

ORIGINAL ARTICLE

## Effect of Low-Level Laser and FM1-43 on Prevention of Ototoxicity in Postnatal Organotypic Cultures of Rat Utricles

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**Objectives:** Low-level laser (LLL) has the capability to alter cellular behavior in the absence of significant heating. The styryl pyridinium dye, FM1-43, is a non-toxic, fluorescent, cationic dye, the fluorescence of which markedly increases after partitioning into membranes. In this study, the effects of LLL and FM1-43 on gentamicin-induced ototoxicity in postnatal organotypic cultures of rat utricles were investigated.

**Materials and Methods:** Organotypic cultures of 4-day-old rat utricular maculae were established. In a series of experiments, utricles were exposed to either irradiation of a LLL (LG group), 10  $\mu$ M FM1-43 (FG group), or both (LFG group), followed by 1 mM of gentamicin treatment for 12 hrs. The results of experimental groups were compared with the control group by confocal laser scanning and scanning electron microscopy.

**Results:** LLL prevented vestibular hair cell ototoxicity. Rapid incubation with FM1-43 dye protected vestibular hair cell damage induced by gentamicin treatment. A substantial additive effect of LLL on the prevention of ototoxicity was noted in combination therapy with FM1-43.

**Conclusion:** The results suggest that there is an additive protective effect of LLL and FM1-43 against gentamicin ototoxicity in postnatal organotypic cultures of rat utricles. LLL may have clinical preventive and therapeutic implications with respect to ototoxicity.

Submitted : 28 May 2011

Accepted : 18 August 2011

### Introduction

The human inner ear is mature at birth with fully differentiated and functioning hair cells that normally do not regenerate if lost due to trauma or ototoxic drugs. Losing inner ear hair cells is one of the most common causes of sensorineural hearing loss and peripheral vestibular disorders. No treatment exists that can restore the sensory epithelia of the human inner ear, despite our current knowledge of the inner ear. From this point of view, the prevention of hair cell loss is an important issue. Until now, many preventative medications have been studied<sup>[1]</sup>, but the effect of laser on the prevention of hair cell loss has not been thoroughly investigated.

Low-level laser (LLL) therapy has a wavelength-dependent capability to alter cellular behavior in the absence of significant heating. LLL has been used

therapeutically for treating wound healing, musculoskeletal pain, dental disease and many more.<sup>[2]</sup> It is especially noticeable that the U.S. Food and Drug Administration have approved the use of LLL for the treatment of carpal tunnel syndrome which is a peripheral neuropathy of the median nerve.<sup>[3]</sup> Nowadays, the effect of LLL on central nervous system including the brain<sup>[3-5]</sup> and visual neurons<sup>[6]</sup> are under investigation by many researchers. However, even now the mechanisms which lead to these effects are not well-established. Mitochondrial respiratory chain components exhibit frequency-dependent action spectra, and many feel that the respiratory chain is central to any effects induced by laser therapy.<sup>[7]</sup> Several studies have revealed that the primary absorption site of LLL irradiation within the neuron is likely the mitochondria. To be more accurate, the cytochrome c oxidase of the mitochondrial membrane

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is presumed to be the primary molecule that absorbs the 600-800 nm wavelength laser energy.<sup>[8]</sup> Cytochrome c oxidase is also known as the complex IV protein which works as a member of the ATP producing cascade.<sup>[9]</sup> Accordingly, when laser light is absorbed, the mitochondria produce ATP and increase the cell metabolism. It is presumed that the increased cell metabolism contribute to the survival of neurons under ischemic stress conditions.<sup>[10]</sup>

Gentamicin is one of the aminoglycosides that causes ototoxicity, but it is still widely used clinically because of its effectiveness and low cost. Gentamicin is known to cause vestibular and cochlear ototoxicity via destruction or damage to the structure and function of the labyrinthine hair cells. The resulting damage from gentamicin can vary from very minimal, to complete loss of vestibular function.<sup>[11]</sup> Various molecules have been used to prevent aminoglycoside-induced ototoxicity such as deferoxamine and MK-801.<sup>[12]</sup> However, there have been no reports on the protective effect of LLL on ototoxicity. Therefore, the aim of this study was to investigate the protective effects of LLL and FM1-43 on gentamicin-induced ototoxicity in postnatal organotypic cultures of rat utricles.

## **Materials and Methods**

### *Animals and experimental groups*

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (7<sup>th</sup> edition, 1996), as formulated by the Institute of Laboratory Animal Resources of the Commission on Life Sciences. Forty, 4-day-old Sprague-Dawley rats were used for this study. Utricles that had been damaged during dissection were discarded and only 70 explants that had been explanted successfully were used in this study. The explants were cultured for 24 hours and then divided into 5 groups as follows: C group (n=10), explants were cultured in media for 36 hours; G group (n=15, 10 for the confocal laser scanning microscope (CLSM), and 5 for double staining with phalloidin-FITC and caspase-3), after 24 hours of culture of the explants, gentamicin (1 mM; Sigma, St. Louis, USA) was added to the culture media for 12 hours; LG group (n=15, 10 for the CLSM, and 5 for double staining), after 24 hours of culture of the explants, the explants were housed in an aluminum-covered box for irradiation with a 632 nm diode laser (LD) at energy densities 16.2 J/cm<sup>2</sup> (30 mW/cm<sup>2</sup>, 90 minutes) for a single time, and then the media was gentamicin-treated as in the G group; FG

group (n=15, 10 for the CLSM, 5 for double staining), after 24 hours of culture of the explants, the explants were treated with 10 μM of FM1-43 for 20 min and then gentamicin-treated as in the G group; and LFG group (n=15, 10 for the CLSM, and 5 for double staining), after 24 hours of culture of the explants, the explants were irradiated using a LD at 632 nm (16.2 J/cm<sup>2</sup>) in an aluminum-covered box, then the media was FM1-43-treated as in the FG group and gentamicin-treated as in the G group.

### *Organ culture*

Our procedure for preparing organotypic cultures was done in a manner similar to those described previously.<sup>[13]</sup> The temporal bone from the rat was removed and the utricular macula was dissected free and the otoconial membrane was gently removed. The dissection was performed in 0.1M Dulbecco's phosphate buffered saline (DPBS; GIBCO, Grand Island, USA) and observed at room temperature under a stereomicroscope (LG-PS2; Olympus, Tokyo, Japan). The explants were placed with their epithelial surfaces facing upward, and anchored to a gelatin and 10% fetal bovine serum (FBS; GIBCO) coated glass bottom dish. The explants were incubated in Dulbecco's modified essential medium (DMEM; GIBCO) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### *Immunohistochemical staining*

To identify live hair cells, FM 1-43 was used to treat each group. FM 1-43 was added directly to culture media for 10 min (final concentration, 10 μM) at the end of the culture period.<sup>[14]</sup> The activity of caspase-3 was observed in each group by double staining the utricles with phalloidin-FITC (Sigma, St. Louis, USA) and a caspase-3 kit (Biovision, Mountain View, USA) to evaluate the degree of apoptosis. The caspase-3 substrate in the kit was used to treat the culture media directly for 1 hour. The utricles were then washed three times in 0.1M DPBS and fixed for 4 hours at 4°C in 4% paraformaldehyde in 0.1M DPBS. After fixation, the utricles were washed three times in DPBS and permeabilized by incubation in 0.1% Triton X-100 for 10 minutes. The utricles were then immersed for 30 min in phalloidin-FITC at a dilution of 1:200.

### *Hair cell counting and statistics*

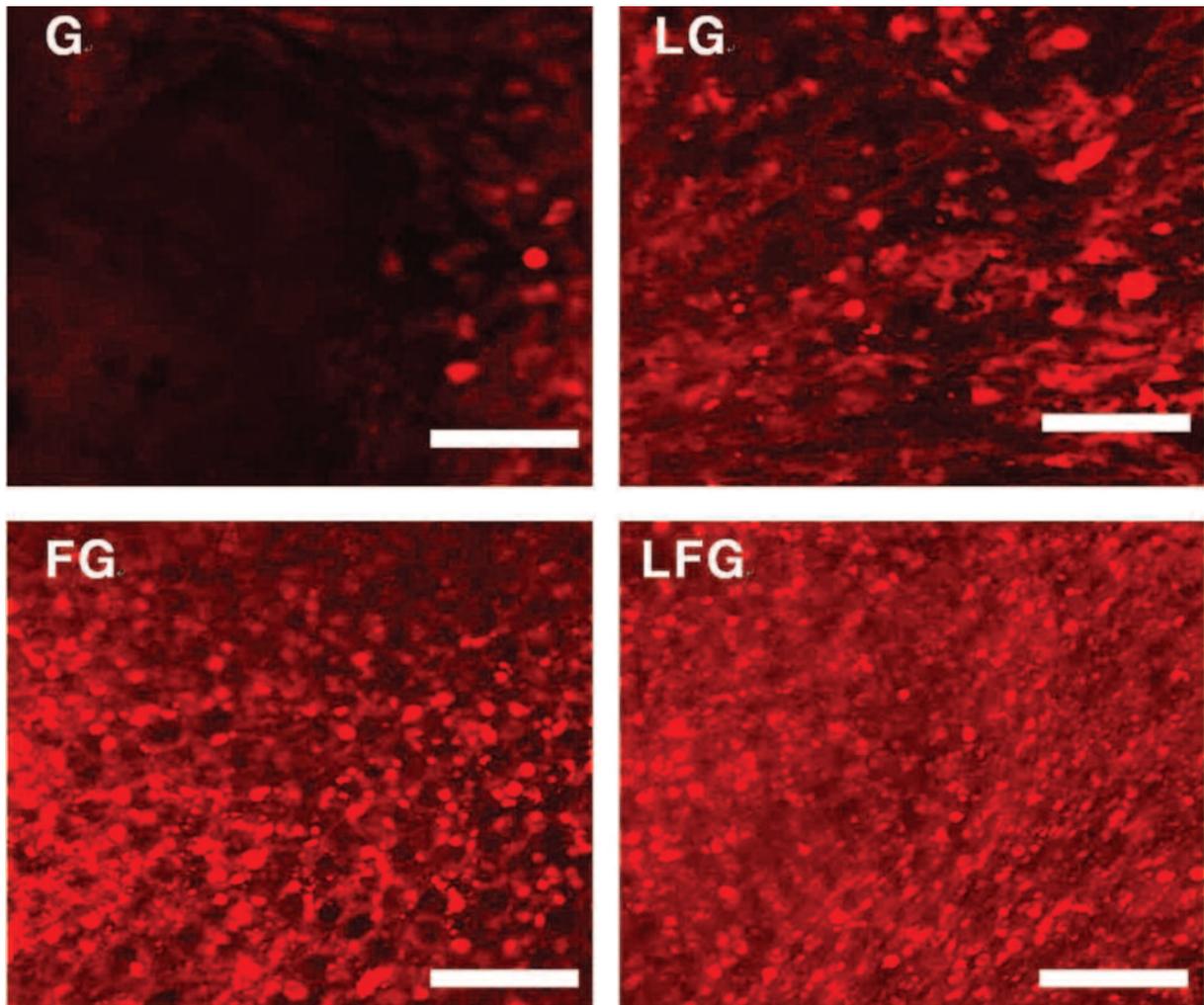
Hair cells were counted under the CLSM (LSM 510 META; Zeiss, Jena, Germany) using x400 magnification (30,000 μm<sup>2</sup>) at the part between the striola region and the peripheral region, where the hair

cells were most dense. Statistical analyses were performed using the Mann-Whitney U test and statistical significance was determined at 95 % ( $p < 0.05$ ).

### **Results**

The hair cells of utricular explants in the DMEM culture media were well-maintained through 24 hours and

further experimental procedures in different groups were carried out under constant conditions. In the G group, hair cell loss was nearly complete after 12 hours (Fig. 1). In the G group, only a few live hair cells were shown when stained with FM1-43 (indicating live hair cells). And relatively more live cells were detected in the LG group. A much larger number of live hair cells were demonstrated in the FG and LFG groups (Fig. 1).



**Figure 1.** In the G group, only a few live hair cells were shown when stained with FM1-43 (indicating live hair cells). And relatively more live cells were detected in the LG group. A much larger number of live hair cells were demonstrated in the FG and LFG groups.

The number of live hair cells which were stained with FM1-43 was  $137.6 \pm 6.5$  in the C group,  $19.9 \pm 3.6$  in the G group,  $36.1 \pm 9.1$  in the LG group,  $117.6 \pm 11.6$  in the FG group, and  $141.3 \pm 12.6$  in the LFG group (Fig. 2). There were significant statistical differences among all five groups ( $p < 0.05$ ); however, the difference in hair cell counts between the C and LFG groups was not significant, suggesting that combined treatment with

FM1-43 and LLL irradiation prevented gentamicin-induced ototoxicity very effectively (Fig. 2). As shown in Fig. 3, the activities of caspase-3 (red fluorescence) were not apparent in the LFG group; however, the number of cells (green fluorescence) with caspase-3 stained fluorescence gradually increased in the FG, LG, and G groups.

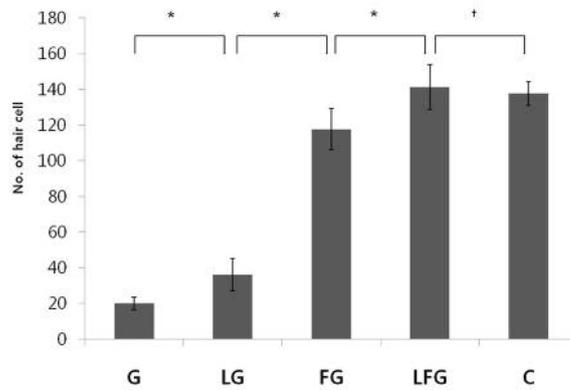


Figure 2. The number of live hair cells which were stained with FM1-43.

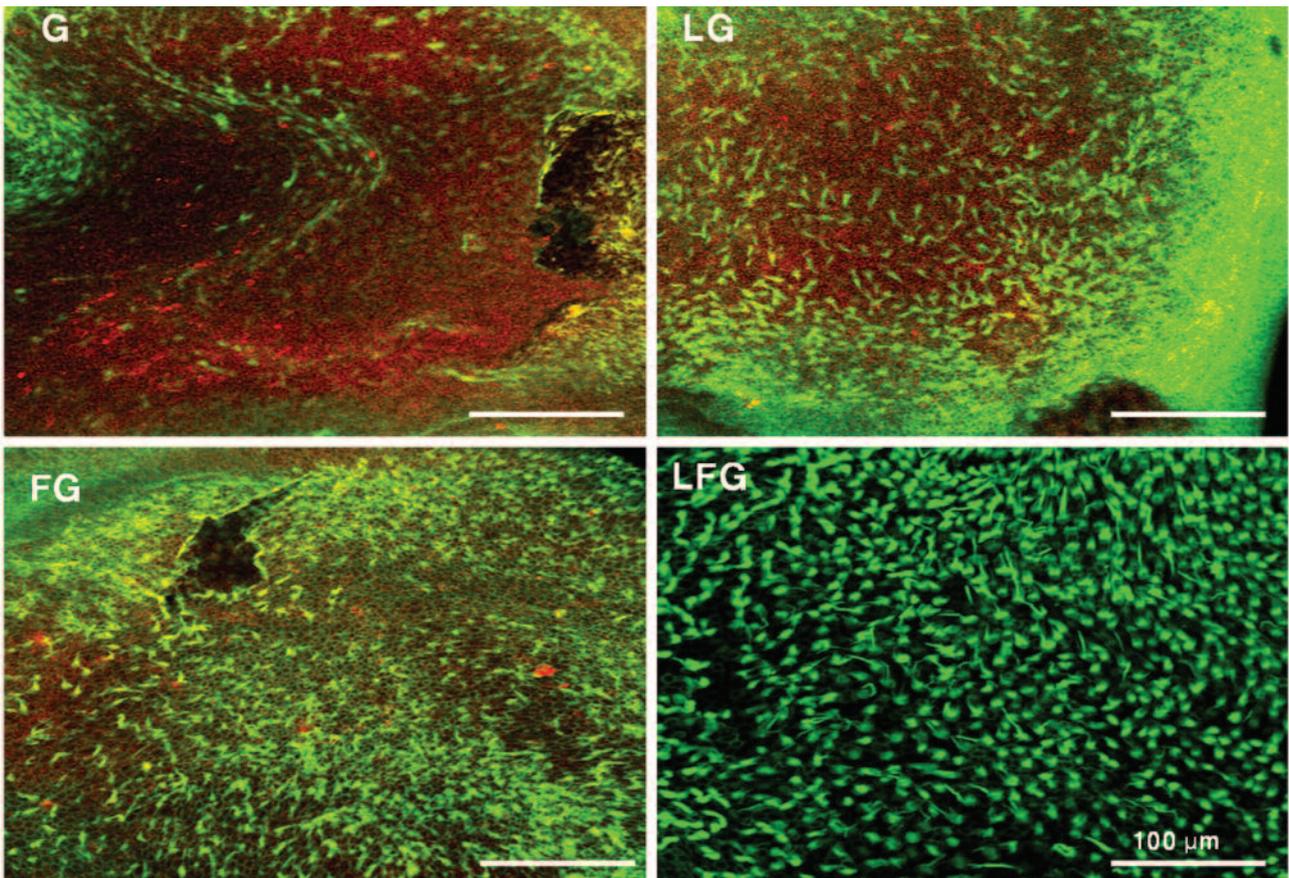


Figure 3. The activities of caspase-3 (red fluorescence) were not apparent in the LFG group; however, the number of cells (green fluorescence) with caspase-3 stained fluorescence gradually increased in the FG, LG, and G groups.

## **Discussion**

The preventive effect of gentamicin ototoxicity by LLL irradiation at 632 nm (16.3 J/cm<sup>2</sup>) to the utricular explants was considerable when compared to the control group. Although this preventive effect was not perfect, this was the first attempt to prevent ototoxicity using LLL. The preventive effect was less compared to the therapeutic effect of LLL, which was observed in our previous study with daily LLL irradiation for 30 days to the gentamicin-damaged utricular explants.<sup>[15]</sup> The results of these two studies may indicate that protection of utricular hair cells from gentamicin-induced ototoxicity can be obtained when LLL is used before and after the administration of aminoglycosides. Further study seems necessary to evaluate the exact synergistic or additive effect of the LLL on the utricular hair cells protection.

The biological effects of LLL are thought to depend largely on well-controlled laser parameters, such as wavelength, waveform, power, dosage per site, duration of irradiation, type of irradiated cells, and time interval between injury and irradiation.<sup>[16-17]</sup> A LD laser at 632 nm was used in this study since it has been reported to be most effective in altering the regeneration rate of rat facial nerves.<sup>[18]</sup> However, more optimal wavelength, fluence, and other parameters should be studied to ascertain the most optimal conditions that can prevent and treat ototoxicity of the inner ear. For example, a higher dose of LLL or near infrared wavelength LLL may have yielded better preventive effects. Our next plan is to find such optimal laser parameters.

The styryl pyridinium dye, FM1-43[N-(3-triethylammonium-propyl)-4-(4-dibutylamono)-styryl] pyridinium dibromide] (FM1-43; Molecular Probes, Eugene, USA), has been widely used to study the synaptic vesicle recycling. It is a non-toxic, fluorescent, cationic dye, the fluorescence of which markedly increases after partitioning into the membrane. FM1-43 quickly fills and stains the cytoplasm of hair cells, but labels only the plasma membrane of adjacent supporting cells. Rapid entry of FM1-43 is inhibited by drugs that block the mechanically-gated transduction channels,<sup>[14]</sup>

suggesting that the dye can itself act as a blocker of the channels. The dye enters the apical pole of sensory hair cells in the mammalian cochlea via the mechano-electrical transducer channel. From the result of FG and LFG group, it seems that ototoxic aminoglycoside antibiotics share the same entry pathway. Although the interaction between FM1-43 and aminoglycoside may be a critical limitation in inner ear research, this dye has a big advantage in that it is non-toxic. The tissue can be stained and cultured repetitively. This allows us to serially observe the change of hair cell population and morphology in the same tissue without sacrificing it. In this study we used the dye mainly to observe hair cells, not to prevent ototoxicity. But against our expectation, the prevention of ototoxicity by FM1-43 was more effective than LLL. Through this study, we would like to point out that the preventative effect of FM1-43 should be seriously considered when using aminoglycosides together with FM1-43.

The main course of aminoglycoside ototoxicity is thought to be the intrinsic pathway of caspase. This pathway activates the chain of stress-activated protein kinases, such as c-JunN-terminal kinase (JNK). JNK damages the membrane of mitochondria by increasing the intracellular Ca<sup>2+</sup> concentration and cytochrome C release. The signal from the mitochondria induces activation of caspase-9 and impairs cells by activating the downstream caspases, such as caspase-3.<sup>[19-21]</sup> In the current study, we have also demonstrated that this gentamicin-induced ototoxicity and LLL induced protection is closely related to the caspase-3 cascade. The full mechanism of LLL in hair cell protection is still not clear. But from the result of this study and former publications it seems that the increased energy metabolism of the mitochondria prevents the cells from entering into the fatal pathway of apoptosis by under regulating the caspase-3 mediated intrinsic pathway.

## **Acknowledgement**

This study was funded by the Medical Research Center Funding (R00-00000-000-00000-0).

## **Conflict of interest**

The authors have no conflict of interest to declare.

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