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Abstract. The primary cause of hearing loss includes damage to cochlear hair cells. Low-level laser therapy (LLLT) has become a popular treatment for damaged nervous systems. Based on the idea that cochlea hair cells and neural cells are from the same developmental origin, the effect of LLLT on hearing loss in animal models is evaluated. Hearing loss animal models were established, and the animals were irradiated by 830-nm diode laser once a day for 10 days. Power density of the laser treatment was 900 mW/cm², and the fluence was 162 to 194 J. The tympanic membrane was evaluated after LLLT. Thresholds of auditory brainstem responses were evaluated before treatment, after gentamicin, and after 10 days of LLLT. Quantitative scanning electron microscopic (SEM) observations were done by counting remaining hair cells. Tympanic membranes were intact at the end of the experiment. No adverse tissue reaction was found. On SEM images, LLLT significantly increased the number of hair cells in middle and basal turns. Hearing was significantly improved by laser irradiation. After LLLT treatment, both the hearing threshold and hair-cell count significantly improved. © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.18.12.128003]

Keywords: laser therapy; cochlea; gentamicin.

1 Introduction

Hearing loss is a major public health issue. It affects about 17 in every 1000 children under age 18, and its prevalence increases with age: 314 in 1000 people above age 65 have a hearing problem and 40% to 50% of people older than 74 have hearing loss. Although hearing loss is rarely life threatening, its influence on our economy and lifestyle is not negligible. The primary causes of hearing loss include damage to the sensory cells (hair cells), supporting cells, and neurons in the cochlea. This is clinically known as sensorineural hearing loss. Because mammalian auditory sensory cells (cells of the organ of Corti) ends mitosis by embryonic day 15, loss of these cells, especially sensory hair cells in the cochlea, leads to permanent hearing loss. Currently, there is no definite treatment for chronic sensorineural hearing loss after the death of hair cells.

Low-level laser therapy (LLLT) has become a popular treatment modality and is finding a variety of uses in medical practice. In the past decade, LLLT has been approved by the United States Food and Drug Administration in treating diseases such as carpal tunnel syndrome and alopecia. Additionally, much research with LLLT in other fields has been reported including nerve regeneration and wound healing. Although these new applications have not been approved by the government, the effects of LLLT, especially on the central nervous system, have been investigated by many studies. Since cochlea hair cells and neural cells are from the same developmental origin, our groups recently studied the effect of LLLT on damaged cochlea hair cells in vitro. The result was promising, revealing improved recovery of hair cells with LLLT. But the encouraging effect of LLLT on hair cells was based on in vitro cultured tissue, and more evidence from living animals seems necessary in order to apply the treatment to human subjects. Also, safety may be a concern when applying a laser to the external auditory canal of living animals and humans. In this article, we aimed to elucidate the in vivo effect of LLLT on hair-cell recovery. We also wanted to see if there was any adverse effect in the ear on applying LLLT to living animals.

The exact mechanism of LLLT on neural-cell recovery and regeneration is still not fully clarified, but there are some theories. The dominant opinion is built around cytochrome c oxidase, which is thought to be the key protein of cell metabolism and repair. It is also one of the three major proteins found in the human body responding to the near-infrared wavelength of light. As with chlorophyll in plants responding to visible light and activating photosynthesis, these proteins can absorb near-infrared wavelength energy and can modulate biochemical reactions in the cells. Cytochrome c oxidase is complex IV in the mitochondrial respiratory chain that consists of five protein complexes that together produce ATP. Solid support for this theory comes from research showing that LLLT enhances ATP production. Increased ATP production may lead to enhanced cell metabolism, promoting the damage-repair process. However, definite evidence is necessary to prove this theory, because many cell signaling pathways can originate from upregulating mitochondrial activity.

In spite of the growing interest in LLLT and hearing restoration, the effect of laser on hearing recovery has not been studied...
thoroughly. The aim of this article was to describe the effect of LLLT on cochlea hair cells in vivo and to overcome the limitations of previous in vitro studies.11

2 Materials and Methods

2.1 Hearing Loss Animal Model Induced by Gentamicin

Healthy adult Sprague–Dawley rats (N = 11, 8-week old, 180 to 200 g) with normal hearing were used as subjects. Gentamicin/furosemide-induced hearing loss models were established in eight rats, according to the modified method previously described.12 Briefly, the animals were treated daily by gentamicin 100 mg/kg intravenously (i.v.) followed 10 min later by furosemide 90 mg/kg i.v. for 2 days. The i.v. injection of ototoxic drugs was performed by inserting a syringe with an Angiocath Plus™ (BD, Franklin Lakes, New Jersey) into the tail vein of the animals with a very slow injection speed of 10 to 20 μl/s. Forty-eight hours after gentamicin and furosemide treatment, hearing loss was confirmed with click auditory brainstem response (ABR).

Three rats were not included in the hearing loss model, in order to monitor if there was any problem with our hearing loss model and experimental technique. The same experimental technique [hearing measurement and scanning electron microscopy (SEM)] was applied to these rats, but the differences was that gentamicin/furosemide and laser were not given to these control animals (C ears, n = 3). These control ears were expected to have normal hearing and normal cochlear hair-cell morphology.

2.2 Auditory Brainstem Response Measurement

Thresholds of ABRs were determined from each ear before treatment (baseline values, day 0), after exposure to the ototoxic drug (day 2), and after the treatment with LLLT (day 12). ABRs were measured with an evoked response signal-processing system (System III, Tucker Davis Technologies, Alachua, Florida). The rats were anesthetized and placed in a soundproof booth. Following anesthesia, needle electrodes were placed subcutaneously at the vertex (active electrode) and beneath each pinna (reference and ground electrodes, respectively).

The click auditory stimuli were delivered through a tube inserted into the ear canal of the rat. Hearing thresholds were determined by assessment of the lowest stimulus level to elicit ABR peaks III at levels from 10 to 90 dB sound pressure level (SPL) in 5-dB steps. One thousand and twenty-four tone presentations were averaged.

2.3 Laser Irradiation

In this in vivo study, 48 h after treatment with the ototoxic drug, the animals were irradiated by 830-nm diode laser (Hi-tech Optoelectronics, Beijing, China) for 60 min every day for 10 days. Laser irradiation was done only in the right ear (GM+L ear, n = 8), and the left ear (GM ear, n = 8) served as the control. The total power output of the laser was set as 200 mW. The power of the laser was checked at the distal end of the optic fiber with a SOLO 2 laser power meter (Newport, Irvine, California) and a XLP12-1S-H2-DO detector head (Newport). The laser energy was delivered by inserting the hollow tube-surrounded laser fiber into the external auditory canal with a distance from the tip of the fiber to the surface of tympanic membrane of around 1 mm (Fig. 1). The core diameter of the optic fiber was 62.5 μm, and when the cladding was included, the diameter was 125 μm. Complete laser parameters are presented in Table 1 as recommended by Jenkins and Carroll.16

The axis of the optic fiber was evaluated through a multidetector computed tomography (CT) scan on a 64-detector scanner (LightSpeed, GE Healthcare, Milwaukee, Wisconsin). Coronal section images were obtained in 0.625-mm slice thicknesses. The optic fiber was positioned so that it was directly aimed at the cochlea. That is, the optic fiber and cochlea was in the same line as in Fig. 1. We have used a quantitatively measurable stereotactic laser aiming device that can consistently place the optic fiber in the external auditory canal. The stereotactic laser aiming system was composed of two measuring scales: a roll plane graduator and a yaw plane rule. Before conducting the main experiment, we took several CT scans with various roll plane and yaw plane angles to figure out the correct roll plane and yaw plane angle which would aim the optic fiber to the cochlea.

In order to calculate the intracochlear penetration rate, we have measured the intracochlear laser power with a fresh rat head. After anesthesia, the rat was decapitated, and the head

Fig. 1 In vivo laser irradiation of the ear and computed tomography (CT) scan demonstrating the direction of the optic fiber. The gentamicin-treated rats were irradiated with 830-nm diode laser through the external auditory canal. (1) Current output of the laser, (2) optic fiber, (3) ear plug supporting the optic fiber, and (4) timer (a). The positional relationship between optic fiber, tympanic membrane, and cochlea was defined through a CT scan. (1) Tip of the optic fiber, (2) tympanic membrane, (3) ossicle in the middle ear, and (4) cochlea (b).
of the rat was sagitally cut in half. After removing the brain and nerve fibers, the cochlea was identified from the medial side of the skull. Laser was irradiated from the external auditory canal in the same way that we have performed the main experiment. The power that was measured from the labyrinthotomy site (medial side of the skull) was considered as the laser power that had penetrated into the cochlea. We have performed the same penetration rate experiment in five different cochleae. From this pilot experiment, we have found that 6.20% ± 1.24% of laser power had penetrated into the cochlea when the optic fiber was placed in the external auditory canal.

### Table 1 Complete parameters of laser treatment applied in the article.

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Value</th>
<th>Additional notes</th>
</tr>
</thead>
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<td></td>
</tr>
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<td>Irradiance at target (mW/cm²)</td>
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<td>Exposure duration (s)</td>
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<td>Radiant exposure (J/cm²) fluence</td>
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<td>Penetration rate of 5% to 6%</td>
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<tr>
<td>Radiant energy (J)</td>
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<td>Number of points irradiated</td>
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<tr>
<td>Area irradiated (cm²)</td>
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<tr>
<td>Application technique</td>
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</tr>
<tr>
<td>Number and frequency of treatment sessions</td>
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</tr>
<tr>
<td>Total radiant energy (J)</td>
<td>356 to 428</td>
<td>Penetration rate of 5% to 6%</td>
</tr>
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</table>

2.5 Histologic Evaluation of the Tympanic Membrane and External Auditory Canal Skin

In order to evaluate microscopic changes of the tympanic membrane and external auditory canal skin, a histologic preparation was made after sacrificing the animals. On the last day of the experiment, after ABR recording, intracardiac perfusions were performed with 4% paraformaldehyde under general anesthesia. After perfusions, the deeply anesthetized animals were decapitated, and the bony external auditory canal and the tympanic membrane were harvested in one piece. The specimens were decalcified in RDO solution (Apex Engineering Products Corporation, Aurora, Illinois) and embedded in paraffin for 5-μm serial sections. The sections were stained with hematoxylin-eosin and examined under light microscopy (Olympus, Tokyo, Japan). The histologic findings were evaluated by one pathologist who was blinded to the group (GM + L ear or GM ear). The epithelial integrity, cell morphology of the skin and subcutaneous tissue, and presence of inflammatory cells and vascular congestion were closely evaluated.

2.6 Scanning Electron Microscopy

On the last day of the experiment, after the ABR recording, intracardiac perfusions and decapitations were performed using the same method mentioned above. The cochleae were removed immediately. The isolated cochleae were then decalcified in RDO solution for 20 min, after which the bony capsule was removed, and the lateral wall tissues (spiral ligament and stria vascularis) as well as the membranous structure were separated under a dissecting microscope. The dissected specimens were rinsed with 0.1 M Dulbecco’s phosphate-buffered saline (DPBS) and post-fixed in 1% osmium tetroxide for 15 min. Afterward, the specimens were gently rinsed with 0.1 M DPBS again and dehydrated in graded ethanol solutions. The specimens were transferred to hexamethyldisilazane for 15 min and dried at room temperature overnight. The dried specimens of the organ of Corti were attached to aluminum stubs with aluminum paint, and then coated with platinum-palladium using E-1030 PT-PD target assembly (Hitachi, Tokyo, Japan). The surfaces of the organs of Corti were examined using S-4300 SEM (Hitachi, Tokyo, Japan).

2.7 Hair-Cell Counting

Quantitative SEM observations of the surface morphology of the organ of Corti were performed by counting retained hair cells from apical to basal turns of the cochlea, based on the method previously described. Different turns of the cochleae were defined according to the percent distance from the apex: 0.0% to 33.3%, 33.3% to 66.6%, and 66.6% to 100.0% from the apex were considered as apical, middle, and basal turns, respectively. Hair cells were counted over a 100-μm longitudinal distance from two separate regions in the representative images that were captured in the central area of each turn. A hair cell was considered absent if the stereociliary bundle was missing.

2.8 Statistical Analysis

Audiometry and hair-cell counting were performed by a single-blinded investigator. Data were analyzed statistically using the Statistical Package for the Social Sciences (SPSS, Inc., an IBM Company, Chicago, Illinois) software. Wilcoxon signed ranks test was used to compare the hair-cell damage and hearing sensitivities.
loss between the experimental groups. Null hypotheses of no difference were rejected if $p$-values were less than 0.05.

3 Results

3.1 Location and Direction of the Optic Fiber

The location and direction of the optic fiber were identified by CT scan. As shown in Fig. 1, the laser fiber, tympanic membrane, and cochlea shared a similar axis with each other. This ensured that the inner ear was directly within the irradiation axis. The CT scan also proved that the tip of the optic fiber did not touch the tympanic membrane. The distance from the tip of the optic fiber to the tympanic membrane was approximately 1 mm. The correct angle that can precisely aim the cochlea was $-7.5$ deg in the roll plane and $14.5$ deg in the yaw plane.

3.2 Endoscopic Findings of the Tympanic Membrane

Until the end of the experiment, all of the tympanic membranes were intact without any clinically significant adverse tissue reaction. That is, there was no evidence of perforation, inflammation, discharge, hemorrhage, swelling, or thickening of the tympanic membrane. But after daily LLLT, the vessels on the tympanic membrane and external auditory canal started to become hyperemic. The vascular congestion was noticed from the first to third days of LLLT and progressed until the end of the experiment. Meanwhile, there was no change in the vasculature of the nonirradiated left ear (Fig. 2).

3.3 Histologic Evaluation of the Tympanic Membrane and External Auditory Canal Skin

After 10 days of LLLT, there was no significant adverse tissue reaction when the tissues were evaluated under the light microscope (Fig. 3). That is, there was no clinically significant difference in the epithelial integrity of the skin between the GM + L and GM ears. The morphologies of the cells in the epithelium and subcutaneous tissue were not different. There was no evidence of inflammation or infection in both the GM + L and GM ears. When the experimental groups were blinded, the pathologist was not able to distinguish between the GM + L and GM ears based on the histologic findings.

Although there was no clinically significant histologic difference between the GM + L and GM ears, a subtle difference was noticed. The tympanic membrane seemed slightly thicker in the GM + L ears when compared with the GM ears. Also, the vessels in the GM + L ears seemed slightly hyperemic when compared with the GM ears. But these findings were not substantial and were not noticed in every specimen of the GM + L ears. The pathologist noticed these findings, but considered it a normal variation. In general, it seemed that there was no difference between the GM + L and GM ears.

3.4 SEM Hair-Cell Count

The hair cells were observed to be absent, and the number of remaining hair cells was calculated in a 100-μm length of each turn (apical, middle, and basal turns) in the SEM images (Fig. 4). The hair-cell morphology was normal in the C ears; there was no absent hair cell. As for the GM ears, the hair cells in the basal turn were mostly damaged.

Fig. 2 Endoscopic findings of the tympanic membrane. The tympanic membrane was photographed every day after laser irradiation with an endoscope. Throughout the experiment, there was no evidence of perforation, inflammation, discharge, hemorrhage, swelling, or thickening of the tympanic membrane. But after daily laser irradiation, the vessels in the tympanic membrane and external auditory canal started to become hyperemic. The vascular congestion was noticed from the first to third days of laser irradiation and progressed until the end of the experiment. Meanwhile, there was no change in thevasculature of the nonirradiated left ear.
while the hair cells were partially conserved in the middle turn (26.7/21.2 cells/100 μm). Although there was no clinically significant histologic difference between the GM and GM+L ears, a subtle difference was noticed. The tympanic membrane seemed slightly thicker in the GM+L ears (b) when compared with the GM ears (a). Also, the vessels in the GM+L ears seemed slightly hyperemic (d) when compared with those of the GM ears (c).

The number of hair cells was summated for all three turns of hair cells in the apical turn between the two groups. When that of the GM+L ears did not improve (57.1 ± 18.0 dB SPL). The hearing was significantly better in the GM+L ears when compared with that of the GM ears (p = 0.023).

### 4 Discussion

In this article, we investigated the effect of LLLT on live hearing loss animals with cochleas artificially damaged by gentamicin and furosemide. The result was interesting, in that both the hearing threshold (as tested by ABR) and hair-cell count (acquired by SEM) significantly improved. Currently, there are only a few studies that have elucidated the effect of LLLT on inner-ear hair-cell recovery.

To the best of our knowledge, this is the first in vivo study that showed improved hearing outcome after gentamicin and furosemide toxicities. Our previous in vitro study had some limitations to consider. One limitation was the organ explantation timing. The cochlear tissue was explanted on postnatal days 3 to 4. Since the cochlea of rodents is still premature and developing at the time of birth, the recovery potential might be enhanced by this timing, partially explaining the results of the previous study. That is, the high-regenerative property of neonatal cochlear hair cells may have been one of the reasons for hair-cell regeneration, together with the effect of LLLT. This issue may be critical in that the encouraging results may not be reproduced in the human ear, which is already mature from birth. However, in this article, live adult rats were the experimental animals, resolving that question clearly. LLLT seems to help adult hair cells which are fully developed and matured to recover from ototoxic damage, irrespective of the intrinsic regenerative power of the cells.

### 3.5 Hearing Outcome

Before gentamicin/furosemide treatment, the hearing threshold was normal in all the animals (<30 dB SPL). After administering gentamicin/furosemide (day 2), the hearing threshold increased to 54.3 ± 11.0 dB SPL in the GM ears and 52.9 ± 12.5 dB SPL in the GM+L ears [Fig. 5(b)]. There was no difference in the degree of hearing loss before starting the LLLT. After daily irradiation treatments for 10 days (day 12), the hearing threshold in the GM+L ears improved to 44.3 ± 12.7 dB SPL. But, the hearing threshold in the GM ears did not improve (57.1 ± 18.0 dB SPL). The hearing was significantly better in the GM+L ears when compared with that of the GM ears (p = 0.023).
Fig. 4 Scanning electron microscopy (SEM) and quantitative hair-cell count results. The hair-cell morphology was normal in the C ears: there were no absent hair cells. As for the GM and the GM + L ears, most of the hair cells in the basal turn were damaged, while the hair cells were partially conserved in the middle turn and relatively intact in the apical turn. But, the number of hair cells was significantly larger in the GM + L ears in the middle turn ($p = 0.028$) and basal turn ($p = 0.042$) when compared with that of the GM ears. Scale bar = 20 μm.

Fig. 5 Total hair-cell count and hearing outcome. When the number of hair cells was summated for all the three turns (a), the total number of hair cells was significantly larger in the GM + L ears when compared with that of the GM ears ($p = 0.034$). As for hearing outcome (b) before gentamicin/furosemide treatment, the hearing threshold was normal in all the animals (<30 dB SPL). After administration of gentamicin/furosemide (day 2), the hearing threshold significantly increased; After daily irradiation treatments for 10 days (day 12), the hearing was significantly better in the GM + L ears when compared with that of the GM ears ($p = 0.023$).
The laser is a very powerful modality in the medical environment. But in order to induce a meaningful biologic effect, the wavelength and power of the laser must be selected carefully. While the wavelength of the laser may be comparable with the chemical composition of a drug, the power of the laser may be comparable with the dosage of the drug. The drug will not be effective, if either the chemical composition or the dosage is incorrect. Also, like a drug overdose, an excessive amount of laser irradiation may lead to destruction rather than promotion.\textsuperscript{21} Therefore, determining the laser parameters to be used is very critical. The parameters of this article were selected based on previous reports and the results of our own previous study. As for the wavelength, it is widely known that there is a typical responsive wavelength for cytochrome c oxidase.\textsuperscript{12} Nevertheless, since this wavelength is within the visible light range and has a low-penetrating rate, it is not easy to deliver laser of this wavelength to the inside of the otic capsule, the bony capsule covering the cochlea.\textsuperscript{11} To maximize the penetration rate, the wavelength of the laser should be between 600 and 1200 nm. This range is called the “optical window” of tissue.\textsuperscript{13} The wavelength with the highest potency of biomodulation among the optical windows is 800 to 830 nm.\textsuperscript{12} Therefore, considering both penetration and biomodulation, the authors selected 830 nm for the \textit{in vivo} treatment of cochlea hair cells.

The second parameter that we have to discuss is the power of the laser. According to previous studies on LLLT\textsuperscript{6,7} and our preliminary trials, the effective therapeutic range was about 7.5 to 10.0 mW. However, unlike these \textit{in vitro} studies, our study was done \textit{in vivo}, and therefore we had to consider the penetration rate. Based on our prior study, the transtympanic penetration rate to the cochlea was 5.5\% in rodents.\textsuperscript{22} To achieve similar LLLT effects as in our previous \textit{in vitro} study, we selected a laser power of 200 mW. Too much power might cause local complications. An expected complication of excessive exposure is local heat formation, which injures the ear canal skin or tympanic membrane. Therefore, we took daily endoscopic pictures at each laser exposure and found no clinically significant adverse tissue reaction, except mild vascular congestion. In order to detect microscopic change that might have been missed during the macroscopic evaluation, we also evaluated the histologic findings of the external ear canal skin and tympanic membrane. But other than mild congestion of the vessels and mild thickening of the tympanic membrane, there was no difference between the GM + L and GM ears. When planning a human trial, the safety of this technique is very important. Currently, we did not find any clinically significant adverse effect with a laser of 200-mW power. But, we believe that studies with higher power are necessary in the future.

With aminoglycosides such as gentamicin, ototoxicity is believed to be induced through the caspase intrinsic pathway. This pathway activates the stress-activated protein kinases such as c-JunN-terminal kinase, increases intracellular Ca\textsuperscript{2+}, and damages mitochondrial membranes, eventually releasing cytochrome c. When the mitochondrial signal activates caspase-9 and the downstream caspases, it induces apoptosis of the cells.\textsuperscript{3-22} As mentioned before, the dominant theory of the LLLT mechanism is related to cytochrome c oxidase that reacts to the optical energy and produces ATPs.\textsuperscript{12,13} A Possible increase in ATP production blocks the cells from fatal pathways by upregulating the caspase-3-mediated pathway.\textsuperscript{11} In the past, there were doubts about the LLLT effect, and the effect of LLLT was regarded as no more than a thermal effect. Nowadays, it has been demonstrated\textsuperscript{26} that ATP production is not a thermal effect. However, the full mechanism of LLLT is still not clear.

Although LLLT seems to be somewhat effective in hair-cell recovery after ototoxic damage, we also found that the effect is not complete, like in other studies.\textsuperscript{11,17} The ABR and hair-cell count did not reach the normal range after full treatment. It seems that when the damage was too severe, as in the basal turn of the ototoxic-damaged cochlea in this article, LLLT did not have any meaningful effect. Also, as in the apical turn of the cochlea, LLLT may have a trivial effect when the ototoxic damage is minor. It seems that LLLT is effective only in a specific group of hair cells in which the damage is not too severe or too small. LLLT cannot rescue the hair cells after they have advanced too far along the apoptosis pathway. Also, it seems that LLLT cannot induce regeneration of hair cells when they have been completely lost. This point is quite discouraging in that most patients with hearing loss have a long history of hearing deterioration.

The eventual goal of our research is the human application of LLLT. In this article, while the outcomes are inspiring, there are a few considerations to take into account before clinical application of this new treatment can begin. First is the different penetration rate of humans; it is thought to be lower than that of rodents,\textsuperscript{22} therefore, more power needs to be delivered to the cochlea in humans but without causing complications. The next thing is local heating; this is presumed to be the only adverse effect.\textsuperscript{17} As mentioned above, there is no damage with laser of power of 200 mW, but there might be significant damage in humans with irradiation of higher power. The last thing to consider is the mechanism of the laser. There is a dominant theory about the mechanism of operation, but to support clinical applications, there should be more evidence for the mechanism. We are planning to do more work on the biomodulation mechanism of LLLT and expecting to find an answer in the near future.

In this article, we investigated the effect of LLLT on live hearing loss animals with cochleas artificially damaged by gentamicin and furosemide. We found that LLLT helps the recovery of hair cells after ototoxic damage: it promotes the survival of hair cells and improves the hearing threshold. But, the effect may not be complete. That is, LLLT is effective only in a specific group of hair cells in which the damage is not too severe or too small. Also, it seems that LLLT cannot induce regeneration of the hair cells when they are completely lost. We believe this promising effect may only be achieved with a laser with a narrow range of wavelength and power. Using a laser with a wavelength or power outside this range may not produce the same results and will probably result in no effect. As for safety issues, the 830-nm laser with power of 200 mW seems safe to use. Although there are some limitations in this article, we believe that LLLT may have some positive effect in hearing recovery after acute ototoxic hearing loss.

Acknowledgments
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