figure 3). The mean SGC density values determined separately for each group are given in Figure 4. Accordingly, the mean density of SGCs in all the four experimental groups was statistically significantly higher than in the control group ($p < 0.05$). There was no statistically significant difference between the remaining groups in terms of SGC density ($p > 0.05$).

Discussion

In this study, based on ABR testing and histopathological findings, we concluded that melatonin was effective in reducing NIHL and noise-induced cochlear damage in a rat model. Rats are often preferred in the investigation the effects of drugs administered for NIHL treatment.22 For the evaluation of hearing loss, both OAE and ABR methods are used.[7-9-20] In addition, hair cells are assessed under fluorescent or electron microscopy to determine which the effects of NIHL and drug treatment.[20] In the present study, we created an AT model in Wistar albino rats and used the ABR method to evaluate the effects of different drugs on hearing loss levels. We also performed histopathological assessment under light microscopy to evaluate the cellular effects on the cochlea. We used Virapath-3.0.14 cell nucleus artificial intelligence analysis program to determine the density of SGCs in the spiral ganglion in an attempt to reduce personal errors during cell counting.

NIHL can occur through two mechanisms including the direct mechanic trauma to the organ of Corti as a result of intense noise exposure and/

Table 2. ABR thresholds over time and between study groups

ABR threshold				p value - intragroup comparison (change over time)			
Groups	Basal (dB)	Day 1 (dB)	Day 28 (dB)	Total	Basal-Day 1	Basal-Day 28	Day1-Day-28
Group 1 (n=16)	6.5 ± 1.6	58.8 ± 8.3	30.0 ± 7.	<0.001	<0.001	<0.001	<0.001
Group 2 (n=16)	6.5 ± 1.3	57.5 ± 10.0	19.4 ± 6.8	<0.001	<0.001	0.003	<0.001
Group 3 (n=16)	6.9 ± 1.6	60.8 ± 10.3	21.8 ± 3.5	<0.001	<0.001	<0.001	<0.001
Group 4 (n=16)	6.6 ± 1.8	58.2 ± 7.9	11.7 ± 3.1	<0.001	<0.001	<0.001	<0.001
Group 5 (n=16)	6.5 ± 1.7	61.8 ± 11.3	11.3 ± 2.3	<0.001	<0.001	<0.001	<0.001
p value -intergroup comparison		NS	0.94	< 0.001 1 vs. 2 0.002 1 vs. 3 0.016 1 vs. 4 <0.001 1 vs. 5 <0.001 2 vs. 3 NS 2 vs. 4 0.046 2 vs. 5 0.031 3 vs. 4 0.006 3 vs. 5 0.004 4 vs. 5 NS			

Group 1: Controls; Group 2: Intratympanic Methylprednisolone; Group 3: Intratympanic Platelet-Rich Plasma; Group 4: Intraperitoneal Melatonin at 24 Hours Before Acoustic Trauma; Group 5: Intraperitoneal Melatonin at 24 Hours After Acoustic Trauma; NS: Not Significant

or metabolic stress due to the increase in oxidative metabolism in the inner ear.[6] Noise exposure reduces cochlear blood flow, causes hypoxia, ischemia, and inflammation, and promotes free radical production. Oxidative DNA then triggers apoptosis, resulting in the deterioration of stereocilia activity in the damaged cochlea, loss of hair cells, and SGHs, which leads to hearing loss.[11-18] Selective OHC loss most commonly occurs within 24 hours and continues for two weeks. Therefore, pharmacological agents used in the treatment of NIHL particularly aim at stopping and/or reversing the aforementioned pathogenetic process.5,8-10 In the current study, we investigated the efficacy of melatonin, PRP, and steroids with previously proven antioxidant and anti-inflammatory effects in preventing and reversing hearing loss in randomized controlled rat groups.

Steroids such as dexamethasone and methylprednisolone are the most popular agents used in the treatment of NIHL. For this purpose, not only intravenous but also ITM steroid administration is preferred.[5-8-10-20] Corticosteroids applied in the treatment of hearing loss due to AT act by binding to glucocorticoid receptors in the stria vascularis, organ of Corti, spiral ligament, and SGCs.[23] Corticosteroids increase cochlear blood flow and reduce hypoxia-ischemia damage by decreasing basal metabolic energy requirement and increasing anti-oxidant enzyme activity.[24] In a study in which dexamethasone was administered via mini-osmotic pumps connected to a cannula into the scala tympani in 26 male albino Hartley guinea pigs, Takemura et al. showed that dexamethasone provided significant hearing protection, and OHC loss was histologically less in the dexamethasone group relative to the control group.[25] In a study by Caliskan et al., the ITM and

systemic steroid administration was compared, and a statistically significant improvement was found in the group administered ITM dexamethasone at 5,000-6,000 Hz and in the group administered systemic methylprednisolone at 6,000-8,000 Hz compared to the control group.[26] In the present study, we applied a single dose of ITM steroid therapy. We achieved a statistically significant improvement in hearing thresholds determined by ABR following a single dose of intratympanic steroid administration compared to the control group. In addition, a single dose of ITM methylprednisolone injection administered following AT appears to reduce SGH loss. However, further studies are necessary to determine the most optimal dose, duration, and administration routes of steroid therapy. PRP has a positive effect on healing due to its high growth factor concentration.[27] In addition, previous animal studies have shown that these positive effects of PRP application also increase nerve damage regeneration.[27] In a study by Yurtsever et al., ITM PRP application was reported to be effective in cisplatin-induced ototoxicity and to reduce hearing loss and cochlear cell damage.[21] Similarly, in the present study, we found that a single dose of ITM PRP application after AT reduced the severity of hearing loss and cochlear cell damage. However, these positive effects of PRP were limited compared to melatonin and steroid treatment. Inner ear damage due to noise, drugs, radiation, and age-related hearing loss (ARHL) are all closely associated to the excessive production of free radicals that damage cochlear hair cells. Since the cochlea has a high aerobic metabolic rate, it is very sensitive to oxidative damage by ROS. The basal layer of the cochlea is the most affected part. Therefore, the main goals of treatment are to eliminate free oxygen radicals, which have an important place in the pathophysiology of hearing loss, and prevent oxidative damage. At this point, melatonin emerges as a very promising molecule. It has also been determined that melatonin has the same effect in the inner ear. Moreover, melatonin metabolites also function as ROS kidnappers so that more ROS can be eliminated, while lipid peroxidation is inhibited by melatonin.[11-14] A number of studies have suggested that melatonin can protect the inner ear from various types of damage. Animal studies on the efficacy of melatonin use in delaying ARHL and preventing cisplatin and gentamicin ototoxicity, radiotherapy-related hearing loss, and high-dose nicotine-induced cochlear hair cell damage, revealed its efficacy in hearing protection.[28-32] In a study by Serra et al., use of 10 mg/kg/day oral melatonin for a 12-month period in the murines was reported to be associated with significant increase in the viable cell density in the spiral ganglion and spiral ligament as well as in the distortion product otoacoustic emissions amplitude values. This suggests that less cell degeneration occurs in the cochlea of animals given oral melatonin, and ARHL development is delayed.[28] This is achieved by melatonin-mediated neutralization of oxidative stress during normal cell aging and the delay in apoptotic process. The glutathione enzyme system constitutes a protective mechanism against the development of NIHL. As the glutathione level increases in cochlear tissues after AT, oxidative stress and sensory epithelial damage decrease in the organ of Corti.33 Karlidag et al. revealed that melatonin applied during noise exposure minimized the decrease in the GSH-Px level in erythrocytes, reduced the production of malondialdehyde, a lipid peroxidation product, and contributed to the protection of hearing thresholds. In light of this information, the authors concluded that melatonin could play an effective role in protection from noise-related cochlear damage.[19] In another study, Bas et al. found that melatonin was more effective than dexamethasone or tacrolimus in reducing noise-related inner ear damage.8 Melatonin can be administered through the oral, IT, intramuscular, or intraperitoneal routes. Demir et al. determined that intratympanic melatonin administration of 0.1 mg/mL once a day for five days was effective against cisplatin-induced ototoxicity, and that transtympanic melatonin administration was effective in ototoxicity and in the treatment of sudden hearing loss.[34] This study has certain limitations. First, we determined hearing thresholds using ABR but did not evaluate frequency-specific hearing thresholds. The determination of frequency-specific hearing thresholds can be more beneficial in terms of revealing the efficacy of treatment. Second, we were not able to examine stereocilia because we performed the histological examination under light microscopy. Further histological studies using scanning

or transmission electron microscopy techniques are needed to provide a better understanding of the cell morphology. There is also a need to determine the optimum time, dose, and frequency of melatonin administration considering that results may differ when the dose and administration method is changed. Human trials to investigate the effect of melatonin against NIHL are also warranted.

Conclusion

We investigated the protective effects of melatonin in the treatment of NIHL by applying intraperitoneal melatonin before and after AT. Similar to previous studies, we found that this application was beneficial both electrophysiologically and histopathologically in the treatment of NIHL. Compared to both methylprednisolone and PRP treatments, the melatonin group had more positive results. However, we did not find a statistically significant difference between the administration of melatonin one day before and one day after AT. To our knowledge, current therapy for AT or noise-induced hearing loss is corticosteroid treatment. Accordingly, to be justified by human studies, our findings seem to indicate that melatonin may be considered an alternative to corticosteroid treatment against NIHL and AT.

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