Research article

Low-level laser therapy for prevention of noise-induced hearing loss in rats

Atsushi Tamura a, Takeshi Matsunobu a,⁎, Kunio Mizutari a, Katsuki Niwa a, Takaomi Kurioka a, Satoko Kawauchi b, Shunichi Satoh b, Sadayuki Hiroi c, Yasushi Satoh d, Masashi Nibuya e, Risa Tamura f, Akihiro Shiotani a

a Department of Otolaryngology-Head and Neck Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan
b Division of Biomedical Information Sciences, National Defense Medical College Research Institute, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan
c Department of Pathology and Laboratory Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan
d Department of Anesthesiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan
e Department of Psychiatry, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan
f Department of Physiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-0042, Japan

HIGHLIGHTS

• LLLT was performed in rats after noise exposure.
• ABR measurement revealed that LLLT accelerated recovery of auditory function.
• Outer hair cell survival rates were significantly elevated in the LLLT group compared to the non-LLLT group.
• Immunoreactivities against iNOS and cleaved-caspase 3 were decreased in the LLLT group compared to the non-LLLT group.

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ABSTRACT

Noninvasive low-level laser therapy (LLLT) is neuroprotective, but the mechanism of this effect is not fully understood. In this study, the use of LLLT as a novel treatment for noise-induced hearing loss (NIHL) is investigated. Sprague–Dawley rats were exposed to intense noise and their right ears were irradiated with an 808 nm diode laser at an output power density of 110 or 165 mW/cm² for a 30 min period for 5 consecutive days. Measurement of the auditory brainstem response revealed an accelerated recovery of auditory function in the groups treated with LLLT compared with the non-treatment group at days 2, 4, 7 and 14 after noise exposure. Morphological observations also revealed a significantly higher outer hair cell survival rate in the LLLT groups. Immunohistochemical analyses for inducible nitric oxide synthase (iNOS) and cleaved caspase-3 were used to examine oxidative stress and apoptosis. Strong immunoreactivities were observed in the inner ear tissues of the non-treatment group, whereas these signals were decreased in the LLLT group at 165 mW/cm² power density. Our findings suggest that LLLT has cytoprotective effects against NIHL via the inhibition of iNOS expression and apoptosis.

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1. Introduction

Exposure to noise is known to initiate a cascade of events, leading to cochlear changes [1,2]. These include microcirculatory changes, such as hypoperfusion and ischemia. The oxidative stress that results from this produces reactive oxygen and nitrogen species (ROS and RNS) [3,4], which then alter the homeostasis in cells. This leads to conditions such as vascular insufficiency and then to apoptosis after caspase-3 activation [5,6].

Laser-induced phototherapy techniques, such as low-level laser therapy (LLLT) have been used as noninvasive treatments for promoting cell regeneration and repair [7,8]. This method is approved by the FDA for healing wounds and treating chronic pain and musculoskeletal complications [9,10]. The output power of the “low-level” laser varies from 10 to 1000 mW/cm² in the continuous mode and the wavelengths extend from the visible (λ = 400 nm) to the infrared (λ = 1000 nm). Near-infrared (near-IR) lasers can penetrate deep into the tissue and the efficacy of transcranial near-IR
laser irradiation for controlling cerebral ischemia has been reported [11].

Recently, several studies have shown that LLLT inhibits inducible nitric oxide synthase (iNOS) and suppresses neurotoxic ROS/RNS in cerebral ischemia and peripheral nerve injury [12,13]. Previous reports indicate that iNOS triggers ROS/RNS production in noise-induced hearing loss (NIHL) [5,6]. In this study, we examined the application of LLLT to prevent NIHL via inhibition of iNOS expression.

2. Materials and methods

2.1. Animals

A total of 34 male Sprague–Dawley rats (150–200 g) with normal tympanic membranes and normal Preyer’s reflexes were used in this study. The animals were randomly divided into three groups to evaluate their auditory function and hair cell count: one non-treatment (noise exposure only) \(n = 3\) and two LLLT groups (110 mW/cm\(^2\), \(n = 6\) and 165 mW/cm\(^2\), \(n = 5\)). The animals were also divided into three groups for immunohistochemistry of iNOS: naïve \(n = 3\), non-treatment (noise exposure only) \(n = 3\) and LLLT (165 mW/cm\(^2\), \(n = 3\)). and for immunohistochemistry of cleaved caspase-3: naïve (no noise exposure) \(n = 3\), non-treatment (noise exposure only) \(n = 3\) and LLLT (165 mW/cm\(^2\), \(n = 3\)). The protocol was reviewed by the National Defense Medical College’s Committee for Ethics in Animal Experiments (Notification No.13088). All experimental protocols were performed in accordance with their guidelines.

2.2. Noise exposure

Rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and medetomidine (1.0 mg/kg) for the entire duration of noise exposure. They were exposed to 1 octave band noise centered at 4 kHz for 5 h (121 dB sound pressure level) in a ventilated sound exposure chamber. The sound chamber was fitted with speakers (Model 2380 A, JBL, Northridge, CA) driven by a noise generator (DANAC-31, Danajapan, Tokyo, Japan) and a power amplifier (D-45, Crown International, Elkhart, IN, USA). Sound levels were calibrated (Type 6224 Precision Sound Level Meter, Aco Instruments, Tokyo, Japan) to ensure uniformity within 1 dB at multiple locations in the sound chamber.

2.3. Auditory brainstem response

Auditory brainstem responses (ABR) were measured using a signal recorder (Synax 1200, NEC, Tokyo, Japan) before, immediately after, and at 2, 4, 7, 14 and 28 days after noise exposure. All subsequent steps were performed under general anesthesia. Stainless steel needle electrodes were placed subcutaneously at the vertex and ventrolateral to the left and right ears. Tone burst stimuli, 0.2 ms rise/fall time and 1 ms flat segments at frequencies of 4, 8, 12, 16 and 20 kHz were generated. The amplitudes were specified by a sound generator and were attenuated by a real-time processor and programmable attenuator (RP2.1 and PA5; Tucker–Davis Technologies, Alachua, FL, USA). The sound stimuli were produced by a coupler-type speaker (ES1spc; Bio Research Center, Nagoya, Japan). ABR waveforms were recorded for 12.8 ms at a sampling rate of 40,000 Hz using 50–5000 Hz bandpass filter settings and waveforms from 256 stimuli at a frequency of 9 Hz were averaged. ABR waveforms were recorded in descending 5 dB increments from the maximum amplitude until no waveform could be visualized. The ABR threshold was defined as the lowest stimulus intensity that produced a wave III or IV. Thresholds obtained immediately before noise exposure were used as the baseline for estimating noise-induced threshold shifts.

2.4. Low-level laser therapy

LLLT was performed under general anesthesia and was initiated within 1 h of noise exposure. An 808 nm CW diode laser beam (B&W Tek Inc., Newark, DE, USA) transmitted through an optical fiber was applied to the right tympanic membrane through the external auditory canal. The optical fiber tip was positioned 6 mm away from the right tympanic membrane. The duration of laser irradiation was 30 min and LLLT was performed for 5 consecutive days on each animal. The laser power was checked using a photodiode-type laser power meter (PD300; Ophir Optronics Ltd., Jerusalem, Israel) before and after irradiation.

2.5. Quantitative assessment of outer hair cell loss

Animals were decapitated under deep anesthesia with pentobarbital (100 mg/kg) 28 days after noise exposure. The bone near the apex was removed and the round and oval windows of the inner ear were opened. This was followed by gentle local perfusion with 2 × 1 mL 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and the tissues were preserved overnight in the fixative. The cochleae were dissected by removing the lateral wall bones and tissues and tectorial membranes. After washing several times with PBS, the remaining parts of the cochleae were incubated in 0.3% Triton X-100 in PBS for 5 min and were washed 3 times with PBS. The organ of Corti was stained for F-actin quantitative assessment with 1% rhodamine-phalloidin (Invitrogen, Carlsbad, CA, USA) for 60 min to outline the hair cells and their stereocilia. After washing several times with PBS, the organ of Corti was dissected and mounted for surface preparation. The tissues were observed under an LSM 510 Meta confocal fluorescence microscope (Carl Zeiss Microlmaging GmbH, Jena, Germany). The cochlea were divided into apical (percentage distance form apex, 0.0% to 33.3%), middle (33.3% to 66.6%) and basal (66.6% to 100.0%) turns [14]. The number of missing outer hair cells (OHCs) was counted in each turn and the percentage of missing outer hair cells was recorded according to a previous report [15].

2.6. Immunohistochemistry for iNOS and cleaved caspase-3

Animals were examined for the presence of iNOS 1 h after noise exposure and for cleaved caspase-3 8 h after exposure in the presence or absence of LLLT. The LLLT group received a single LLLT session (165 mW/cm\(^2\)). After decapitation under deep pentobarbital anesthesia (100 mg/kg) the temporal bones were quickly removed and the bullae were opened. The samples were then subsequently transferred to 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The bone near the apex and the round and oval windows were opened. This was followed by gentle local perfusion with 2 × 1 mL 4% paraformaldehyde in 0.1 M PBS. The tissues were kept in the fixative overnight and decalcified in 0.1 M ethylenediaminetetraacetic acid (EDTA, pH 7.2) for 14 days at 4 °C. After incubation in 15% sucrose solution for 36 h, 8 μm frozen sections were prepared. Following 3 washes with PBS, the sections were incubated in 0.3% Triton X-100 in PBS for 15 min, washed 3 times and incubated in a blocking solution of 0.25% casein in PBS (Dako, Glostrup, Denmark) to block any non-specific reactions. Immunolabeling was carried out overnight at 4 °C with rabbit polyclonal iNOS antibody (1:1000; BML-SA200, Enzo Life Sciences, Farmingdale, NY, USA) or rabbit polyclonal cleaved caspase-3 antibody (1:100; #9661, Cell Signaling Technology, Danvers, MA, USA). The sections were rinsed in PBS and were subsequently incubated with a secondary antibody (1:200; Alexa Fluor 488 goat anti-rabbit, Invitrogen, Eugene,
Fig. 1. Auditory brainstem response threshold shift is attenuated by LLLT.
Low-level laser therapy (LLLT) attenuates the noise-induced threshold shift. In the 165 mW/cm² group, LLLT significantly attenuated the noise-induced threshold shift at 8, 16 and 20 kHz at day 14. The values represent the mean ± SD. (*p < 0.05)

OR, USA) for 1 h at room temperature. After washing with PBS 3 times, the sections were stained for F-actin with 1% rhodamine-phalloidin for 60 min. Following 3 washes with PBS, the sections were mounted on slides containing an anti-fade medium (VECTORSHIELD with DAPI; Vector laboratories, Burlingame, CA, USA). The images of the immunolabeled specimens were obtained with a confocal fluorescence microscope.

2.7. Data analysis

The ABR threshold shift and the ratios of missing OHCs in the groups were compared using a one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison tests. All data are presented as means ± SD. Differences with a p-value < 0.05 were considered significant.

Fig. 2. LLLT attenuates loss of OHCs 28 days after noise exposure.
Representative images of outer hair cells (OHCs) stained with rhodamine-phalloidin 28 days after noise exposure with or without LLLT. Numbers 1–3 indicate the rows of OHCs. Arrows indicate loss of OHCs, which was partially attenuated in the LLLT groups. Scale bar = 50 μm
3. Results

3.1. Auditory brainstem response threshold shift is attenuated by LLLT

Immediately after noise exposure (day 0), the threshold shifts were found in all three groups (Fig. 1). At day 2, the threshold shift was significantly lower in the LLLT groups. A one-way ANOVA confirmed this, indicating a significant difference in these and the controls at 8, 12, 16 and 20 kHz ($p < 0.05$ for each). The LLLT decreased the threshold shift at 12, 16 and 20 kHz (one-way ANOVA, $p < 0.05$ for each) at days 4 and 7 and at 8, 16 and 20 kHz (one-way ANOVA, $p < 0.05$ for each) at day 14. Statistically significant differences in threshold shift were not found at day 28, although there was a
continued trend in which the threshold shifts appeared to be somewhat smaller in the laser treated group at 12, 16 and 20 kHz (one-way ANOVA, \( p < 0.10 \) for each). These results indicate that LLLT treatment accelerates recovery of auditory function.

3.2. LLLT attenuates loss of OHCs 28 days after noise exposure

Missing OHCs were quantified in F-actin-labeled surface preparations (Fig. 2). The inner hair cells (IHCs) in the LLLT groups and controls were well preserved after sound exposure (data not shown). At 28 days after noise exposure, the average OHC losses in the basal turn of the non-treatment and LLLT groups (110 mW/cm\(^2\) and 165 mW/cm\(^2\)) were 20.0, 2.0 and 2.2%, respectively. The average OHC losses in the middle turn of the non-treatment and LLLT groups were 30.2, 2.8 and 2.2%, respectively. The average OHC losses in the apical turn of the non-treatment and LLLT groups were 32.7, 4.6 and 8.3%, respectively. The percentages of the average OHC losses in the basal, middle and apical turn in the both LLLT groups

![Image](image_url)

**Fig. 4.** LLLT attenuates apoptosis at 8 h after noise exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cleaved caspase-3 (green), F-actin (red), DAPI (blue).

In the organ of Corti, strong immunoreactivity for cleaved caspase-3 was detected in the OHCs (arrows) in the non-treatment group. In contrast, less immunoreactivity was observed in the LLLT group. Scale bar = 20 μm.

In the lateral wall, strong immunoreactivity for cleaved caspase-3 was detected in the fibrocytes of the spiral ligament (arrows) in the non-treatment group. In contrast, less immunoreactivity was observed in the LLLT group. Scale bar = 50 μm.
were significantly lower than the non-treatment group (one-way ANOVA, \( p < 0.01 \) for each). These results indicate that LLLT treatment attenuates loss of OHCs 28 days after noise exposure.

3.3. LLLT attenuates iNOS immunohistochemistry 1 h after noise exposure

The expression of iNOS after noise exposure was also evaluated. Based on the results from the auditory function analysis, a laser power density of only 165 mW/cm\(^2\) was used in the immunohistochemical analysis. At 1 h after noise exposure, strong immunoreactivity for iNOS was observed in the non-treatment group in the organ of Corti (Fig. 3A) and in the fibrocytes of the lateral wall (Fig. 3B), whereas less immunoreactivity was observed in the LLLT group (Fig. 3A and B). This indicates that LLLT treatment attenuated expression of iNOS 1 h after noise exposure.

3.4. LLLT attenuates apoptosis 8 h after noise exposure

At 8 h after noise exposure, strong immunoreactivities for cleaved caspase-3 were observed in the organ of Corti (Fig. 4A) and in the fibrocytes of the lateral wall (Fig. 4B), whereas less immunoreactivity was observed in the LLLT group (Fig. 4A and B). This result indicates that LLLT treatment attenuates apoptosis 8 h after noise exposure.

4. Discussion

Our results indicate that LLLT protects against NIHL via inhibition of iNOS expression. Our immunohistochemical studies also show that LLLT led to significant decreases of cleaved caspase-3 expression in the OHCs. This indicates that LLLT reduces oxidative stress and inhibits caspase-3-mediated apoptosis. Nitric oxide (NO), which is synthesized by nitric oxide synthase (NOS) plays an essential role in the physiological functioning of the inner ear. Recent evidence has suggested that excessive NO production causes hearing impairment [16,17] and iNOS catalyzes the production of large amounts of NO [18,19]. Consistent with our results, it was reported that LLLT inhibits expression of iNOS, which results in inhibition of neurotoxic ROS/RNS [12,13]. This study has shown that LLLT prevents cochlea damage if started within 1 h of noise exposure.

We observed immunoreactivities for iNOS and cleaved caspase-3 not only in OHCs but also in supporting cells of the organ of Corti and fibrocytes in the spiral ligament after noise exposure. We observed that LLLT inhibited these immunoreactivities dramatically. Thus, LLLT may protect these cells, which results in decreased OHCs loss. Supporting cells play a critical role in maintaining the structure of hair cells [16]. The spiral ligament plays a crucial role in maintaining electrochemical homeostasis in the cochlea [17].

In this study, the output power density of LLLT (110 mW/cm\(^2\) and 165 mW/cm\(^2\)) was used in a rat noise-exposure model. This was done according to the description by Rhee et al. [14]. The ABR measurement revealed that the threshold shifts of both LLLT groups were significantly smaller than the non-treatment group on days 4, 7 and 14. Although no significant differences were found between the non-treatment and LLLT groups on day 28, histological data showed that LLLT attenuated loss of OHCs. Thus, LLLT could accelerate recovery of auditory function after noise exposure.

Recent studies have also suggested that LLLT could be used to treat Meniere’s disease and chronic tinnitus [20,21] and no side effects were observed [14,20,21]. As with the previous report [14], we did not observe perforation of the tympanic membrane in our experiment. In conclusion, LLLT is a promising therapeutic approach for the treatment of noise-induced hearing loss.

Conflict of interest

The authors have no conflicts of interest to disclose.

References