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Abstract. We investigated the effect of low-level laser radiation on rescuing hair cells of the cochlea after acute acoustic trauma and hearing loss. Nine rats were exposed to noise. Starting the following day, the left ears (NL ears) of the rats were irradiated at an energy output of 100 to 165 mW/cm^2 for 60 min for 12 days in a row. The right ears (N ears) were considered as the control group. Frequency-specific hearing levels were measured before the noise exposure and also after the 1st, 3rd to 5th, 8th to 10th and 12th irradiations. After the 12th treatment, hair cells were observed using a scanning electron microscope. Compared to initial hearing levels at all frequencies, thresholds increased markedly after noise exposure. After the 12th irradiation, hearing threshold was significantly lower for the NL ears compared to the N ears. When observed using an electron microscope, the number of hair cells in the middle turn of the NL ears was significantly larger than that of the N ears. Our findings suggest that low-level laser irradiation promotes recovery of hearing thresholds after acute acoustic trauma. [DOI: 10.1117/1.JBO.17.6.068002]

Keywords: acute acoustic trauma; auditory brainstem response; cochlea; hair cell; low-level laser therapy; noise-induced hearing loss.

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1 Introduction

Among the various cells in the ear, hair cells play the most important role in sound perception. While sound energy is a mechanical vibration, through the hair cells, it is transformed into electrical energy which can be perceived as sound by the brain. Because such a crucial role is played by the hair cells, damage to the inner ear is displayed as hair cell loss and reduced hearing abilities. In humans, hair cells normally cannot regenerate when lost, resulting in permanent hearing impairment. Much research has been carried out to find ways to restore hearing, but no definite treatment has been established yet.

Nowadays, one of the most common factors that cause hearing disorders is noise trauma. Noise is an increasing hazard and it is pervasive, which makes it difficult to take precautions and prevent noise-induced hearing loss (NIHL). A number of studies have been carried out regarding the effects of noise on hair cells and have reported that exposure to intense noise initiates a cascade of free radical formation and apoptosis which ultimately induces hair cell loss.[1] The oxidative stress that results from these changes produces a variety of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the superoxide anion (O_{2-}) and the hydroxyl radical (OH).[2–5] Production of these ROS alters the homeostasis in cells and the imbalance brings forth conditions such as vascular insufficiency, ultimately resulting in necrosis or apoptosis of the hair cells.[6,7]

Already applied in numerous areas, laser-induced phototherapy is widely applied as a noninvasive treatment promoting cell regeneration and repair processes. Interest in low-level laser therapy (LLLT) has been growing, especially for the past decade, and the number of studies and clinical trials performed has increased markedly. The low-level laser has been discovered to be effective in wound healing, and has been found to enhance various biological processes.[8–12] It is especially notable that LLLT has been approved by the U.S. Food and Drug Administration for the treatment of several diseases, including carpal tunnel syndrome[13–15] and alopecia.[16]

Although the exact mechanism of laser therapy is not fully clarified, it has been documented that once absorbed, the light can modulate biochemical reactions in cells and stimulate mitochondrial respiration, enhancing the production of molecular oxygen and adenosine triphosphate (ATP) synthesis.[17–19] As with chlorophyll in plants responding to light and activating photosynthesis, there are three major proteins in the human body that respond to infrared wavelengths of light.[20] Of these proteins, cytochrome c oxidase is presumed to be the key protein in cell regeneration and recovery. Cytochrome c oxidase is a protein that is mostly found in the membrane of the mitochondria and is one of the five protein complexes (complex IV) in the mitochondrial membrane that are responsible for ATP production.[21] It has been reported that irradiation with LLLT is closely related to increased production of ATP.[22] Increased ATP production may lead to enhanced cell metabolism, promoting the damage-repair process. Overall, low-level laser irradiation increases cell proliferation, thus positively modulating cell repair processes.[23,24]

Despite the sharply growing interest in low-level lasers and the variety of their use in different fields, the effects of lasers on

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hearing recovery has not been thoroughly investigated. In the present study, the ability to recover hearing thresholds after an acute acoustic trauma was assessed using an 830-nm diode laser.

2 Materials and Methods

2.1 *Animals*

Adult male Sprague Dawley rats weighing approximately 200 g were divided into control ears (C, $n = 6$), noise only ears (N, $n = 16$) and a combination of noise and laser ears (NL, $n = 16$). N and NL were the bilateral ears of the same rat: the left ear was treated (NL) while the right ear (N) was not. The control ears were on individuals different from the N and NL rats. For anesthetics, zolazepam (Zoletil, Virbac, Carros Cedex, France) and xylazine (Rompun, Bayer, Leverkusen, Germany) were mixed in a 4:1 ratio (0.1 ml/100 g). The animals were anesthetized before each auditory-brainstem-response (ABR) recording and laser irradiation. The N ears were exposed to a 116-dB sound pressure level (SPL) noise, causing noise-induced hearing loss and no other intervention was applied except for routine ABR hearing tests. For NL ears, the animals were exposed to the same noise-induced hearing loss but were treated with daily laser irradiation for 12 days. Hearing was checked in NL ears according to the same schedule as for N ears. Animals were sacrificed after 12 days of treatment for a scanning electron microscope (SEM) study. No intervention was done to the C group. Only the baseline hearing threshold was checked and the cochlea was harvested as in the other groups to serve as the control.

2.2 *Acute Acoustic Trauma*

A trapezoid-shaped acryl reverberation chamber was designed with a loudspeaker CP800Ti (Beyma, Valencia, Spain) attached on top. The noise was generated with a type 1027 sine random generator (Bruel and Kjaer, Naerum, Denmark) and amplified with a R300 plus amplifier (Inter-M corp, Seoul, Korea). A real-time, frequency-specific sound-level meter (2250, Bruel and Kjaer) was used to calibrate the noise generator and confirmed that the amplifier projected the exact settings of the generator. The rats were placed in small, separate cages to prevent defensive behaviors such as blockage of the ears and were set inside the noise box. The rats were given a one-time exposure to a narrow band noise of 116 dB SPL centered at a frequency of 16 kHz (bandwidth 1 kHz) for 6 h.

2.3 *Auditory Brainstem Response Recordings*

Auditory brainstem responses (ABRs) were recorded using a signal-processing system (System III, Tucker Davis Technologies, Alachua, FL) with tone-burst, frequency-specific stimulus-generation modules. The animals were placed in a soundproof booth and three electrodes were inserted subcutaneously, one at the vertex and the other two ventrolateral to each ear, beneath the pinna (active, reference and ground electrodes, respectively). The tone-burst auditory stimuli were delivered through a tube inserted into the ear canal of the rat and the measurements of ABR were taken at 4, 8, 12, 16 and 32 kHz to observe changes in hearing thresholds. Hearing thresholds were determined by assessment of the lowest stimulus level to elicit ABR peaks III at levels from 10 to 90 dB SPL in five-dB steps. 1024 tone presentations were averaged. The ABRs were measured before the noise exposure for control values to confirm that they were functional in the normal range, and also after the 1st, 3rd to 5th, 8th to 10th and 12th irradiations to keep up with the changes in the hearing thresholds.

2.4 *Laser Irradiation Treatment*

An 830-nm diode laser (Hi-Tech Optoelectronics, Beijing, China) was used to irradiate the ears. The following day after the noise exposure, the rats were irradiated at their left ears for 60 min at an energy density of 100 to 165 mW/cm^2 for 12 days in a row. The power of the laser was checked at the distal end of the optic fiber every day before each irradiation with a SOLO 2 laser power meter (Newport, Irvine, CA) and a XLP12-1S-H2-DO detector head (Newport). The optic fiber (core fiber 62.5 μm/cladding 125 μm) was delivered through a hollow tube into the external acoustic canal so that the distance from the tip of the fiber to the surface of the tympanic membrane was around 1 mm. Laser irradiation was done only in the left ear (NL ear) and the right ear (N ear) served as the control.

2.5 *SEM Study*

After 12 consecutive irradiations over 12 days, ABRs of the rats were recorded and the rats were sacrificed under general anesthesia. Intracardiac perfusions were done using 20 min of 0.1 M phosphate-buffered saline (PBS) and 20 min of 4% paraformaldehyde (PFA). The animals were then decapitated and the cochleae were harvested. The harvested cochleae were fixed in 2% glutaraldehyde overnight and then were rinsed with 0.1 M PBS. The samples were then postfixed with 1% osmium tetroxide for 3 to 5 min and were gently rinsed again with 0.1 M PBS. After being dehydrated in a series of graded ethanol concentrations, a 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 coated with platinum-palladium using an E-1030 PT-PD target assembly (Hitachi). The surfaces of the basilar membrane with hair cells were examined using an S-4300 scanning electron microscopy (Hitachi). The cochleae were quantitatively analyzed by counting the number of remaining hair cells. The cochleae were divided into apical (percent distance form apex, 0.0% to 33.3%), middle (33.3% to 66.6%) and basal (66.6% to 100.0%) turns and the hair cells were counted in each turn at ×600 magnification. According to the frequency place map of Viberg, the 12-kHz region is mapped to the middle turn[25] and we have looked at this region and adjacent regions to count hair cells of the middle turn. But in some samples in which the exact region of interest was damaged or lost due to the limitation of our technique, a nearby region of interest may have been included in the cell count. The number of hair cells over 200 μm of the basilar membrane was averaged for each group. A hair cell was considered as absent if the bundle of stereocilia was missing. A total of six ears for the control group and 16 ears for each of the N and NL groups were prepared for the SEM study. But due to delicate dissection procedures, some cochleae were partially damaged, making it difficult to evaluate the whole turn of the cochlea. The mean number of samples that were successfully evaluated under the SEM was 6.7 ± 4.7 for each turn of each group.

2.6 *Statistical Analysis*

All data were analyzed statistically by the paired-samples t-test and independent-samples t-test using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) software. Data were expressed as mean $\pm$ standard deviation and differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 *Threshold Shifts*

Hearing thresholds of the animals were 16.3 ± 5.1, 15.8 ± 4.9 and 17.2 ± 5.4 dB SPL at, respectively, 8, 12 and 16 kHz, before the noise exposure. 24 h after the noise exposure, the ABR thresholds were increased markedly to 58.3 ± 8.7, 55.0 ± 14.9, 71.3 ± 16.3, 60.6 ± 6.8 and 55.3 ± 11.2 dB SPL on the N ear, and 54.4 ± 8.8, 52.2 ± 11.5, 70.9 ± 6.4, 60.6 ± 6.3 and 52.5 ± 8.0 on the NL ear at, respectively, 4, 8, 12, 16 and 32 kHz. The hearing threshold was almost the same at this time point between N and NL ears. Signs of change were observed after three to five days of irradiation (Fig. 1). After the 8th to 10th irradiation, the thresholds of the NL ears had recovered to 40.6 ± 5.8, 36.3 ± 10.6, 48.4 ± 8.7, 43.9 ± 7.0 and 37.8 ± 6.3 dB SPL, respectively, at 4, 8, 12, 16 and 32 kHz. On the 8th to 10th day, thresholds of the N ears measured 54.4 ± 7.3, 49.7 ± 15.5, 62.5 ± 16.4, 55.6 ± 12.4 and 46.9 ± 13.1 dB SPL, respectively, at 4, 8, 12, 16 and 32 kHz. On 8th to 10th day, significant differences were found at all five frequencies [$4(p = 0.002)$, $8(p < 0.001)$, $12(p = 0.0092)$, $16(p = 0.023)$ and 32 khz ($p = 0.009$)]. After the 12th irradiation, the thresholds of the NL ears had recovered to 27.2 ± 4.4, 26.9 ± 7.3, 38.1 ± 14.6, 30.0 ± 9.3 and 29.7 ± 6.2 dB SPL, respectively, at 4, 8, 12, 16 and 32 kHz. On the 12th day, thresholds of the N ears measured 46.7 ± 16.2, 45.3 ± 17.3, 59.1 ± 18.5, 50.6 ± 12.6 and 45.6 ± 12.0 dB SPL, respectively, at 4, 8, 12, 16 and 32 kHz. The hearing threshold was significantly better for the NL ears when compared to the N ears at all five frequencies [$4(p = 0.007)$, $8(p < 0.001)$, $12(p = 0.002)$, $16(p = 0.001)$ and 32 khz ($p < 0.001$)].

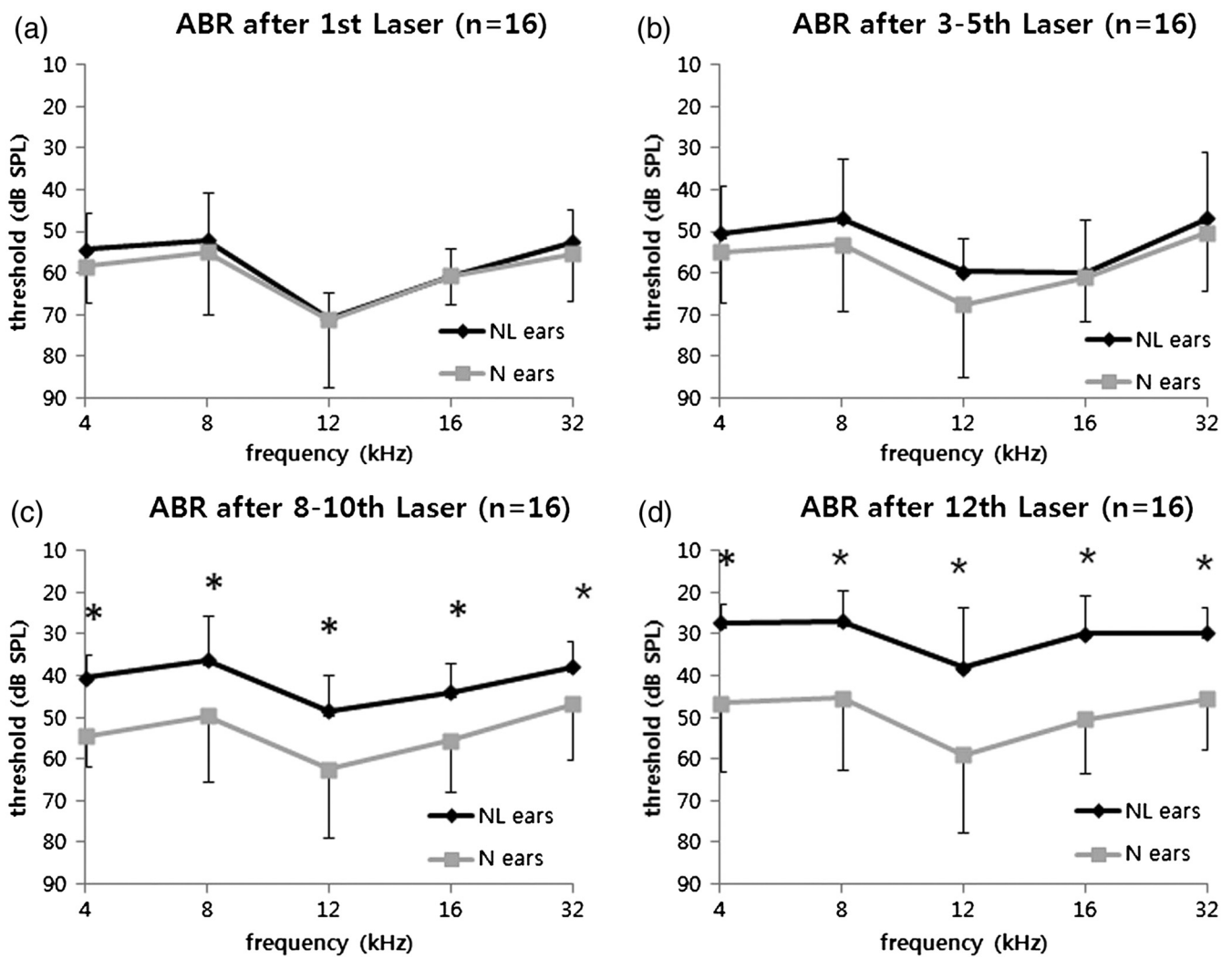

Fig. 1 Hearing threshold changes after repeated low-level laser treatment (LLLT). 24 h after noise exposure (after the first LLLT), the ABR thresholds were increased markedly to 50 to 80 dB SPL for both N (noise only) and NL (noise and laser) ears. The hearing threshold between N and NL ears were almost the same until this time point. Signs of change were observed after five days of irradiation. After the 8th to 10th irradiation, a significant difference was found for all frequencies. After the 12th irradiation, the hearing threshold was significantly better on the NL ears when compared to the N ears at all five frequencies. $*p < 0.05$.

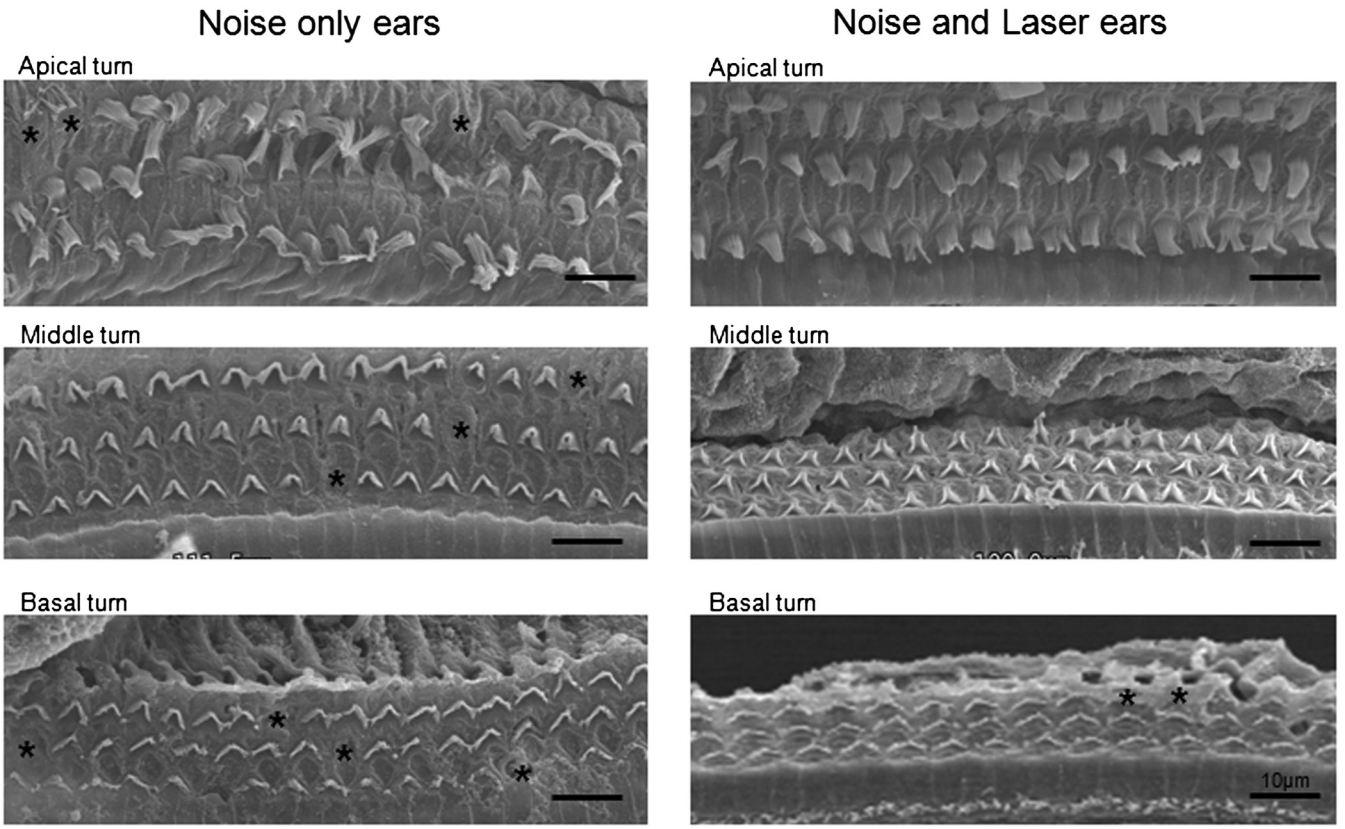

Fig. 2 Representative scanning-electron-microscopic images of the hair cells in the N (noise only) and NL (noise and laser) ears. Missing hair cells (steocilia) were observed throughout the whole cochlea. But more hair cells were missing in N ears when compared to NL ears.

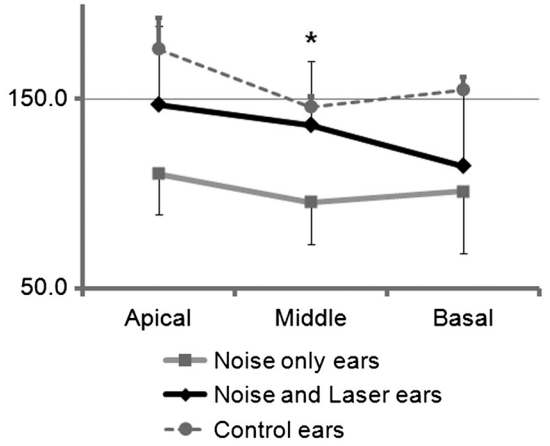

Fig. 3 Number of hair cells observed by scanning electron microscopy. The number of hair cells of the NL (noise and laser) ears was larger than that of the N (noise only) ears, and this difference was statistically significant in the middle-turn ($*p < 0.05$).The number of hair cells of the C (control) ears was significantly larger than that of the N ears in the apical, middle and the basal turn. The number of hair cells of the C ears was also significantly larger than that of the NL ears in the basal turn. But the number of hair cells of the C ear in the apical and middle-turn was similar to that of the NL ears.

3.2 *SEM Cell Count*

A representative image of the hair cells in the N and NL ears are presented in Fig. 2. The number of hair cells of the N ears was 110.3 ± 21.1, 95.2 ± 21.7 and 101.1 ± 32.4, respectively, for apical, mid, and basal turns. The cochleae of the NL ears displayed better results, having hair-cell counts of 147.2 ± 41.1, 136.2 ± 33.5 and 114.7 ± 38.7 for, respectively, apical, mid, and basal turns (Fig. 3). The number of hair cells of the NL ears was larger than that of the N ears, and this difference was statistically significant in the middle turn ($p = 0.032$). The number of hair cells was also larger in the apical ($p = 0.188$) and basal turn ($p = 0.439$) of the NL ears when compared to that of the N ears, but did not reach statistical significance.

In the cochleae of rats that received neither noise nor the laser (C ears), the hair-cell count was 176.5 ± 16.3, 146.0 ± 5.6 and 154.8 ± 6.7 cells at, respectively, the apical, mid, and basal turns. The number of hair cells of the C ears was significantly larger than that of the N ears in the apical ($p = 0.035$), middle ($p = 0.015$) and the basal turn ($p = 0.002$). The number of hair cells of the C ears was also significantly larger than that of the NL ears in the basal turn ($p = 0.010$). But the number of hair cells of the C ears in the apical ($p = 0.375$) and middle turn ($p = 0.627$) was similar to that of the NL ears.

4 Discussion

Through this study we found that LLLT may have a positive effect on hair-cell recovery after acute acoustic trauma. The hearing threshold became lower after repeated laser irradiation, and the final hearing result was significantly better than that of the untreated ears. We also found that the number of hair cells was greater in the NL ears when compared to that of the N ears. It is noticeable that both hearing threshold and cell counts improved markedly in the most intensely simulated region (middle-turn 12-kHz region). As in the neural cells of the central nervous system, these results suggest that LLLT helps

the recovery process of the cochlear cell after acute cell damage. This is the first *in vivo* study in the literature that has reported the hearing-recovery potential of LLLT in living animals. Considering that there is no definitive treatment for acute acoustic trauma in humans, we hope that LLLT evolves as a new treatment modality for noise-induced acute hearing loss.

According to the knowledge we have learned from neural cells, LLLT significantly enhances the energy metabolism of mitochondria. Enhanced ATP production from the mitochondria may help cells overcome noxious damage caused by acute acoustic trauma. The mechanism of acute acoustic trauma is not yet fully understood, but the noise trauma may be justified as abrupt damage done to inner-ear structures by disproportionate kinetic sound energy that overwhelms the physical resistance of inner-ear tissues.[26] The damage to the inner ear drives free radical production and reduces cochlear blood flow, causes excitotoxic neural swelling, and induces both necrotic and apoptotic cell death in the organ of Corti.[27,28] The hydroxyl (OH) radicals also initiate lipid peroxidation, which leads to oxidative lipid deterioration and damage to proteins embedded in cell membranes. It has been reported that the stria vascularis (lateral wall), outer hair cells, and inner hair cells of the cochlea are targeted heavily.[3] Innate antioxidative mechanisms may cope with daily unadventurous free radicals. But when the acute trauma is too big and overwhelms the innate antioxidative protection mechanism, cells may fall into a cascade of degeneration. It is well known that chronic hair-cell loss is not reversible after complete loss. But we may have a short time window between acute acoustic trauma and hair cell loss, when the antioxidative protection mechanism struggles with the overwhelming free radicals. In fact, the peak levels of ROS production have been found to occur in the hair cells 7 to 10 days after noise exposure, and treatments initiated within one to three days post noise were reported to attenuate later production of ROS.[29,30] It is presumed that the LLLT and enhanced ATP production pushes the balance between free radical and antioxidant to a state more favorable for cell recovery.

This hypothesis is quite similar to that of antioxidant medications. In previous studies, prevention of NIHL has been reported with a variety of antioxidants, glutamate antagonists and nitric-oxide synthase (NOS) inhibitors.[31] Pharmacological effects of NIHL prevention were found for chemical compounds such as *N*-acetylcysteine (NAC), *D*-methionine and ebselen, and also for natural products like the flavonoid baicalein, ginsenoside Rb1 and Korean red ginseng.[32–35] However, no definitive treatment modality has been established, especially for post-exposure treatments that will be more applicable in clinical therapies. As opposed to the preventative effects of pretreatments, previous reports on post-exposure treatments have yielded disappointing results.[36] The result of this study is interesting in that we improved the final hearing outcome by using LLLT one day after the acute acoustic trauma. One of the pharmacologic medications that has been studied most for post-exposure treatment of acute acoustic trauma is NAC.[37] The mechanism of NAC is also presumed to enhance the antioxidative mechanisms of the mitochondria. Although not confirmed, the mechanism of hair-cell recovery may be similar between the LLLT and NAC. But the mechanism of LLLT has never been studied in cochlea hair cells and the specific mechanism of cochlear-hair-cell recovery requires future verification.

LLLT seems to have a beneficial effect on hair-cell recovery, but we have also observed that the effect is not complete. That is, even after 12 days of LLLT, hearing did not reach levels present before acute acoustic trauma. Also, the hair-cell count of the NL ears did not reach that of the control ears. This incomplete effect of LLLT in hearing recovery has also been reported in our previous *in vitro* studies.[38,39] It seems that LLLT does help hair-cell recovery but does not cause complete recovery from hair-cell damage. The limited effects of LLLT may be enhanced by combining LLLT with other pharmacologic antioxidant or steroid medications. Combination treatment may have an additive or maybe a synergistic effect and should be studied to improve treatment results.

Although the results of this study are encouraging, there are some points requiring further discussion before this new treatment can be applied to humans. For instance the penetration rate of the laser into the cochlea needs further verification. The hair cells are covered by several layers of bone and soft tissue. According to our prior study, the transtympanic penetration rate of laser light into the cochlea was 5.5% in rodents and 2.8% in humans.[40] Since the power of the laser was 100 to 165 mW/cm^2 at the end of the optic fiber, the energy that reached the cochlea in our study is presumed to be around 5.5 to 9.1 mW/cm^2. In order to deliver the same amount of energy in the human cochlea, a much stronger laser will be needed. Theoretically, the power should be about 324 mW/cm^2. Also the scattering effect of tympanic membrane, middle-ear mucosa, and otic capsular bone may be quite different between rodents and humans. These factors should be considered when deciding if LLLT ought to be applied to humans.

Another issue is the possibility of local heating. The only presumable adverse effect of LLLT is heat production. Since the tympanic membrane is the structure closest to the optical fiber tip, it is the structure that may be most vulnerable to heat damage. During our pilot study, when the optical-fiber tip directly contacted the tympanic membrane, tympanic membrane perforation due to heat was observed in one animal. This problem was easily controlled by adopting an ear-plugging device that holds the optical fiber 1 mm away from the tympanic membrane. But if we use 324 mW/cm^2 of laser to irradiate the human ear, as has been calculated theoretically, this may result in excessive heating of the tympanic membrane and external auditory canal skin. Since the laser power settings have not been finely adjusted in this study, it is not clear if we would be able to apply lower intensity and longer duration therapy to accomplish the same results with less heat production. We are currently working on this issue and hope to find an answer in the near future. Also, there might be a low end beyond which extended duration does not ever accomplish the desired therapeutic endpoint. These issues require further clarification before moving on to human clinical trials.

5 Conclusion

The present study yielded positive results on restoring hearing levels in animals after a noise-induced hearing loss. It seems that low-level laser therapy promotes hair-cell recovery of hearing thresholds.

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