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The Preventive Role of Low-Level Laser Therapy in Cochlear Outer Hair Cell Damage Due to Noise Exposure in Guinea Pigs

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Abstract

Objectives: By considering the necessity prevention of hearing loss secondary to noise exposure, this research investigated the protective role of low-level laser (LLL) therapy in the prevention of cochlear outer hair cell (OHC) damage and death due to noise exposure in guinea-pigs.

Materials and Methods: In this study, a total of 30 male albino guinea-pigs (290±10 g) were used and randomly divided into three groups in order to evaluate hair cell counts and apoptosis of cochlear hair cells, including noise, control, and LLL therapy group each containing 10 pigs. The right outer ear canal of the LLL therapy group was exposed to165 mW/cm^2 LLL for five successive days for just half an hour per day. Then, the LLL therapy and noise groups were exposed to 3-6 kHz octave band noise at 120 dBSPL. Twenty-one days after noise exposure, the animals of all groups were killed for the count of their OHCs and immunohistochemistry for caspase-3 experiments.

Results: The percentage of OHCs in the base and middle turns of the cochlea was significantly lower in the noise group compared to the control and low laser therapy groups ($P<0.05$). The expression of caspase-3 significantly differed in the noise group in comparison to control and low laser groups regarding the experiment of immunohistochemistry ($P<0.05$).

Conclusions: The findings indicated that LLL was useful in the protection of the cochlear OHCs and could help in the prevention of hair-cell apoptosis.

Keywords: Low-level laser, Caspase-3, Guinea-pigs, Apoptosis

Introduction

One of the main prevalent and hidden natural pollutants is the noise which can have adverse effects on the tissue and structure of the organs of animals and humans. Even exposure to any type of noise with a intensity level of 85 dB or more can be a source of the environmental stressor that leads to changes in nervous, cardiovascular, acoustic, and endocrine systems (1).

Exposure to noise starts a cascade of occurrences that lead to cochlear alternations in the hearing system (2,3). These consist of microcirculatory alterations such as ischemia and hypoperfusion. The oxidative stress originating from this process not only produces reactive nitrogen species (RNS) but also reactive oxygen species (ROS) and thus changing the homeostasis in cells (4,5). This oxidative stress makes damage to cells and their vital structures, and consequently, alters the balance between the molecular oxides and antioxidants in favor of oxidation (6-8). This results in circumstances such as vascular insufficiency and then cell death after the activation of caspase-3 (9,10).

Different strategies can be applied (e.g., antioxidant and anti-inflammatory strategies, apoptosis inhibitors, and the like) for the protection of cochlear hair cells when exposed to various damages such as drugs, noise, and the like (11).

On the other hand, low-level laser (LLL) is considered as an unaggressive strategy that has been increasingly used in medicine (12).

It has been applied for healing the nerves, preventing tissue damage, and promoting wound (13). In addition, LLL can change the biochemical reaction and cell aerobic respiration. This aerobic respiration can affect the electron transport chain in mitochondria and lead to an increase in oxygen and adenosine triphosphate (ATP) production (14,15).

Furthermore, LLL can halter the route of the nitric oxide signaling enzyme and the expression of caspase-3, leading to a decrease in ROS production and apoptosis (16,17).

The application of the ear protector has some limitations in the industrial environment and the activation of the endogenous protective system has been known as a remedy in the prevention of damage to different tissues and cells. Given that no study has so far evaluated the impact of LLL on cochlear hair cells, this research focused on the use of

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Key Messages

- The low-level laser has a protective and therapeutic effect on cochlear outer hair cell damage due to noise exposure in guinea pigs.

LLL as a persevering factor in cochlear tissues. In other words, this research investigated the protective role of laser irradiation on the prevention of cochlear outer hair cell (OHC) damage and death because of noise exposure in guinea-pigs. Thus, the damaged OHCs were counted and immunohistochemistry of caspase-3 was examined to investigate whether LLL has any effect on the prevention of cell damage and cell death, respectively.

Materials and Methods

Animals

In general, 30 male albino guinea-pigs (290 ± 10 g) with normal tympanic membranes were used in this study. These guinea-pigs were randomly assigned to LLL therapy (LLLT), noise, and control groups each consisting of 10 cases.

LLL Therapy

LLL irradiation was accomplished under general anesthesia. An 808 nm CW diode laser beam with an intensity level of 165 mw/cm^2 was transmitted by means of a handpiece probe to the tympanic membrane of the right ear via the outer ear canal to the LLLT group. Laser irradiation via LLL was lasted for thirty minutes for each animal and was carried out for five consecutive days. The power of the laser was controlled by applying a photodiode-kind of a laser power meter that was attached to the laser therapy device (LASMIK, Russia) before and after the irradiation (18).

Noise Exposure

The animals in both LLLT and noise groups were exposed to 1-octave band noise (3-6 kHz) centered at 4 kHz for 6 hours (120 dB sound pressure level) in an acoustic chamber. This chamber was fitted with a mono-speaker (Euroshine) that was hung from the ceiling driven by a power amplifier and a sound stimulus generator (Benaphone Electronic, Iran). Every animal in each group was exposed separately. Furthermore, the animals had easy access to food and water. In fact, both groups had the same situation with regard to lighting, temperature, humidity, and the like (19).

The Count of the Outer Hair Cell

Six guinea-pigs from both laser and noise groups were euthanized 21 days after noise exposure by means of intraperitoneal anesthesia with ketamine (40 mg/kg) and xylazine (10 mg/kg). Moreover, three guinea-pigs in the control group were decapitated, and then accessibility to the cochlea was possible by opening the bulla. The bone near the apex was removed and the oval and round windows of the inner ear were holed, and this process was continued via gentle local perfusion with 2×1 mL 4% paraformaldehyde in a one-tenth mole phosphate-buffered saline (PBS, pH = 7.4). Afterward, the samples were maintained in the fixative solution for one night. Then, the process of decalcification was done in 0.1 methylene diamine tetraacetic acid (EDTA, pH = 7.4) for 14 days at 4°C.

After carrying out decalcification, the following procedure was done successively. First, the samples were dehydrated by alcohol and then cleared by xylol. Next, these samples were embedded by paraffin, molded, and sliced by means of microtome with a thickness rate of 5 µm. Then, these slices were put on the lams that were soaked by albumin sticks. The paraffin was removed to color the tissues. Then, they became clear and hydrated, and the samples were stained by hematoxylin and eosin (H&E). The nucleus and cytoplasm became blue and pink by means of applying H&E in order to count the OHCs (20).

The cochlea was divided into three parts. The first part is the apical turn, the percentage distance of its turn to the apex is 0.0%-33.3%. The second part is called the middle turn and the percentage distance of its turn to the apex is 33.3%%-66.6%, and the third part is called the base turn and the percentage distance of its turn to the apex is 66.6%-100.0% (18). The damaged OHCs were counted in the middle and base turn and at last missing OHCs were expressed according to the percentage (21). The OHCs were identified by means of the nucleus as a marker of the presence of cells and were counted in the middle and base turn by superimposing a 100 × 100 µm box on the middle and base turn cochlea image and counting cells within each box (Figure 1) according to (22).

Immunohistochemistry for Caspase-3

The existence of caspase-3 was examined 21 days after noise exposure in all three groups although only the LLLT group received LLL. In fact, this group received LLL (165 mW/cm^2) for five consecutive days for just half an hour before exposure to noise. The temporal bones of guinea-pigs were quickly removed and their bullae were opened when they were decapitated under deep anesthesia by means of ketamine (40 mg/kg) and xylazine (10 mg/kg). These samples were put in the 4% paraformaldehyde in one-tenth mole PBS (pH = 7.4). The bone that was near the apex was holed, and the oval and round windows were opened. Then, this process was continued via gentle local perfusion with 2×1 mL 4% paraformaldehyde in 0.1 M PBS. These samples were maintained in the solution of fixative for one night. Next, the process of decalcification was done in 0.1 EDTA (pH = 7.4) for fourteen days at 4°C. Then, 5-µm sections were incubated in 15% solution of sucrose for 36 hours. These sections were incubated in

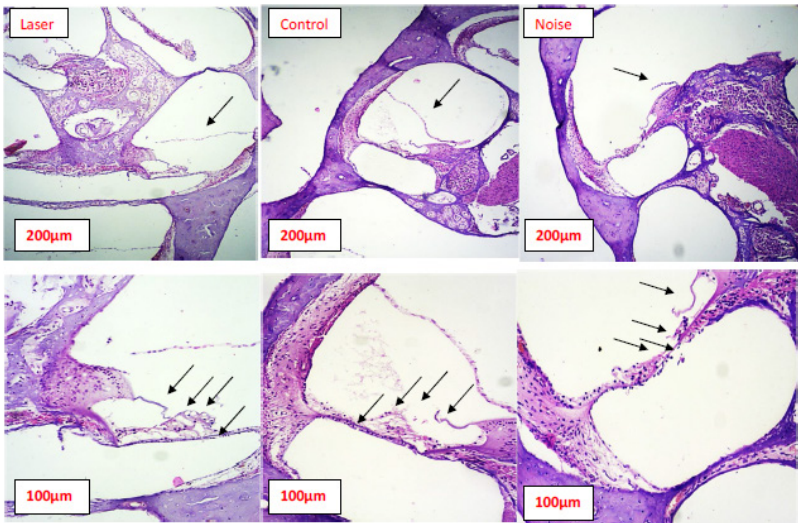

Figure 1. Histological Images of Cochlea Stained by the Hematoxylin and Eosin Method in Different Study Groups.

0.3% Triton X-100 in the PBS for 15 minutes after washing for three times with the PBS. Furthermore, they were washed 3 times and incubated in a blocking solution of 0.25% casein in the PBS (Dako, Glostrup, Denmark) to avoid any non-specific reactions. Immune labeling was done for one night at 4 °C with caspase-3 antibody (1:100). These samples were washed in the PBS and incubated by applying a secondary antibody (1:150) for 90 minutes. After washing for 3 times with the PBS, the samples were put on slides including DAPI as an anti-fade medium. Finally, the samples were observed by a fluorescent microscope by applying the lens of 400 for confirming the markers (23).

Statistical Analysis

The statistical analysis was accomplished using SPSS software. The one-way analysis of variance test (ANOVA) was applied for analyzing the differences among the groups, and $P<0.05$ was considered as a significant factor. In addition, Tukey's multiple comparison test was applied for multiple comparisons among the groups. All values are expressed as the mean and standard deviation (SD).

Results

Outer Hair Cell Count

The mean percentage of the OHC loss in the three groups were compared after 21 days of exposure to noise. The mean percentage (SDs) of hair cell loss in the middle and basal turn of cochlea in the control, laser, and noise groups were 8.33 (4.93), 12.67 (3.05), and 21.67(3.05), respectively (Figure 2).

Based on the results (Table 1), there was no significant difference between the number of the hair cell loss in the LLL and control groups ($P>0.05$). However, a significant difference was found in the hair cell loss between the noise group and the two other groups ($P<0.05$).

Immunohistochemistry for Caspase-3

The mean percentage of the expression of caspase-3 in the three groups was compared after 21 days of noise exposure. The mean percentage (SDs) of the expression of caspase-3 in the middle and basal turn of cochlea in the control, laser, and noise groups were 10.20 (1.93), 15.17 (4.48), and 42.60 (2.68), respectively (Figure 3).

After 21 days of noise exposure, strong immunoreactivities for the expression of caspase-3 were found in the Corti organ in the noise group. However, less immunoreactivity was observed in the LLLT and control groups. Therefore, the results (Table 2) demonstrated a significant difference between the noise group and the two other groups ($P<0.05$). Based on this finding, LLLT reduced apoptosis 21 days after exposure to noise (Figure 4).

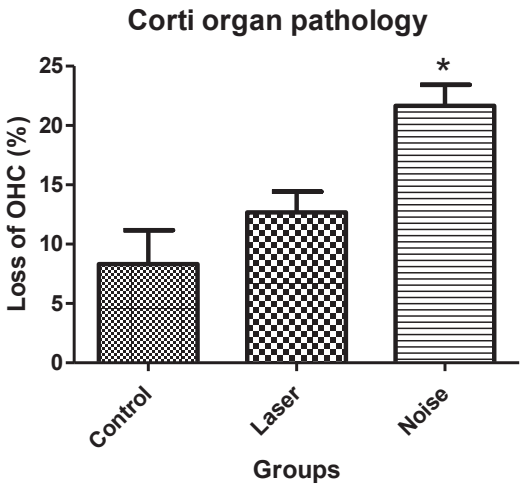

Figure 2. The Loss of OHC in the Three Groups. *Note*. OHC: Outer hair cell. SD: Standard deviation. Data are demonstrated as means ± SD. * The mean difference is significant at the 0.05 level.

Table 1. Tukey Post Hoc Comparison of Means Following ANOVA in All Groups

	Mean Difference	q	Significant $P < 0.05$	Summary	95% CI of Difference
Control vs. laser	- 4.333	1.982	No	NS	-11.89 to 3.23
Control vs. noise	- 13.33	6.100	Yes	Significant	-20.89 to -5.76
Laser vs. noise	- 9.00	4.117	Yes	Significant	-16.56 to -1.43

Note. ANOVA: Analysis of variance; CI: Confidence interval; NS: Not significant.

Discussion

The present study investigated the impact of the LLL on the prevention of cochlear cell damage and cell death after exposure to high noise by counting the OHCs and the expression of caspase-3.

The findings of this research indicated that applying the LLL led to a reduction in the OHC damage and even the expression of caspase-3 in cochlea decreased significantly in the LLL group in comparison to that expression in the noise group.

The Corti organ with a complex structure of different kinds of cells has been located on the basal membrane of the cochlea. The most important types of these cells are hair cells consisting of three or four rows of OHCs and one row of inner hair cells. Inner hair cells are responsible for the transduction of the vibration of the basal membrane to the neural spike. OHCs have a cell membrane that is responsible for reinforcing the vibrations of the basal membrane (24,25). After noise exposure, ROS/RNS formed in the hair cells. The OHCs in the base region of the cochlea are more vulnerable in comparison to those cells in the apex region. Moreover, inner hair cells are less exposed to the damages of oxidative stress compared to OHCs (26).

The LLL has recently been applied for the protection of damage in different tissues in medical sciences. By considering the radioprotective impacts of the LLL in another research, Esmaeeli Djavid et al studied the effect of the LLL (685 and 830 nanometers) on normal NIH3T3 cells and Hela cancer cells that were exposed to radiotherapy. The results demonstrated that applying the LLL (685 nanometers) before radiotherapy could halter the clonogenic growth of cancer cells. Furthermore, this LLL (830 nm) could protect normal cells from the damages of radiotherapy (27).

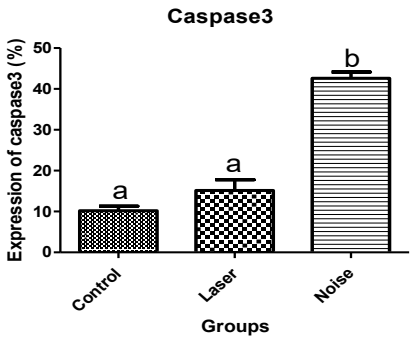

Figure 3. Caspase-3 Expression in the Three Groups. *Note*. SD: Standard deviation. Data are represented as means ± SD. (a) The mean difference is not significant at the 0.05 level. (b) The mean difference is significant at the 0.05 level.

Dos Reis et al evaluated the impact of the LLL on the function of leg muscles before and after exercise of 27 sport men. The LLL (830 nm) was used before and after the exercises and the results indicated that this therapy could reduce muscle fatigue both before and after doing these exercises (28). Likewise, Baroni et al studied the protective effect of the LLL on the muscles of 36 sportsmen after knee exercises. The LLL (810 nm) was used before exercises and it was found that this therapy was effective in reducing damages to muscles, provided that it was used before doing the exercises (29). In addition, Neto and Westphalen examined the effect of the LLL on the prevention and treatment of mouth mucositis in patients suffering from breast cancer. In this research, the LLL (660 nm) was used and the finding revealed that applying the LLL decreased inflammations in the stomach and mucositis in the mouth in patients after chemical therapy (30).

In another study, Cheng et al investigated the effect of the LLL on the avoidance of the apoptosis of endothelial cells using the endothelial cells of human beings and the LLL (660 nm). Based on their results, applying LLL led to a molecular mechanism that could protect endothelial cells after the inflammation (31).

Ghadimi et al examined the impact of the combination of LLL and using fluoride on the prevention of tooth decay. The results indicated that the simultaneous use of LLL and fluoride had a useful effect on the prevention of tooth decay (32).

As discussed in the previous above-mentioned studies, LLL was used in different tissues and before any intervention. The present study was similar to these

Table 1. Tukey Post Hoc Comparison of Means Following ANOVA in All Groups

	Mean Difference	q	Significant $P < 0.05$	Summary	95% CI of Difference
Control vs. laser	- 4.967	2.673	No	NS	-13.03 to 3.094
Control vs. noise	- 32.40	17.44	Yes	Significant	-40.46 to -24.34
Laser vs. noise	- 27.43	14.77	Yes	Significant	-35.49 to -19.37

Note. ANOVA: Analysis of variance; CI: Confidence interval; NS: Not significant.

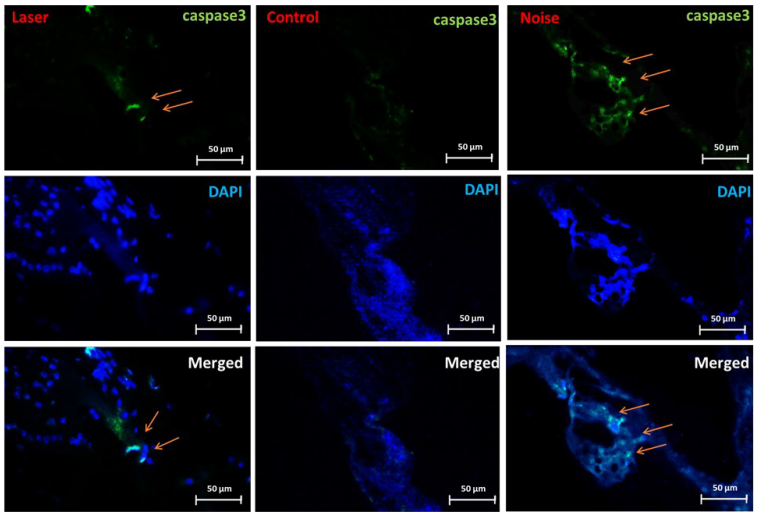

Figure 3. Caspase-3 Expression in the Three Groups. *Note*. SD: Standard deviation. Data are represented as means ± SD. (a) The mean difference is not significant at the 0.05 level. (b) The mean difference is significant at the 0.05 level.

studies in that LLL was used as a preventive factor and before noise exposure. Moreover, the results of this study are in line with those of previous studies, representing that the use of LLL had a positive effect on the prevention of the next damage. However, the only difference was because LLL was applied in cochlear tissues in this study. In other words, this research examined the preventive and preserving role of LLL in cochlear OHCs. In fact, cell damage and cell death in the cochlear OHCs can be decreased when applying LLL. As the results of the counting of outer hair cell and expression of caspase-3 indicated that using LLL was effective.

The possible mechanisms for this effect of the LLL can be discussed as follows.

Cellular signaling pathways have been regulated by means of the cellular redox state in which they control gene expression. In fact, signaling pathways can be activated and inhibited by the modulation of the cellular redox state.

The cellular and molecular mechanisms of LLL propose that photons have been absorbed by the mitochondria. The low levels of ROS and more ATP production have been stimulated by these photons, and then transcription factors have been activated (e.g., NF-kB) that induce various gene transcript products which are responsible for the useful impacts of LLLT.

By considering the results and observations, it seems that by the activation of transcription factors by means of LLLT, protective proteins have been up-regulated, which are anti-apoptotic and lead to cell survival.

Another possibility is that the use of LLLT leads to the relief of the blockade of cytochrome c oxidase by any inhibitory molecules and thus it can also reduce the probability of apoptosis in many circumstances.

In the future, the findings of this research will be used for preparing workmen when they are exposed to noise in industries. In addition, more research is required in this regard, particularly in applying different dozes of LLL for achieving more effective parameters on tissues, the study of the combination of LLL and antioxidant and its function on cochlear tissues, and the evaluation of the impacts of LLL on human beings. The lack of suitable instruments for the delivery of the light of the laser to the tympanic membrane (the optical fiber), the expensive cost, and the lack of an antibody for doing histological and immunohistochemistry experiments were some of the limitations of this research.

Conclusions

In conclusion, the use of LLL led to a reduction in oxidative stress. Moreover, it inhibited the expression of caspase-3 which decreased the sings of apoptosis and cell damage. Finally, the protective impact of the LLL on the tissue of cochlea was observed in addition to the avoidance of apoptosis.

Authors' Contribution

AZ, GM, MA, and SJ had basic contributions to theoretical search and research design. AZ and GM played a significant role in preparing tools for the research, data collection, and drafting. MA and SJ notably participated in data analysis and drafting. All authors agreed to be accountable for all aspects of the work regarding ensuring that questions related to the accuracy or integrity of any part of the work are investigated and resolved appropriately.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The project was in accordance with the ethical principles and national norms and standards for conducting animal research in Iran (IR.TUMS.FNM.REC.1397.049). All experimental protocols were in line with this approved guideline.

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