ORIGINAL ARTICLE

Effect of low-level laser therapy on cochlear hair cell recovery after gentamicin-induced ototoxicity

Chung-Ku Rhee · Peijie He · Jae Yun Jung · Jin-Chul Ahn · Phil-Sang Chung · Myung-Whan Suh

Abstract Cochlear hair cells are the sensory receptors of the auditory system. It is well established that antibiotic drugs such as gentamicin can damage hair cells and cause hearing loss. Rescuing hair cells after ototoxic injury is an important issue in hearing recovery. Although many studies have indicated a positive effect of low-level laser therapy (LLLT) on neural cell survival, there has been no study on the effects of LLLT on cochlear hair cells. Therefore, the aim of this study was to elucidate the effects of LLLT on hair cell survival following gentamicin exposure in organotypic cultures of the cochlea of rats. The cochlea cultures were then divided into a control group ($n=8$), a laser-only group ($n=8$), a gentamicin-only group ($n=8$) and a gentamicin plus laser group ($n=7$). The control cultures were allowed to grow continuously for 11 days. The laser-only cultures were irradiated with a laser with a wavelength of 810 nm at 8 mW/cm^2 for 60 min per day (0.48 J/cm^2) for 6 days. The gentamicin groups were exposed to 1 mM gentamicin for 48 h and allowed to recover (gentamicin-only group) or allowed to recover with daily irradiation (gentamicin plus laser group). The hair cells in all groups were stained with FM1-43 and counted every 3 days. The number of hair cells was significantly larger in the gentamicin plus laser group than in the gentamicin-only group. The number of hair cells was larger in the laser-only group than in the control group, but the difference did not reach statistical significance. These results suggest that LLLT may promote hair cell survival following gentamicin damage in the cochlea. This is the first study in the literature that has demonstrated the beneficial effect of LLLT on the recovery of cochlear hair cells.

Keywords Low-level laser therapy · Cochlea explants · Hair cell · Regeneration · Hearing loss

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Introduction

Low-level laser therapy (LLLT) has been used in various fields of medicine, including wound healing, musculoskeletal pain, and dental disease. In fact, LLLT has been approved by the US Food and Drug Administration (FDA) for the treatment of several diseases including carpal tunnel syndrome [1–3] and alopecia [4]. Carpal tunnel syndrome is a peripheral neural disorder caused by compression of the median nerve traveling through the carpel tunnel by the wrist ligaments. It has been reported that neural function significantly improves after LLLT [5]. Although such applications have not yet been approved by the FDA, the effects of LLLT on the central nervous system are also being studied by many researchers [6–11].

Several hypotheses have been proposed for the mechanism of action of LLLT. The prevailing opinion is that the respiratory chain plays a central role in the effect induced by laser therapy. As with chlorophyll in plants responding to light and activating photosynthesis, there are three major proteins in the

human body that respond to the infrared wavelengths of light [12]. 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 [13]. It has been reported that LLLT irradiation increases the production of ATP [14]. Increased ATP production may lead to enhanced cell metabolism, promoting the damage recovery process.

Cochlear hair cells are the sensory receptors of the auditory system. It is well established that antibiotic drugs such as gentamicin can cause hair cell damage and hearing loss. Rescuing hair cells after ototoxic injury is an important issue in hearing recovery. Currently there is no study in the literature that has studied the effect of LLLT on the recovery of cochlear hair cells. However, the effect of LLLT on neural cell recovery has been reported by several authors [6, 7, 11]. In one of these studies, neurologic performance rapidly recovered in LLLT-treated rabbits after brain infarction, but not in control rabbits [8]. It has also been reported that a significantly larger number of proliferating cells and migrating neuroblasts can be found in the rat brain after LLLT [6]. According to these and many other studies, it seems that LLLT has a positive effect on neuronal cell recovery after cell damage [6–9, 11–13]. Considering that cochlear hair cells also originate from the neural ectoderm, we thought that the recovery effect may also apply to the cochlear hair cells. Accordingly, in this study we damaged cultured cochlea hair cells ex vivo with gentamicin and investigated the effect of LLLT on their recovery.

Materials and methods

Cochlear organotypic culture

The procedures in this study were reviewed and approved by the Animal Use Committee of the College of Medicine, Dankook University. All experimental procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

The organ culture procedures were similar to procedures described previously [15]. Sprague-Dawley rats were decapitated on postnatal day 5 and the cochleas were carefully excised. The lateral wall and cochlear nerve were excised leaving the basilar membrane. Only the middle turn of the cochlea was used for further analysis. A 50-μl drop of type I rat-tail collagen (Becton Dickinson, Franklin Lakes, NJ) was placed on a 35-mm glass-bottom culture dish (BioCoat, Becton Dickinson) and allowed to gel for approximately 1 h. Rat-tail collagen was mixed with 0.02 N acetic acid in a 1:100 ratio prior to use. Dulbecco's modified Eagle's

medium supplied with 1× N1 medium supplement (Sigma-Aldrich, St. Louis, MO), 10% FBS, and 100 U/ml penicillin was added to the culture dish. The cochlea was positioned on the collagen-coated dish as a flat surface preparation with the hair facing upward. The cochlea explants were placed in an incubator (37°C, atmosphere containing 5% CO_2) for 48 h (days 1–3). After 2 days culture in the full medium, the cochlea explants were usually well attached to the culture dish. The cochleas were then divided into a control group (*n*= 8 from five rats), laser-only group (*n*=8 from five rats), gentamicin-only group (*n*=8 from four rats) and gentamicin plus laser group (*n*=7 from five rats). In the gentamicin-only and gentamicin plus laser groups, the medium was replaced on the third day of culture with new medium containing 1 mM gentamicin (Sigma-Aldrich, St. Louis, MO) and incubated for another 48 h (days 3–5). The concentration and duration of gentamicin exposure in this study was based on the previously published procedures [16, 17]. After 48 h exposure to gentamicin, the gentamicin was washed out and the samples in the laser-only and gentamicin plus laser groups were irradiated with LLLT daily for 6 days and were evaluated every 3 days. Control samples, cultured in normal medium, were also evaluated every 3 days concurrently with the actively treated samples.

Laser irradiation

Starting on day 5, the gentamicin plus laser and laser-only samples were exposed to an 810-nm diode laser (LAS-30A, TNL, Incheon, Korea) for 60 min daily for 6 days. The optical fiber was placed perpendicularly 50 cm away from the stage. Using a laser power meter, the contour of equal laser power was drawn, and three to five dishes containing cochlea cultures were placed within the contour so that the energy delivered to the cochleas would be equal. The laser was set at an energy output of 8 mW/cm^2 (60 min, 0.48 J/cm^2) and the irradiation was performed under sterile conditions on a clean bench (see Fig. 1).

Confocal microscopy

The stain FM1-43 (Molecular Probes, Eugene, OR), a specific dye for identifying live sensory cells [18], was used to stain the live hair cells in the cochlea cultures. Briefly, the cochlea explants were immersed in culture medium containing 2 μg/ml FM1-43 at 37°C for 5 min. The cochlea explants were then viewed by confocal microscopy using LSM-510-META apparatus (Carl Zeiss, Oberkochen, Germany) with an excitation wavelength of 514 nm from an argon laser, and a 560-nm long-pass barrier filter. After each observation the dishes were washed out and recultured for the next observation. There was no residual dye in the medium at the time of LLLT, since we washed out the dye and cultured the sample in fresh
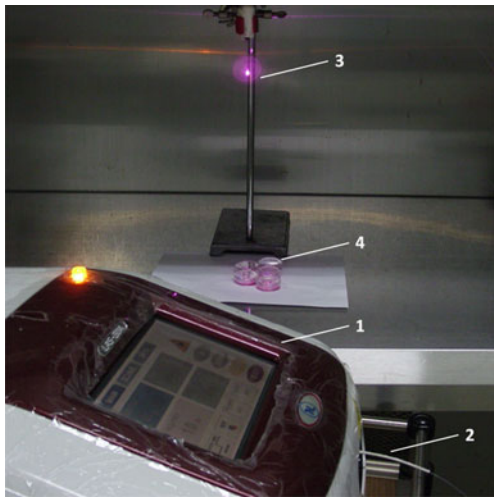
Fig. 1 Laser irradiation of the cochlea cultures. The cochlea explants were irradiated with a diode laser at 810 nm on a sterilized clean bench. The optical fiber was placed perpendicularly 50 cm away from the stage. Using a laser power meter, the contour of equal laser power was drawn. Dishes containing cochlea cultures were placed within the contour, so that the energy delivered to the cochleas would be equal (*1* laser device, *2* proximal end of the optical fiber, *3* distal end of the optical fiber; *4* cochlea cultures attached to dishes)

medium after each confocal observation. FM1-43 is a dye that can stain hair cells, similar to myosin VIIa or phalloidine, but an important difference is that cells can survive even after being stained with this vital dye. This property allowed us to observe the live cells in the cultures under the confocal microscope and reculture them every 3 days.

Hair cell counting

FM1-43 labeled the cytoplasm of the hair cells allowing easy identification of hair cells that were present versus those that were missing. To quantify hair cell loss in the cochlea explants after the various treatments, hair cells in the ×200 confocal images were counted over a 200-μm longitudinal distance from two separate regions in the middle turn of each cochlea. A mean value was determined for each experimental condition. For relative objectivity, the preprocessing of the original confocal images involved two steps done using the Scion Image for Windows program (NIH, Frederick, MD) before counting the cells. At first, the density was calibrated to make the empty background absolutely black (0) and the hair cells absolutely white (255). The small disparity in gain and brightness between different images was successfully calibrated by this process. Second, density slicing was done to allow only signals with a density value higher than 110–160 to be considered visible. Signals weaker than this value were considered as noise. Also, only the visible signals larger than 6 μm in the shortest diameter were considered hair cell signals. Non-hair-cell signals from the limbus and cochlear nerve fibers were excluded manually. In this second step, hair cell signals which qualified for counting were easily identified objectively and accurately.

Statistical analysis

The hair cells were counted by a single investigator. Data were analyzed statistically using the Statistical Package for the Social Sciences software (SPSS, IBM, Somers, NY). In order to compare the serial changes in hair cell numbers, repeated measures analysis of variance (RM ANOVA) was used. Differences were considered statistically significant when $p < 0.05$.

Results

Figure 2 shows the serial follow-up of the cochlea cultures following gentamicin treatment and/or LLLT and staining with FM1-43. The hair cells gradually grew out in the efferent direction during the 11 days of culture, breaking from the regular rows of cells. Although the hair cells migrated and the signals became slightly noisy after 11 days of culture, FM1-43-stained hair cells were identified with no problem. Over the 11-day period, more live hair cells were seen in the laser-only group and the gentamicin plus laser group than in the control group and gentamicin-only group, respectively. High-power views of the hair cells (Figs. 3 and 4) confirmed that the number of hair cells increased after daily laser irradiation. It was also noticeable that while a large number of hair cells were identified in the laser-only group (Fig. 3) before laser treatment, 48 h after incubation with gentamicin, the number of FM1-43-labeled hair cells had dramatically decreased in gentamicin plus laser group (Fig. 4) before laser treatment. Numerous hexagonal signal losses were observed after gentamicin treatment, which were assumed to represent the cell wall of the lost hair cells (Fig. 4).

Figure 5 shows the longitudinal changes in the number of hair cells over 11 days. The mean numbers of hair cells in the control group were 67.5±18.6, 59.9±31.6, and 54.0±25.9 (per 200 μm) on days 5, 8, and 11, respectively. The mean numbers of hair cells in the laser-only group were 70.9±28.4, 77.1±32.0, and 80.0±29.2 (per 200 μm) on days 5, 8, and 11, respectively. Although the number of hair cells seemed to be larger in the laser-only group than in the control group, the RM ANOVA analysis revealed no significant difference ($p = 0.234$). The group × time interaction was also not significant ($p = 0.204$), i.e. the decrease in the number of hair cells in the control group (shown by the steeper gradient) was not significantly different from that in the laser group (Fig. 5a).

The mean numbers of hair cells in the gentamicin-only group were 27.3±13.2, 23.8±5.6, and 25.0±9.1 (per 200 μm)

Fig. 2 Serial follow-up of the cochlea cultures. The upper series (a) are original images and the lower series (b) are images after two preprocessing steps using the Scion Image for Windows program. The cochleas were evaluated by confocal microscope every 3 days for 6 days. Over the 11-day period, more live hair cells can be seen in the laser-only group (*L group*) and the gentamicin plus laser group (*GL group*) than in the control group (*C group*) and gentamicin-only group (*G group*), respectively
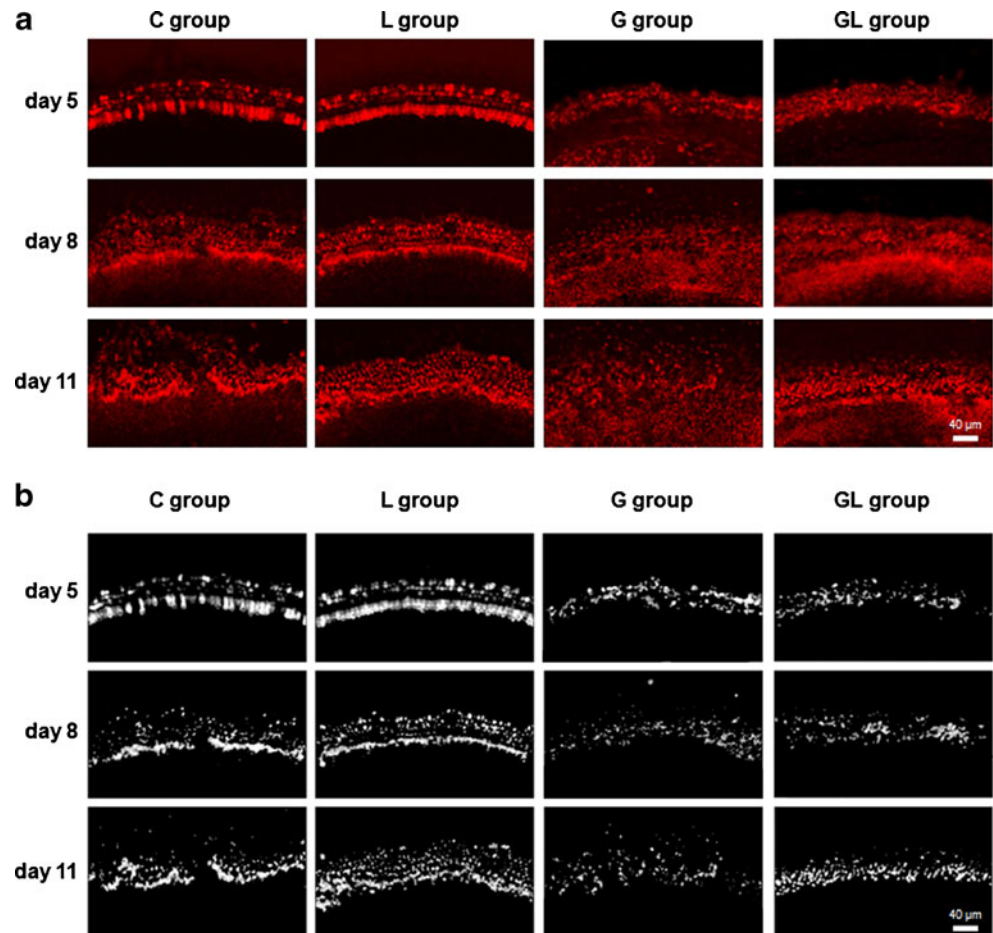
on days 5, 8, and 11, respectively. The mean numbers of hair cells in the gentamicin plus laser group were 37.3±12.9, 36.0± 23.7, and 58.6±27.8 (per 200 μm) on days 5, 8, and 11, respectively. The number of hair cells was significantly larger in the gentamicin plus laser group than in the gentamicin-only group (p=0.0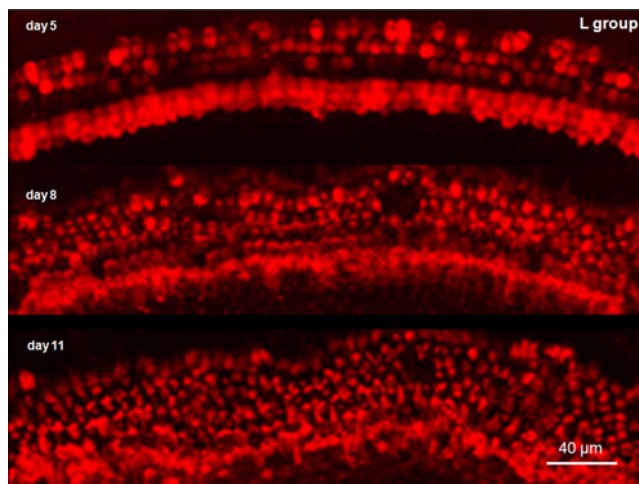28, RM ANOVA) indicating a significant effect of LLLT. The group × time interaction was also significant (p< 0.001), showing that the increase in the number of hair cells in the gentamicin plus laser group was significantly different from that in the gentamicin-only group (Fig. 5b).

Fig. 3 High-power views of the hair cells in the laser group. The number of hair cells is increased after daily laser irradiation
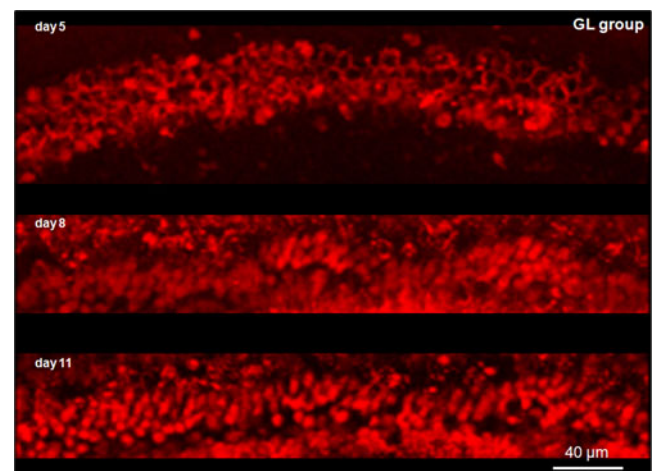

Fig. 4 High-power views of the hair cells in the gentamicin plus laser group. Immediately after gentamicin damage (day 5) only a small number of hair cells can be seen. The hexagonal signal losses are assumed to represent the cell wall of the lost hair cells. By 3 and 9 days of laser irradiation, the number of hair cells has increased significantly
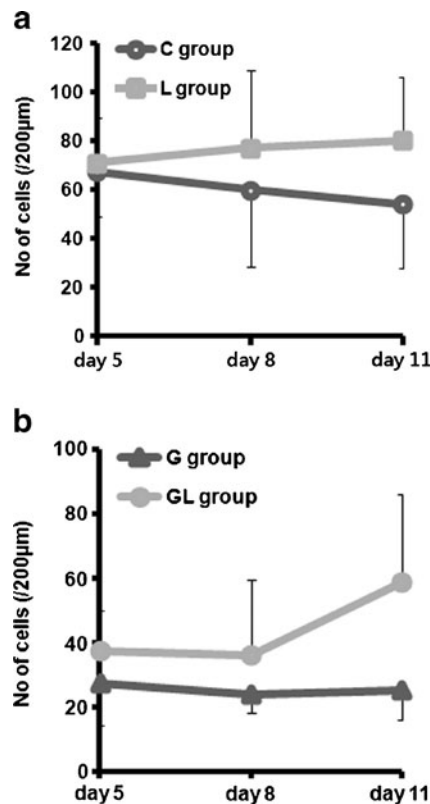
Fig. 5 Longitudinal changes in the number of hair cells over 17 days. FM1-43-labeled hair cells were counted from the micrographs. **a** The number of hair cells was larger in the laser-only group (*L group*) than in the control group (*C group*). **b** The number of hair cells was significantly larger in the gentamicin plus laser group (*GL group*), and the number of hair cells in the gentamicin plus laser group showed an increasing tendency which was significantly different from that in the gentamicin-only group (*G group*)

Discussion

In this study, we found that the number of hair cells was significantly larger in the gentamicin plus laser group than that in the gentamicin-only group after LLLT. When strictly focusing on the gentamicin-treated groups, it seems LLLT had a beneficial effect on the recovery of cochlear hair cells after acute hair cell loss. Live hair cells in the cultured samples were observed not only in the same sample, but also approximately the same location of the sample. Hence, the increases in the number of hair cells occurred in exactly the same area of each sample. The results may be more convincing based on this factor. To our knowledge, this is the first study that has demonstrated the beneficial effect of LLLT on the recovery of cochlear hair cells. The reason why we did not find a significant effect of LLLT in the laser-only group is not clear, but it seems that the regenerative effect of LLLT is maximal only when there has been significant damage to the hair cells. The therapeutic effect of laser

irradiation may have been smaller because the hair cells were already healthy in the laser-only group.

The laser parameters wavelength and power used in this study were carefully chosen based on previous reports, as well as the results of our own pilot study. The wavelength is especially critical in changing the biology of cells. It has been known that cytochrome *c* oxidase is most responsive to wavelengths shorter than 600 nm [12]. However, this wavelength is within the range of visible light and is very poor at penetrating tissues such as the tympanic membrane and otic capsule. When designing this study, we wanted to be sure that the laser parameters could be used in future in vivo animal studies, as well as in human clinical trials. The wavelength range 600–1,200 nm is the so-called "optical window" of tissue [13], which means that the penetration rate is maximal in this range. The wavelength that has the highest biomodulation potency in the "optical window" is reported to be around 800–830 nm [12]. Accordingly, when both the tissue penetration rate and the biomodulation potency are considered, it appears that 810 nm is the optimal wavelength for cochlear hair cell treatment.

The second important parameter seems to be the power of the laser. While the wavelength of the laser may be considered as the medicine, the power of irradiation may be considered as the dose of the medicine [13]. As in medication overdose, an excessive amount of laser irradiation is known to prohibit rather than promote cell recovery [19]. Based on other publications [20] and our preliminary trials, we determined that 8 mW/cm^2 was within the therapeutic range. Since laser treatment may have different biomodulatory properties based on the wavelength and power setting used, the beneficial effect of LLLT found in this study may have been critically dependent on the specific wavelength and power setting used.

Although the results of this study are encouraging, there are several points requiring further discussion. First, the recovery potential of cochlear hair cells may have been augmented due to the timing of organ explantation. The results of this study were based on ex vivo cultures of postnatal day 5 rat cochleas. While the cochlear hair cells of humans are fully differentiated and mature at the time of birth, the cochlea is still premature and developing during this period in rodents [21]. Premature cochlea cells may be more multipotent, thus favoring regeneration and/or recovery. Accordingly, the effect of LLLT on fully differentiated and mature hair cells may be quite different from that seen in this study. However, it is very difficult to culture adult rat cochleas and the majority of studies involving ex vivo cochlea cultures in the literature used the same technique as used here. Second, the exact mechanism of LLLT needs to be studied more thoroughly. Our understanding of the mechanism involved in the action of LLLT on cell recovery is still quite shallow. Moreover, most of the current knowledge is based on neural

cells of the central nervous system. The mechanism of bio-modulation in cochlea hair cells has never been studied and needs investigation.

Conclusion

The results of this study suggest that the LLLT irradiation of cochlear hair cells may have a beneficial effect on their recovery after gentamicin ototoxicity.

Conflicts of Interest   None.

References

1. FDA (2009) Application K081166. Food and Drug Administration, Rockville, MD
2. FDA (2002) Application K020657. Food and Drug Administration, Rockville, MD
3. FDA (2003) Application K030226. Food and Drug Administration, Rockville, MD
4. FDA (2010) Application K091496. Food and Drug Administration. Silver Spring, MD
5. Dincer U, Cakar E, Kiralp MZ, Kilac H, Dursun H (2009) The effectiveness of conservative treatments of carpal tunnel syndrome: splinting, ultrasound, and low-level laser therapies. Photomed Laser Surg 27(1):119–125. doi:10.1089/pho.2008.2211
6. Oron A, Oron U, Chen J, Eilam A, Zhang C, Sadeh M, Lampl Y, Streeter J, DeTaboada L, Chopp M (2006) Low-level laser therapy applied transcranially to rats after induction of stroke significantly reduces long-term neurological deficits. Stroke 37(10):2620–2624. doi:10.1161/01.STR.0000242775.14642.b8
7. Detaboada L, Ilic S, Leichliter-Martha S, Oron U, Oron A, Streeter J (2006) Transcranial application of low-energy laser irradiation improves neurological deficits in rats following acute stroke. Lasers Surg Med 38(1):70–73. doi:10.1002/lsm.20256
8. Lapchak PA, Salgado KF, Chao CH, Zivin JA (2007) Transcranial near-infrared light therapy improves motor function following embolic strokes in rabbits: an extended therapeutic window study using continuous and pulse frequency delivery modes. Neuroscience 148(4):907–914. doi:10.1016/j.neuroscience.2007.07.002
9. Naeser MA, Saltmarche A, Krengel MH, Hamblin MR, Knight JA (2011) Improved cognitive function after transcranial, light-emitting diode treatments in chronic, traumatic brain injury: two case reports. Photomed Laser Surg 29(5)351–358. doi:10.1089/pho.2010.2814
10. McCarthy TJ, De Taboada L, Hildebrandt PK, Ziemer EL, Richieri SP, Streeter J (2010) Long-term safety of single and multiple infrared transcranial laser treatments in Sprague-Dawley rats. Photomed Laser Surg 28(5):663–667. doi:10.1089/pho.2009.2581
11. Zivin JA, Albers GW, Bornstein N, Chippendale T, Dahlof B, Devlin T, Fisher M, Hacke W, Holt W, Ilic S, Kasner S, Lew R, Nash M, Perez J, Rymer M, Schellinger P, Schneider D, Schwab S, Veltkamp R, Walker M, Streeter J (2009) Effectiveness and safety of transcranial laser therapy for acute ischemic stroke. Stroke 40 (4):1359–1364. doi:10.1161/STROKEAHA.109.547547
12. Wong-Riley MT, Liang HL, Eells JT, Chance B, Henry MM, Buchmann E, Kane M, Whelan HT (2005) Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: role of cytochrome c oxidase. J Biol Chem 280(6):4761–4771. doi:10.1074/jbc.M409650200
13. Huang YY, Chen AC, Carroll JD, Hamblin MR (2009) Biphasic dose response in low level light therapy. Dose Response 7(4):358–383. doi:10.2203/dose-response.09-027.Hamblin
14. Oron U, Ilic S, De Taboada L, Streeter J (2007) Ga-As (808 nm) laser irradiation enhances ATP production in human neuronal cells in culture. Photomed Laser Surg 25(3):180–182. doi:10.1089/pho.2007.2064
15. Zheng JL, Gao WQ (1996) Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. Eur J Neurosci 8 (9):1897–1905
16. Kim JB, Jung JY, Ahn JC, Rhee CK, Oh YH (2009) Preventive and therapeutic effects of low level laser irradiation on gentamicin-induced vestibulotoxicity in rat utricles. Korean J Otolaryngol Head Neck Surg 52(1):19–28
17. Chung YW, Ahn JC, Lim ES, Kim YS, Lee SH, Lee MY, Rhee CK (2007) A promotive effect of low-level laser on hair cell regeneration following gentamicin induced ototoxicity in postnatal organotypic culture of rat utricles. Korean J Otolaryngol-Head Neck Surg 50 (1):25–30
18. Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, Corwin JT, Corey DP (2003) Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 23(10):4054–4065
19. Sommer AP, Pinheiro AL, Mester AR, Franke RP, Whelan HT (2001) Biostimulatory windows in low-intensity laser activation: lasers, scanners, and NASA's light-emitting diode array system. J Clin Laser Med Surg 19(1):29–33. doi:10.1089/104454701750066910
20. Mohammed IF, Al-Mustawfi N, Kaka LN (2007) Promotion of regenerative processes in injured peripheral nerve induced by low-level laser therapy. Photomed Laser Surg 25(2):107–111. doi:10.1089/pho.2006.1090
21. Freeman S, Geal-Dor M, Sohmer H (1999) Development of inner ear (cochlear and vestibular) function in the fetus-neonate. J Basic Clin Physiol Pharmacol 10(3):173–189