ORIGINAL ARTICLE

Melatonin Prevents Noise Induced Hearing Threshold Shift and Hair Cell Loss in Rat

Sungdo Jung, Chan Woong Bahk, Myung-Whan Suh, Jae-Yun Jung

Department of ORL-HNS, College of Medicine, Dankook University College of Medicine, Cheonan, Korea (SJ, MWS, JYJ)
Medical Laser Research Center, Dankook University College of Medicine, Cheonan, Korea (CWB)
University of Illinois Urbana-Champaign, Illinois, USA (CWB)

Objective: Noise causes damage of the auditory system leading to hearing loss. The aim of this study was to investigate preventive role in noise induced hearing loss (permanent threshold shift model) and anti-apoptotic effects of two different dose of melatonin.

Materials and Methods: Forty-four adult Sprague-Dawley rats were randomly divided into six groups. Narrow band noise for 6 hours was used to induce noise induced permanent threshold shift model. We used two different doses (0.4mg/kg, 4mg/kg) of melatonin, which were all injected intraperitonially (IP) four times. IP injection was given one day before, immediately before first and second noise exposure and one time between first and second noise exposure (total four times). Group NO was exposed to noise and treated with vehicle. Group NL and NH was exposed to noise and treated with either low dose (NL) or high dose (NH) melatonin. The hearing thresholds change and orphological assessment for hair cell damage were performed. Apoptosis activity was also compared using Western blotting of caspase 3 in each group.

Results: We observed consistent threshold increase up to 90 dB SPL until 3 weeks with current noise exposure method. Rats treated with melatonin showed less hearing loss after noise exposure than untreated rats. Outer Hair cell loss was also attenuated in melatonin treated rats under SEM observation. Melatonin reduced caspase 3 activity after noise exposure. However, there was no difference between two different doses (NL, NH).

Conclusion: Preventive treatment with melatonin attenuates hearing threshold shift by reducing outer hair cell damage. This protective effect was shown to with reduced proapoptotic caspase 3 protein. We could not find additional protective effects with higher dose compared to lower dose in this study.

Submitted : 16 April 2012 *Accepted : 7 May 2012*

Introduction

Apoptosis is known as the predominant mode of noise induced hair cell loss, and reactive oxygen species (ROS) play an important role as a initiating promoter during apoptosis.[1] The emergence of ROS and byproducts in the cochlear tissue after noise has been observed in various animal species.[2-4]

Melatonin, which is a secretary product of the pineal gland, was recently found to be an powerful antioxidant and a free radical scavenger.[5] Low-dose melatonin was shown to protect the inner ear from ototoxicity, but, high dose melatonin showed facilitation of amikacin-induced ototoxicity, possibly via the vasodilatory effect, leading to an increased accumulation of amikacin in the inner ear.[6] On the other hand, ischemic damage is one of possible causative mechanism in noise damaged ear.[7] We can expect that possible vasodilatory effect with high dose may bring additional hearing protective effect in noise

Corresponding address:
Jae Yun Jung, M.D. PhD.
Associate Professor of ORL-HNS,
Coll. of Medicine, Dankook University
16-5 Anseo-Dong, Cheonan, Chungnam 330-715, Korea
Phone: 82-41-550-3973, Fax: 82-41-556-1090
E-mail: jjkingy2k@gmail.com

induced hearing loss (unlike ototoxic drug induced hearing loss). We tried low and high dose melatonin for prevention to see if we could achieve additional advantage with higher dose through relieving vasoconstriction which is known as one of noise damage mechanism.

Materials and Methods

Animals

Forty-four adult Sprague-Dawley (SD) rats weighing approximately 200 gm (Narabio, Korea) with normal Preyer reflexes were included in the study. All animal experiments were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes approved by the Animal Ethics

Committee, Dankook University, College of Medicine. Adult male SD rats were allowed some adaptation period with normal food and water. The rats were fed a standard diet and maintained on a 12 hours light-dark cycle. The ambient noise level was 35-45 dB. Forty-four rats were divided into six groups. The distribution of animals and administered substances according to groups is shown in Table. 1.

Noise Exposure

An acryl noise box was designed with a Beyma Loudspeaker CP800Ti (Beyma, Spain) attached on top. The rats were placed in small, separate wire cages fit for rat, to prevent defensive behaviors such as blockage of the ears and were set inside the noise box. The animals were given a two-time exposure to a narrow band noise of 120 dB SPL centered at the frequency of 16 kHz for 6 hours with one day interval. No anesthesia was used during noise exposure and individual cages was placed 15 inches from the speaker.

Chemicals

Melatonin (M5250) was purchased from Sigma Chemical Co. St. Louis Missouri (USA). Melatonin was dissolved in ethanol and then further diluted in saline to make a final concentration of 5% ethanol. Melatonin of 0.4mg/kg and 4mg/kg concentration were used for low dose group (LM, NL) and high dose group (HM, NH) respectively. Drug of either concentration was all injected intraperitonially (IP) four times (one day before, immediately before first and second noise exposure and one time during the interval).

Auditory Brainstem Response

During auditory brainstem response (ABR), rats were anesthetized with Zoletil (Virbac Laboratories, France) and Rumpun (Bayer, Korea). Otoscopic evaluations were made to exclude any outer and middle ear problem before ABR analysis. Testing for ABR was performed using a Smart EP high-frequency software/hardware package (System III, Tucker Davis Technologies) with system-calibrated high frequency transducers. Insert ear tips (3.5 mm, Nicolet Biomedical, Inc.) were lightly coated with ophthalmic ointment and placed in the ear canal. Subdermal platinum needle electrodes (F-E2, 48 inches, Grass Telefactor, Inc.) were placed with the active electrode at the vertex, the reference electrode ventrolateral to the test ear, and the ground electrode contralateral to the test ear in the ventrolateral position.

Table 1. Details of experimental groups. Forty-four rats were divided into six groups. Four rats were sacrificed for western blot assay for each group. ABR: Auditory Brainstem Response

	Animals (n)	Noise	Melatonin
Control group (C)	6	No	No
High dose melatonin group (HM)	6	No	4.0 mg/kg/day
Low dose melatonin group (LM)	6	No	0.4 mg/kg/day
Noise only group (NO)	6	Yes	No
Noise + high dose melatonin (NH)	10	Yes	4.0 mg/kg/day
Noise + low dose melatonin (NL)	10	Yes	0.4 mg/kg/day

Auditory stimulus was either click or pure-tone with continuous presentation and a rectangular envelope. Stimulus duration was 100 msec for click and 5000 msec for pure-tone stimuli, at a rate of 19.3/sec. Eight hundred sweeps were recorded and averaged per stimulus. Tonal data were collected at 4, 8, 12, 16 and 32 kHz. Threshold was defined in 5-dB steps as determined by the presence of wave forms I, III, and V. It was not unusual to lose the detection of peaks I or V approaching threshold levels. Rats with normal ABR values (mean value ± 5 dB at each acoustic stimulus) were entered into noise protection studies. All audiometric data were collected and analyzed by a clinically certified audiologist blinded to the treatment groups.

Western blot analysis

Animals for Western blot analysis were sacrificed under general anesthesia 4 hours after second noise exposure. The cochlea lysates of 4 rats from each group were used for assay. The cochleae were washed with PBS and then homogenized in 1 mL Radio Immuno Precipitation (RIPA) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 1 mM EGTA, 0.5% Deoxycholate) with 100 μ g/mL phenylmethylsulfonyl fluoride and 1 μ g/mL leupeptin to estimate the caspase-3 activation and the cell suspensions, cultures were clarified by centrifugation at 15,000 rpm for 30 min. The pellets were re-extracted and the supernatants were pooled. The amount of protein was estimated by a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Tissues lysates that contained 20 μg of total protein were load onto 12% sodium dodesyl sulfate (SDS)-polyacrylamide gel and then the proteins were transferred to polyvinylidine difluoride (PVDF) membranes (Amersham, Arlington Heights, IL, USA). Each membrane was blocked with blocking buffer (5% skim milk in TTBS: 137 mM NaCl, 0.1% Tween 20 and 20 mM Tris-HCl, pH 7.6) for 1 hr at room temperature and the membranes were incubated with the primary antibody (anti-caspase-3 1:1,000, anti-GAPDH 1:1,000, Santacruz, CA, USA), and then the membranes were incubated at room temperature for 1 hr with a secondary antibody conjugated with HRP (1:10,000, diluted in 5% skim milk). The signal was detected using an ECL Western blotting detection system (GE Healthcare, Amersham, UK).

Scanning electron microscopy and outer hair cell counting

Animals for SEM were sacrificed under general anesthesia at 21th day after second noise exposure. Intracardiac perfusions were performed with 0.1 M phosphate-buffered saline (PBS) for 20 minutes and then 4% paraformaldehyde (PFA) for another 20 minutes. The animals were then decapitated and the cochleae were removed. Microperfusions were performed once more on the harvested cochleae and afterwards, a diamond burr dissecting drill (Saeshin Precision Co., LTD., Daegu, Korea) was used to remove the bone and the lateral wall (spiral ligament and stria vascularis) under a dissection microscope (Olympus Corporation, Tokyo, Japan). The separated cochleae were fixed in 2% glutaraldehyde overnight and then were rinsed with 0.1 M PBS. The samples were then postfixed with a 1% osmium tetroxide for 3 to 5 min and were gently rinsed again with 0.1 M PBS. After being dehydrated in graded series of ethanol, the critical-point dryer (Hitachi, Tokyo, Japan) was used to fully dehydrate the specimens. The prepared cochlea samples were then attached to aluminum stubs and were sputter-coated with platinum-palladium using E-1030 PT-PD target assembly (Hitachi, Tokyo, Japan). The surfaces of the basilar membrane with hair cells were examined using an S-4300 scanning electron microscopy (Hitachi, Tokyo, Japan).

Besides morphological observations, the cochleae were also quantitatively analyzed by recording the number of remaining hair cells. The cochleae were divided into apical, middle and basal turns at approximately 0°~270°, 270°~ 540° and 540° ~ 810°, respectively, and the hair cells were counted at three different sites of each turns at x500 magnification. A hair cell was considered as absent if the bundle of stereocilia was missing.

Statistical Analysis

All data were analyzed statistically by repeated measures analysis of variance (RM-ANOVA) &

Mann-Whitney test using the Statistical Package for the Social Sciences (SPSS, Chicago, USA) software. Data were expressed as mean±SD and differences were considered statistically significant when $p<0.05$.

Results

Auditory Brainstem Response Threshold Shifts

The ABR threshold levels of each group before the noise stimulus were not significant different between the groups (Fig. 1). ABR test of NO group measured 1 day after noise exposure showed threshold shift with 12 kHz most affected. The rats which were treated with melatonin had less threshold increase than NO group when measured 1 day after noise. This difference was most noticeable at 4 kHz hearing threshold which is relatively lower frequency in rat. The control group and drug administered group without noise (HM, LM: data not shown here) showed normal hearing.

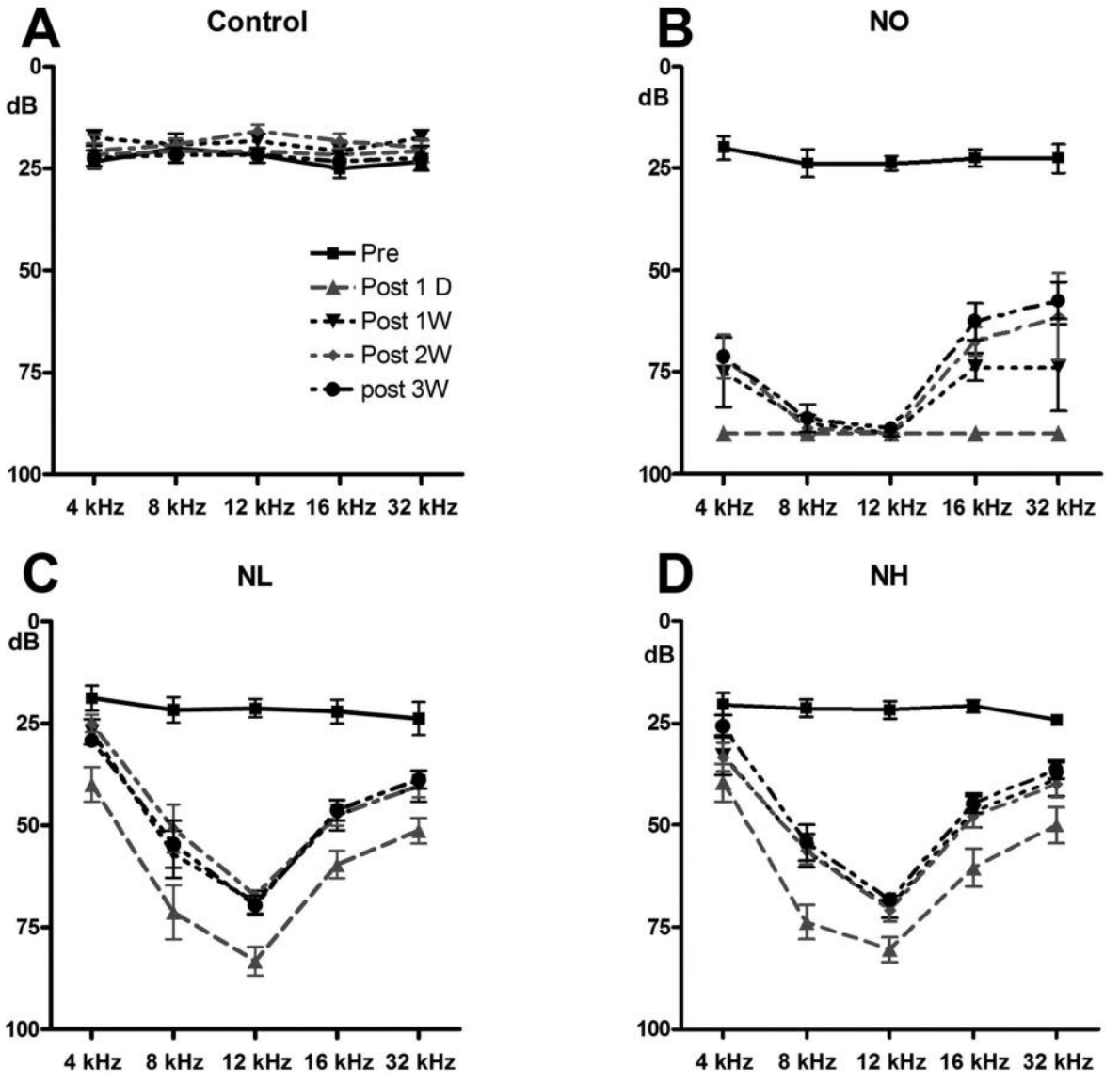

Figure 1. Result of auditory brainstem response threshold. Hearings of all groups prior to noise exposure were normal. Control group showed no change in hearing after 3 weeks **(A)**. On the other hand, a day after noise exposure, the NO group showed signs of scale out over all frequencies **(B)**, and while the melatonin administered group also experienced hearing loss, it was significantly($p<0.01$) less than that of the NO group. After 3 weeks, measurements after hearing has stabilized showed that it recovered to post 1 day levels. Nevertheless, hearing loss still remained in the exposure groups **(B, C, D)**. The NO group, compared to the melatonin administered groups **(C, D)** suffered significantly ($p<0.01$) more severe hearing loss.

ABR threshold in NO group showed slow threshold decrease with 3 weeks threshold 62.5dB at 16 kHz and 57.5dB at 32 kHz, but no change at 12 kHz.

The rats treated with melatonin (NH, NL group) showed threshold decrease at all frequency when tested one week, 2 weeks and 3 weeks after noise exposure.

Biggest threshold change was observed at first one week interval. However, there was no difference between the two groups.

Outer hair cell number counting

The cochlear structures of all groups not exposed to noise appeared normal and well defined under SEM observation. When we observed noise exposed group at 21 days after noise exposure, various degrees of hair cell damage including missing stereocilia were detected throughout all three rows (Fig. 2).

SEM observations revealed no significant differences in the outer hair cell number among the groups without noise exposure (control, LM and HM groups). Number of outer hair cells measured after noise exposure showed remarkable decrease compared to the groups with no exposure ($p<0.01$). Melatonin treated rat (NL, NH group) showed more outer hair cell than untreated rat (NO group) after noise exposure, which was statistically significant. Outer hair cell counts difference between these two treated groups was not statistically significant (Fig. 3).

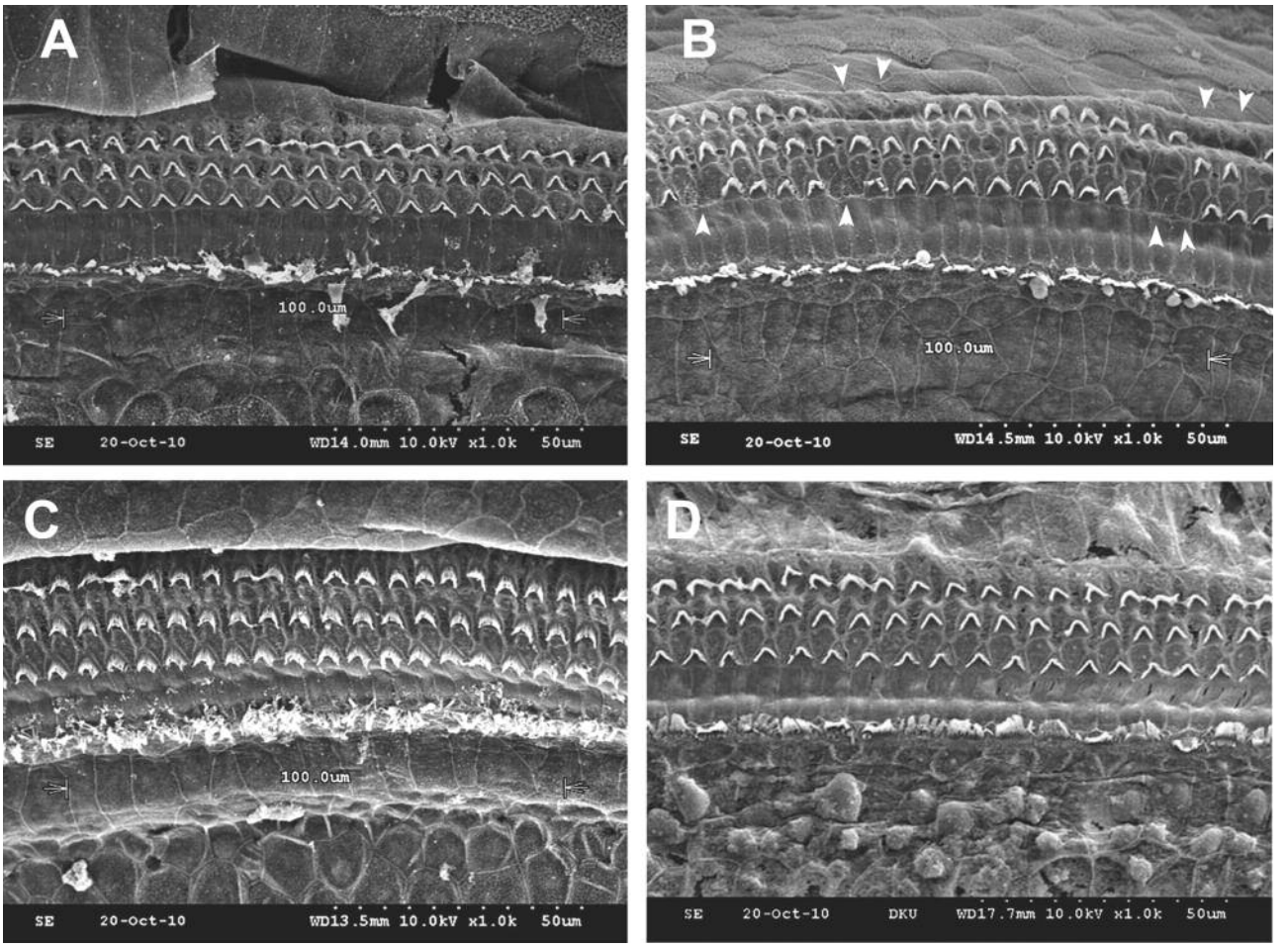

Figure 2. Scanning electron microscopy finding. **A:** Representative SEM findings from Group C establish normal outer hair cell contours. **B:** SEM findings from Group NO shows scattered outer hair cell stereocilia damages (arrowheads). **C, D:** SEM findings from Group NH and NL show less outer hair cell damage.

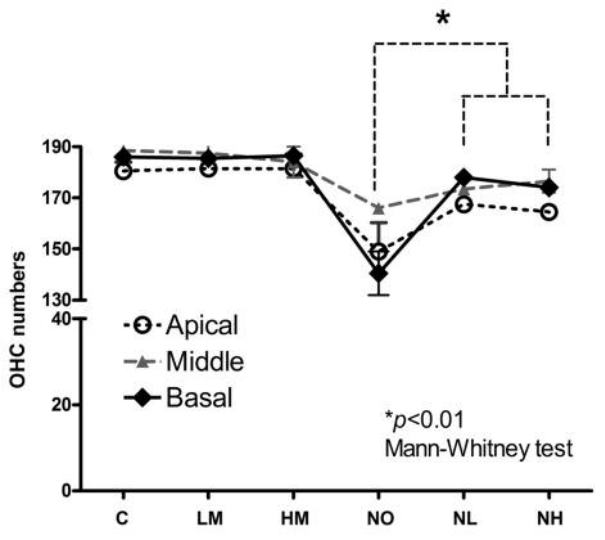

Figure 3. Average outer hair cell (OHC) number in each group. The cochleae were divided into apical, middle and basal turns at approximately 0°~270°, 270°~ 540° and 540° ~ 810°, respectively, and the hair cells were counted at three different sites of each turns at x500 magnification. SEM observations revealed no significant differences in the number of outer hair cells between the control, LM and HM groups. Animals with noise exposure (NO) showed significant OHC loss. But the group (NL, NH) which had preventive melatonin medication showed significant lower OHC damage compared to NO group regardless of melatonin dosage. OHC numbers of NH and NL groups showed no statistically significant difference.

Western blotting

Western blot analysis following RIPA extraction demonstrated the presence of caspase 3 as 32 kDa bands in the groups. Cleaved caspase 3 protein was most abundant in noise exposed rat with no melatonin (group NO). Cochlea from the rat from group NL and NH (melatonin treated rats) showed very weak signal of cleaved Caspase 3, which was comparable with signals of groups HM, LM, control (Fig. 4).

Discussion

Recently reviewed evidence about noise induced hearing loss (NIHL) made clear that noise exposure can cause mechanical or metabolic as well as neuronal damages, driving mitochondrial activity and free radical productions, reducing cochlear blood flow, causing excitotoxic neural swelling, and inducing both necrotic and apoptotic cell deaths in the organ of Corti.[8, 9] This complex process of hair cell death primarily involves two different pathways: cell death through either TNF receptors or the generation of oxidative stress and the mitochondrial activation pathway. The two pathways are related, converging at the caspase activation phase, especially that of caspase 3.[10]

Melatonin is a natural occurring compound with well-known antioxidant properties. In addition, the effects of melatonin on mitochondrial homeostasis have been discovered in the last decade. Melatonin has been used to protect cells from the cytotoxic activities of free radicals in vitro and in vivo studies.[11, 12] Regarding dose dependent manner of protection from apoptotic activity, Sahna et al. used low (0.4mg/kg per day) and high (4mg/kg per day) doses of melatonin on ischemia-reperfusion arrhythmias in rats concerning which mechanisms were based on free radical formation.[13] In this study, no differences were detected between the low and high doses of melatonin, and both doses were effective in protecting from these conditions, while higher concentrations did not

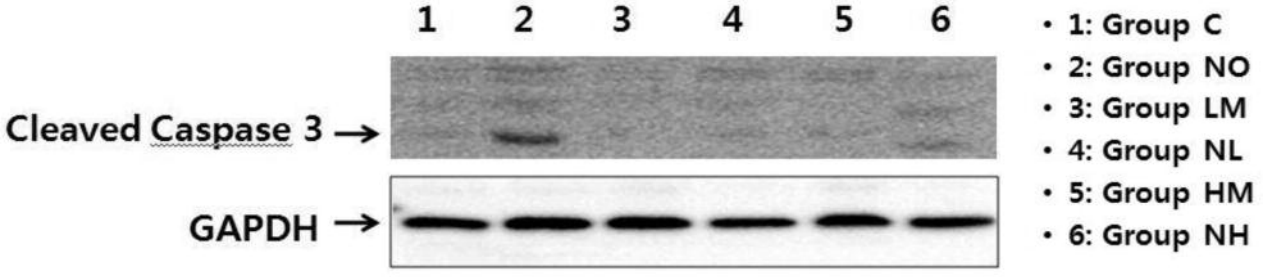

Figure 4. The protein of caspase 3 on western blotting with rat cochleae lysate. Noise exposure increased caspase 3 in group NO. Treatment with melatonin (both low dose and high dose) showed remarkable decrease of caspase 3 activity after noise exposure (group NL, NH).

increase its beneficial effect. In other words, the antioxidant effects of these doses were similar. But if high dose melatonin has another arm of protection from noise damage, we can expect different story. Another study using two different dose of melatonin in ototoxic damage rat model showed accelerated amikacin induced ototoxicity. The authors speculated higher dose of melatonin enhanced ototoxic drug accumulation through vasodilatory effect. If this is the case, vasodilatory effect of high dose melatonin in NIHL is expected to handle ischemic mechanism after noise exposure along with its original antiapoptotic effect.

We investigated the effect of melatonin by analyzing cell survival and also the levels of the caspase 3 expressions in noise-induced hair cell death. For the results, the number of outer hair cells decreased in the noise exposed groups and the survival of hair cells was enhanced when melatonin was added. Caspase 3 is an important protein for the apoptosis of outer hair cells because it processes caspases further downstream in the pathway. It has been identified as a key mediator of apoptosis in mammalian cells. It cleaves and activates caspases 6, 7, and 9, and the protein itself is processed by caspase 8, 9, and 10.[14, 15] Activation of the upstream initiator caspase is related to caspase 8 and 9, and this activates downstream effector caspase such as caspase-3. The sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.[16, 17] This study demonstrated that melatonin could suppress the apoptosis induced by noise in the outer hair cells. We also demonstrated that one of the protective effects of melatonin was the attenuation of the caspase 3 expression.

Conclusion

Melatonin prevented hearing threshold shift and outer hair cell regardless of two different drug concentration. This protective effect is presumably through reduced caspase dependent apoptosis process, considering western blot data. Although no additional effect with higher dose in this study, further study is required to investigate vasodilatation effect and possible role in noise damage with higher dose melatonin.

Acknowledgments

The present research was conducted by the research fund of Dankook University in 2010.

References

1. Forge A, Li L. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. Hear Res. 2000; 139:97-115.

2. Ohinata Y, Miller JM, Altschuler RA, Schacht J. Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea. Brain Res. 2000; 878:163-73.

3. Ohlemiller KK, Dugan LL. Elevation of reactive oxygen species following ischemia-reperfusion in mouse cochlea observed in vivo. Audiol Neurootol. 1999; 4:219-28.

4. Yamane H, Nakai Y, Takayama M, Iguchi H, Nakagawa T, Kojima A. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur Arch Otorhinolaryngol. 1995; 252:504-8.

5. Hardeland R. Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. Endocrine. 2005; 27:119-30.

6. Erdem T, Ozturan O, Iraz M, Miman MC, Olmez E. Dose-dependent dual effect of melatonin on ototoxicity induced by amikacin in adult rats. Eur Arch Otorhinolaryngol. 2005; 262(4):314-21.

7. Shaddock LC, Hamernik RP, Axelsson A. Effect of high intensity impulse noise on the vascular system of the chinchilla cochlea. Ann Otol Rhinol Laryngol. 1985; 94:87-92.

8. Kujawa SG, Liberman MC. Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. J Neurosci. 2006; 26:2115-23.

9. Hamernik RP, Turrentine G, Roberto M, Salvi R, Henderson D. Anatomical correlates of impulse noise-induced mechanical damage in the cochlea. Hear Res. 1984; 13:229-47.

10. Coleman JK, Littlesunday C, Jackson R, Meyer T. AM-111 protects against permanent hearing loss from impulse noise trauma. Hear Res. 2007; 226:70-8.

11. Leon J, Acuna-Castroviejo D, Sainz RM, Mayo JC, Tan DX, Reiter RJ. Melatonin and mitochondrial function. Life Sci. 2004; 75:765-90.

12. Hardeland R, Reiter RJ, Poeggeler B, Tan DX. The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. Neurosci Biobehav Rev. 1993; 17:347-57.

13. Sahna E, Olmez E, Acet A. Effects of physiological and pharmacological concentrations of melatonin on ischemia-reperfusion arrhythmias in rats: can the incidence of sudden cardiac death be reduced? J Pineal Res. 2002; 32:194-8.

14. Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, et al. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. 1997; 278:294-8.

15. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997; 326:1-16.

16. Kirsch DG, Doseff A, Chau BN, Lim DS, de Souza-Pinto NC, Hansford R, et al. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem. 1999; 274:21155-61.

17. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004; 305: 626-9.