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Noise-induced hearing loss is a common cause of hearing loss. The effects of low-level laser therapy (LLLT) have been investigated from various perspectives, including in wound healing, inflammation reduction, and n erve regeneration, as well as in hearing research. A promising feature of the laser is its capability to penetrate soft tissue; depending on the wavelength, laser energy can penetrate into the deepest part of the body without damaging non-target soft tissues. Based on this idea, we developed bilateral transtympanic LLLT, which uses simultaneous laser irradiation in both ears, and evaluated the effects of bilateral LLLT on cochlear damage caused by noise overexposure. Thus, the purpose of this research was to assess the benefits of simultaneous bilateral LLLT compared with unilateral LLLT and a control. Eighteen Sprague-Dawley rats were exposed to narrow-band noise at 115dB SPL for 6 h. Multiple auditory brainstem responses were measured after each low-level laser irradiation, and cochlear hair cells were counted after the 15th such irradiation. The penetration depth of the 808 laser was also measured after sacrifice. Approximately 5% of the laser energy reached the contralateral cochlea. Both bilateral and unilateral LLLT decreased the hearing threshold after noise overstimulation in the rat model. The bilateral LLLT group showed faster functional recovery at all tested frequencies compared with the unilateral LLLT group. However, there was no difference in the endpoint ABR results or final hair cell survival, which was analyzed histologically.

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Conflict of Interest: None

Running title: Bilateral Low-Level Laser Therapy on Cochlear

Abstract

Noise-induced hearing loss is a common cause of hearing loss. The effects of low-level laser therapy (LLLT) have been investigated from various perspectives, including in wound healing, inflammation reduction, and nerve regeneration, as well as in hearing research. A promising feature of the laser is its capability to penetrate soft tissue; depending on the wavelength, laser energy can penetrate into the deepest part of the body without damaging non-target soft tissues. Based on this idea, we developed bilateral transtympanic LLLT, which uses simultaneous laser irradiation in both ears, and evaluated the effects of bilateral LLLT on cochlear damage caused by noise overexposure. Thus, the purpose of this research was to assess the benefits of simultaneous bilateral LLLT compared with unilateral LLLT and a control. Eighteen Sprague-Dawley rats were exposed to narrow-band noise at 115 dB SPL for 6 h. Multiple auditory brainstem responses were measured after each low-level laser irradiation, and cochlear hair cells were counted after the 15th such irradiation. The penetration depth of the 808 laser was also measured after sacrifice. Approximately 5% of the laser energy reached the contralateral cochlea. Both bilateral and unilateral LLLT decreased the hearing threshold after noise overstimulation in the rat model. The bilateral LLLT group showed faster functional recovery at all tested frequencies compared with the unilateral LLLT group. However, there was no difference in the endpoint ABR results or final hair cell survival, which was analyzed histologically.

Keywords

: Bilateral LLLT; Noise induced hearing loss; ABR; hair cell survival

Introduction

Hearing loss, caused by diverse factors, is an important public health issue. In particular, noise overexposure is considered harmful to hearing function. Intense noise can cause damage to the hair cells by increasing oxidative stress, which produces various reactive oxygen species (ROS), such as the superoxide anion (O_2^-) (Yamane et al. 1995) and hydrogen peroxide (H_2O_2) (Ohinata et al. 2000).

Noise exposure can cause a temporary threshold shift (TTS) or a permanent threshold shift (PTS) that will not recover. The type of threshold shift is determined by the intensity and duration of exposure. Several studies with similar levels of noise and exposure times (>100 dB, >6 h) have reported that a PTS occurred after few minutes or hours of such noise exposure (Buck 1981; Hu et al. 2000; Hu et al. 2006). Both TTS and PTS can occur simultaneously at different frequencies in one cochlea. According to recent research, damage to the auditory neurons, such as at the ribbon synapse and postsynaptic receptors, was found following noise exposure, even after recovery of the hearing threshold (Kujawa & Liberman 2009).

Low-level laser therapy (LLLT) has been used as a treatment for various symptoms, and its use has been increasing because of its non-invasive nature. After it was approved by the United States Food and Drug Administration, applications of LLLT have widened in the research area, including in studies of wound healing (Anneroth et al. 1988; Grossman et al. 1998; Kana & Hutschenreiter 1981), inflammation reduction (Boschi et al. 2008; Ferreira et al. 2005), and nerve regeneration (Miloro et al. 2002; Mohammed & Kaka 2007). Effects of LLLT have also been reported in the area of hearing research. Some studies have demonstrated significant effects in reducing tinnitus and increasing auditory neuron activation (Littlefield et al. 2010; Medalha et al. 2012; Park et al. 2013). Recently, our group reported a promising recovery effect of LLLT on cochlear hair cells in an animal study (Rhee et al. 2012b). Tamura et al. (2015) also reported a cytoprotective effect of LLLT in cochlear hair cells against noise overstimulation (Tamura et al. 2015).

One useful feature of the laser is its penetrating capability in soft tissue; depending on the wavelength, laser energy can penetrate into deep parts of the body without damaging non-target soft tissues. This enables the delivery of laser energy from multiple points, which may lead to faster or increased effects of the laser in the target area. In our previous animal experiments, we found improvements in the hearing threshold not only in the laser-irradiated but also in the contralateral ear (Rhee et al. 2012b). This suggests that unilateral LLLT may affect the contralateral auditory organs. Thus, here we measured the degree of laser penetration in the contralateral ear of SD rats and assessed the benefit of simultaneous bilateral LLLT compared with unilateral LLLT and a control.

Materials and methods

Animal

Male Sprague Dawley (SD) rats (180 - 200 g) were used in this study. Eighteen rats were randomly divided into three different groups (noise only [n=6], unilateral laser [n=6], and bilateral laser [n=6]). All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals (7th edition, 1996), as formulated by the Institute of Laboratory Animal Resources of the Commission on Life Sciences. All procedures were approved by the Institutional Animal Care and Use Committee for the Dankook University (DKU-15-048).

Acute acoustic trauma

The acoustic stimulus was a narrow band of noise which has frequency information centered at 16 kHz with 1 kHz of bandwidth (116 dB SPL). Rats were placed on individual cages to prevent defensive behaviors and these cages were placed in acryl reverberant chamber with a speaker CP800Ti (Beyma, Balencia, Spain) attached on top. The traumatic stimulus was generated with a type 1027 sine random generator (Bruel and Kjaer, Denmark) and amplified with a R300 plus amplifier (inter-Mcorp, Seoul, Korea) during 6 hours. For real time monitoring, frequency-specific sound level meter (Sound Level Meter – Type 2250, Bruel and Kjaer, Denmark) was used to monitor noise level in the chamber (placed on the floor) every hour so that consistent intensity (116 dB SPL) was maintained during noise exposure.

Auditory Brainstem Response Measurement

Auditory brainstem responses were measured to identify degree of hearing loss and recovery. The evoked response signal-processing system (System III, Tucker Davis Technologies, Alachua, Florida) was used for ABR measurement. Animals were anesthetized with Zolazepam (Zoletil, Virbac, Carros Cedex, France) and Xylazine (Rompun, Bayer, Leverkusen, Germany) and placed in sound proof chamber. Three of needle electrodes were inserted at vertex (active) and beneath of each pinna (reference and ground), subcutaneously. The tone-burst stimuli (4, 8, 12, 16, and 32 kHz) were used for measurement and total 1,024 responses were averaged. Responses were measured in 5 dB steps of decrement from 90 to 10 dB SPL and were determined as a threshold with the presence of peak I. Hearing thresholds were obtained before and after noise exposure. ABR measurement was also performed during and after laser irradiations (after 3^{rd}, 6^{th}, 9^{th}, 12^{th}, and 15^{th} laser irradiations).

Laser irradiation Treatment

An 808-nm diode laser (Wontec, South Korea) was used for laser therapy. Each rat in experimental group was anesthetized and irradiated for 60 mins with laser (165 ㎽/㎠, 594 joule) for 15 days. The density of laser was calibrated with a laser power meter (FieldMax II-To, Coherent, USA) and detect sensor (Powermax, Coherant, USA). The optic fiber (core fiber 62.5 ㎛ / cladding 125 ㎛) was attached to a hollow tube and placed to external ear canal which makes a distance between fiber tip and tympanic membrane within 1 mm. Laser irradiation was presented to both right and left ear simultaneously for bilateral group and only right ear for unilateral group. Noise only group was anesthetized and optic fiber was placed to external ear canal without power. The detailed information of laser described in Table 1.

Measurement of laser energy in the contralateral ear

Laser energy was measured from the contralateral side of ear with 808 laser irradiation (calibrated as 165 mW) in SD Rat to confirm the delivery of laser energy to the contralateral cochlea. Rat was sacrificed in CO2 chamber and was decapitated. Skin and pinna of test ear (contralateral side from the laser irradiation) were removed and cochlea was exposed. The exposed contralateral cochlea was placed just above the laser detector and laser was irradiated from the ipsilateral external canal with protocol explained above.

Hair cell count

For the quantitative analysis of outer hair cells (OHCs), whole mounts of the organ of Corti were prepared. Intracardiac perfusion was performed using 4 % Paraformaldehyde (PFA) followed by 0.9 % normal saline then cochlea was harvested. The harvested cochlea was fixed in 4 % PFA overnight. After washing with 0.1 M Posphate-buffered saline (PBS), cochlea was decalcified with ethylenediaminetetracetic acid (0.5 M EDTA, pH 8.0) and was dissected as three parts. Prepared samples were stained with Phalloidin (Phalloidin-FITC, Sigma, USA) and rinsed again with 1x PBS. Prepared sample was carefully examined under a confocal microscopy (LSM 510 META, Zeiss, Germary) at a magnification of 400X.

We chose three representative areas for the quantitative analysis OHC, which were 20, 50, and 80 % distanced from the apex that represent 4, 12, and 32 kHz respectively (Viberg &

Canlon, 2004). Hair cells within 200 ㎛ length were counted in each representative area. The morphometric analysis software Image J (http://rsb.info.nih.goz/ij/) was used to count a number of cells in each section.

Statistical analysis

All data were analyzed statistically using the Statistical Package for the Social Sciences software 19 version (SPSS, IBM, Somers, USA). Tuckey post hoc test following Two-way analysis of variance (ANOVA) was used to determine a difference of hearing threshold for ABR measurement and number of hair cell.

Results

Energy from the 808 laser was detected in the contralateral ear

Laser energy was measured in the contralateral ear using the 808 laser. With no medium between the laser probe and detector, the energy level shown on the detector was the same as the output from the laser, showing “good” calibration status of the machines (Fig. 1A). Around 6 mW of laser energy was detected (Fig. 1B), and the maximum level of laser energy penetrating the contralateral ear was 8 mW (Fig. 1C). This result suggests that some laser energy irradiated in one ear is delivered to the other ear (contralateral ear).

Hearing loss after noise overstimulation

ABRs were measured before noise exposure to determine the baseline hearing threshold. Mean values (SDs) were 18.61 (5.37), 16.11 (5.57), 16.94 (6.67), 16.11 (5.3), and 16.39 (6.14) at frequencies of 4, 8, 12, 16, and 32 kHz, respectively (Fig. 2A). At 24 h after noise exposure, ABRs were measured again to confirm the degree of hearing loss. Hearing thresholds were increased markedly after noise exposure. Mean values (SD) were 51.11 (6.08), 57.78 (8.44), 60.28 (6.96), 63.06 (4.79), and 60.56 (4.82) at frequencies of 4, 8, 12, 16, and 32 kHz, respectively (Fig. 2B). Thus, these results indicate that overstimulation with a stimulus of 115 dB SPL can cause PTS.

LLLT improved hearing recovery in the bilateral and unilateral treated groups

After the sixth laser irradiation, there was a significant difference in the hearing threshold at 16 and 32 kHz between the noise-only and the bilateral laser-treated groups (p = 0.001 at 16 kHz and 0.046 at 32 kHz; Fig. 2D). After the ninth laser irradiation, significant differences existed at all test frequencies between the noise- only and the bilateral laser-treated group (p = 0.009 at 4 kHz, 0.04 at 8 kHz, <0.001 at 12 kHz, 0.001 at 16 kHz, and <0.001 at 32 kHz). The response of the unilateral laser-treated group was significantly different from that of the noise-only group at 32 kHz (Fig. 2E) after the ninth laser irradiation. The difference between the unilateral and the noise-only group increased to 12 kHz and 16 kHz after the twelfth laser irradiation, and the bilateral-treated group showed difference at all frequencies except 8 kHz (Fig. 2F). Finally, after the 15^{th} laser irradiation, the hearing threshold at all test frequencies was significant different in the noise-only compared with the bilateral laser-treated group (p < 0.001 at 4 kHz, 0.005 at 8 kHz, < 0.001 at 12 kHz, < 0.001 at 16 kHz, and < 0.001 at 32 kHz), and the difference between the unilateral group and noise-only group increased to 4 kHz (Fig. 2G). This result showed that both bilateral and unilateral LLLT could reduce the hearing threshold in the SD rat model after noise overstimulation. However, complete recovery of the hearing threshold (to the baseline level) was not achieved.

Bilateral laser therapy resulted in faster hearing threshold recovery than did unilateral laser therapy

A significant difference in the threshold between the bilateral group and the noise-only group was observed from the point of the sixth laser irradiation (at 16 kHz and 32 kHz). In contrast, significant differences between the unilateral group and the noise-only group were observed from the points of the ninth and twelfth laser irradiations (at 32 kHz and 16 kHz; Fig. 3D, E). Furthermore, compared with the hearing threshold recovery in the bilateral group at 4 kHz, 8 kHz, and 12 kHz after the ninth laser irradiation, hearing threshold recovery in the unilateral group at these frequencies (at 4 kHz and 12 kHz) was observed after the twelfth and 15^{th} laser irradiations (Fig. 3A-C), respectively. At 8 kHz, there was no significant difference between the unilateral group and the noise-only group at any time point. This result indicated that despite the absence of differences in the extent of hearing recovery between the unilateral and bilateral LLLT groups, the bilateral simultaneous application of LLLT induced faster (up to 3 days) recovery of the hearing threshold after noise-induced hearing loss than did unilateral LLLT.

Laser-treated group showed better outer hair cell (OHC) preservation in the basal turn

A confocal image of a whole mount of three representative areas is presented in Figure 4. At the apex and the middle area, the averaged numbers of OHCs were similar across the three experiment groups (73.67, 72, and 70.33 at the apex, and 71, 72.67, and 73 at the middle in the bilateral, unilateral, and noise-only groups, respectively; Fig. 3). However, average numbers of OHCs at the basal turn differed among the groups (72.67, 67.5, and 59 in the bilateral, unilateral, and noise-only groups, respectively), and both the bilateral and unilateral laser groups showed larger number of OHCs than did the noise-only group (p = 0.0052 and 0.0006, respectively; Fig. 4).

Discussion

Cochlear damage can be variable, and a hearing threshold shift can occur abruptly or progressively, depending on the intensity and duration of noise overstimulation (Clark 1991). In the results of the present study, we found permanent threshold shifts in almost every frequency region examined. This result is consistent with our previous study (Rhee et al. 2012a). The results demonstrate that a high level of noise can cause PTS in this rat model. We observed slight improvements in the hearing threshold at low-frequency regions (4 and 8 kHz) with no treatment, which could be explained as a TTS, because it was not the main target frequency (Clark 1991) of the acoustic overstimulation applied in the current study. Increases in hearing threshold after noise exposure as both PTS and TTS could be a result of loss or dysfunction of outer hair cell electromotility, which contributes to hearing sensitivity by amplifying the incoming stimulus (Liberman et al. 2002). However, in the present study, we found that loss of hearing function was not obviously correlated with the histopathology of the OHCs. For such unrevealed functional loss, some other mechanism of TTS or PTS, such as dispersal of presynaptic ribbons and postsynaptic receptors, which connect the inner hair cells and spiral ganglion (Furman et al. 2013) may be involved.

Application of LLLT after noise overstimulation induced recovery of hearing function, similar to our previous study (Rhee et al. 2012b). This protection is considered to be related to the inhibition of iNOS and caspase 3 expression (Tamura et al. 2015), but the details of the underlying mechanism remain unclear. Also, it may be explained by the balance of free radicals and antioxidants. Before hair cell death, ROS levels increase as a result of noise overexposure. Movement of electrons in hair cells releases energy for converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP) by phosphorylation. During this process, superoxide is generated as an intermediate. When the use of oxygen is increased by noise exposure, the generation rate of superoxide is also increased by the activity of the mitochondria (Evans & Halliwell 1999). During noise exposure, mitochondria are strongly stimulated, and they produce excessive superoxide as a byproduct. Superoxide can react with other molecules in cochlear hair cells, resulting in molecular damage. Decreased cochlear blood flow due to noise exposure can also contribute to a deficiency of oxygen in the cochlea. Increased ROS can damage DNA, lipids, and proteins, leading to hair cell death (Evans & Halliwell 1999).

Despite the low penetration level in the contralateral ear, we found faster hearing recovery in the bilateral LLLT group than in the unilateral LLLT group. Additional laser energy may improve the speed of hearing recovery by prompting the endo-organs of the contralateral cochlea. It may be that the penetrated laser energy directly affected the contralateral cochlea as an activator of cell metabolism. Additionally, the amount of penetrated laser energy in the middle of the head, which would be more than that reaching the contralateral cochlea, could have sufficient influence to activate the cochlear nerve or auditory pathway in the midbrain. That said, the bilateral LLLT group did not show better recovery of hearing threshold than the unilateral LLLT group did after the 15th laser irradiation. This limited effect might be explained by the destruction of the most vulnerable auditory pathways after noise exposure, such as synaptic ribbons (Kujawa & Liberman 2009). Relatively normal morphologies of outer hair cells after noise overexposure supports this hidden damage theory because the functional loss was dramatic compared with the apparently limited hair cell loss found in the histology. There may be additional mechanisms responsible for the functional loss of hearing after noise overexposure, such as synaptic degeneration (Kujawa & Liberman 2009).

The faster effect of bilateral LLLT versus unilateral LLLT is promising for clinical use. Most treatments of hearing loss due to different insults require early intervention (Ward 1960). There are critical periods that increases the success of treatment outcome, resulting in more favorable prognoses (Chen et al. 2007). With bilateral LLLT, a shorter time was required to achieve a desirable outcome; thus, there is higher chance of staying within the “golden time” for the treatment of hearing loss. Transcanal LLLT treatment can lead to middle ear complications, such as acute inflammation and perforation of tympanic membrane (Moon et al. 2016). Applying bilateral LLLT might reduce the possibility of complications while increasing the effect because the laser energy is delivered from two different sites, similar to the protocol for transcranial LLLT. Multiple site laser irradiation has been used for transcranial laser therapy by several groups (Barrett & Gonzalez-Lima 2013; Schiffer et al. 2009). These studies reported improvements in cognitive and emotional functioning in the brain, with no side effects due to laser irradiation, using lower laser power and irradiating from multiple sites. As such, if estimating the exact location of the cochlea is possible, we may be able to deliver energy to the cochlea from multiple sites transcranially. However, no methodology for transcranial aiming toward the cochlea has yet been established.

To apply bilateral LLLT in the clinic, some practical issues must be considered. Because of anatomical differences between humans and rodents, the effects of laser energy on the contralateral side would be different. The larger distance from one ear to the other may limit the delivery of laser energy; however, the beneficial effect of bilateral LLLT would be expected to remain if the mechanism involves targeting the brainstem. Increasing the power of the laser may be another approach to deliver energy to the other ear, but this could cause side effects, resulting in local burning and tympanic perforation. Thus, increasing the power of transcanal laser irradiation should be considered carefully before clinical application.

Conclusions

The present study showed positive effects of bilateral low-level laser therapy after noise-induced hearing loss in an animal model. The results suggest that the use of bilateral low-level laser therapy in the clinical setting may improve the therapeutic effects on hearing while minimizing side effects.

Acknowledgement

This study was supported by a grant of the Ministry of Science, ICT and Future Planning grant funded by the Korea government (2012K1A4A3053142, NRF-2014R1A1A1038199)

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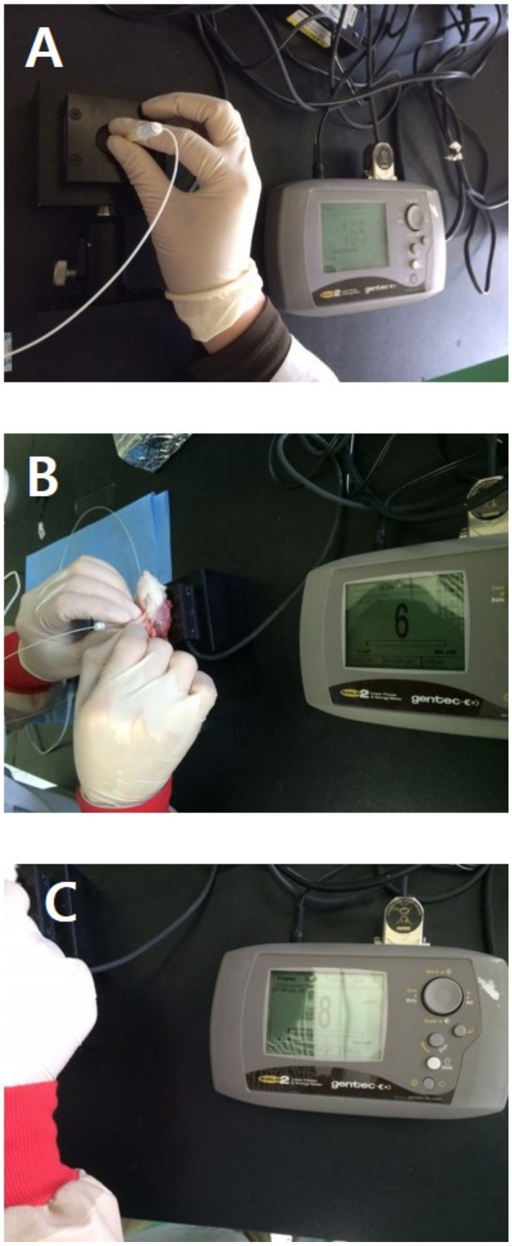

Figure 1. Measurement of the penetration depth of 808 laser energy in the SD rat. The pinna and skin of the test side were removed (A). Bullas and nearby muscles were also removed to expose the cochlea (white circle) (B). The laser energy was set at an intensity of 165 mW (C). Penetration depth was measured, and the amount of penetrating laser energy reached 8 mW (D, E).

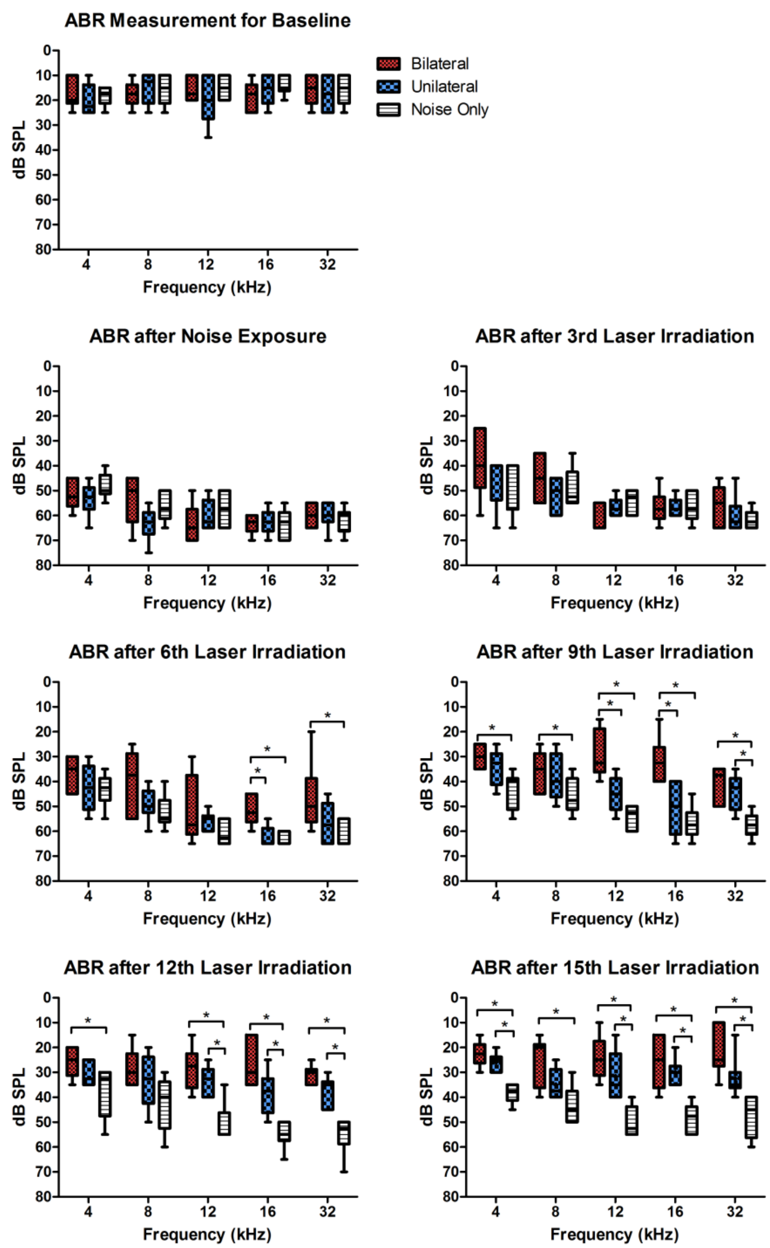

Figure 2. Changes in hearing threshold after noise exposure and low-level laser irradiation. After noise exposure, hearing thresholds at all tested frequencies increased in all test groups (A, B). After the sixth laser irradiation, the bilateral laser group showed significant improvement at 16 and 32 kHz (*$p < 0.05$) (D). These differences were expanded to all tested frequencies after the ninth laser irradiation and were maintained until the 15th laser irradiation, except at 8 kHz (E, F, and G).

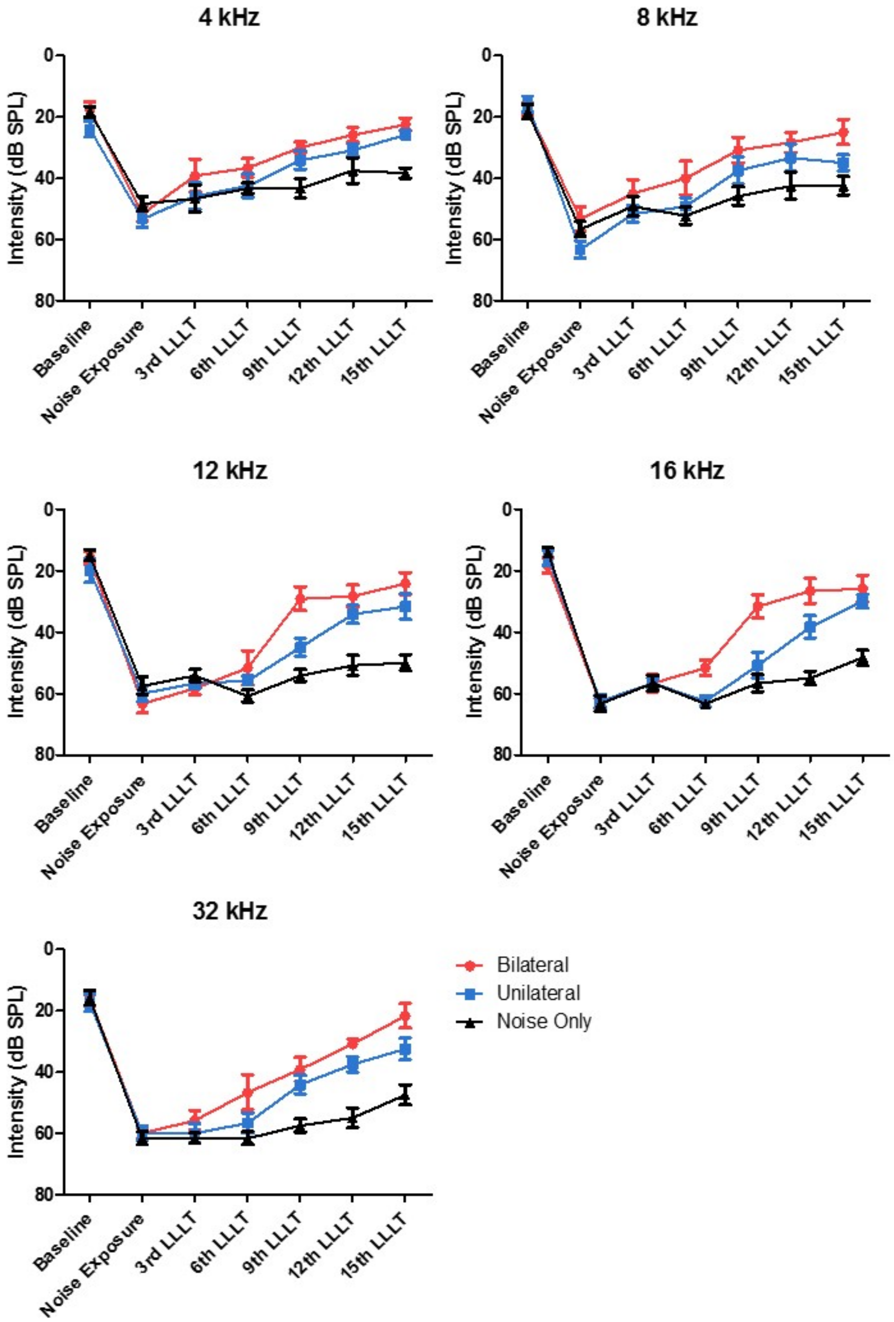

Figure 3. Changes in hearing threshold at each ABR measurement. At every tested frequency, the result of the bilateral LLLT group showed faster hearing recovery than the unilateral LLLT group (B: baseline, NE: noise exposure).

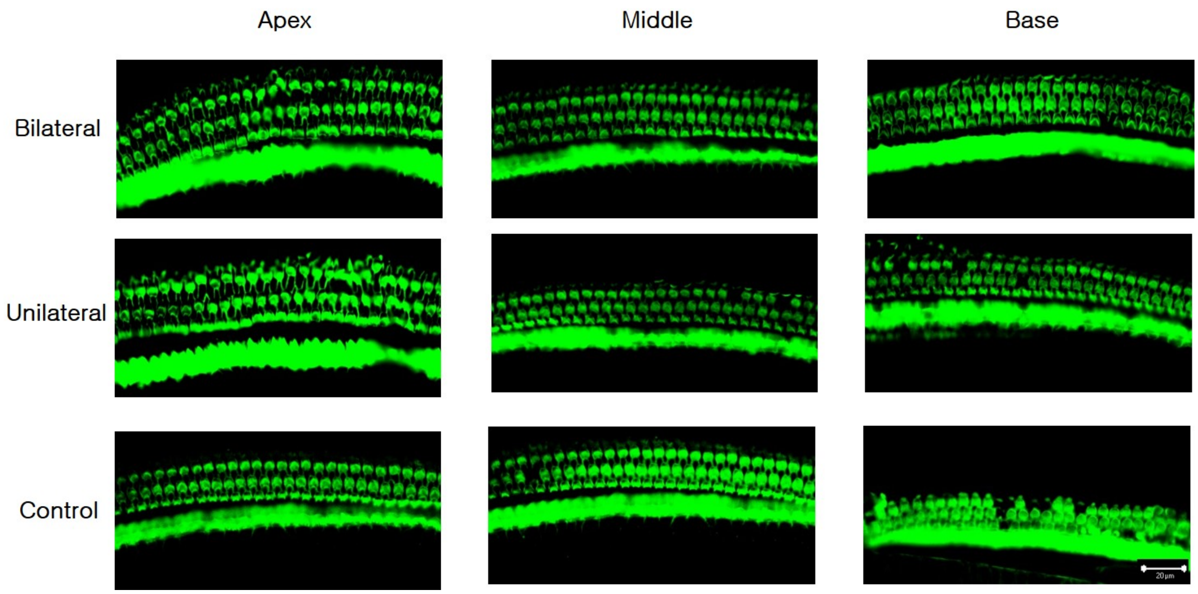

Figure 4. Representative confocal images of hair cells at three different locations (apex, middle, and base) in each experimental group. Missing hair cells were observed only at the base part of the cochlea in the noise-only group.

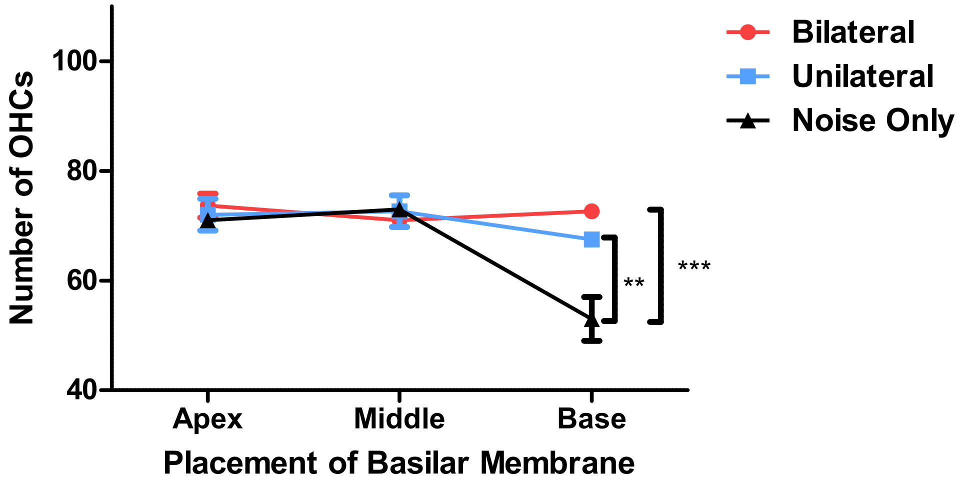

Figure 5. Numbers of OHCs in three parts of the basilar membrane in each group. The bilateral and unilateral laser groups showed significantly larger numbers of OHCs at the base part of the basilar membrane (***p* < 0.01, ****p* < 0.001).

Table 1. Laser (Photobiomodulation) parameter

Parameter	Laser group (Bilateral and Unilateral)
Power (mW)	185
Beam spot size at target (cm2)	0.22
Irradiance at target (mW/cm2) power density	841
Exposure duration (s)	3600
Radiant exposure (J/cm2) fluence	2700
Radiant energy (J)	594
Number of points irradiated	1
Area irradiated (cm2)	0.22
Application technique	Through tympanic membrane
Number and frequency of treatment sessions	Once a day for 15 days
Total radiant energy (J)	8,910

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