低出力 Nd:YAG レーザー照射がヒト歯肉線維芽細胞に与える影響

― 創傷治癒に関与する因子の検討 ―

大久保　美佐

Effect of low-level Nd:YAG laser irradiation on human gingival fibroblast:

Study on factors involved in wound healing

Misa OHKUBO

日本歯科大学大学院生命歯学研究科歯科臨床系専攻

（指導：沼部　幸博教授）

研究指導：村樫　悦子講師

（日本歯科大学生命歯学部歯周病学講座）

The Nippon Dental University, Graduate School of Life Dentistry at Tokyo

(Director: Prof. Yukihiro NUMABE)

Conducted by Assistant Prof. Etsuko MURAKASHI

The Nippon Dental University, School of Life Dentistry at Tokyo

Department of Periodontology

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著者名：Misa OHKUBO, Etsuko MURAKASHI, Hitomi ISHIGURO, Hiroko TAKEUCHI-IGARASHI, Yukihiro NUMABE

この論文を大久保美佐の博士（歯学）学位論文として提出することを
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村樫　悦子　㊞

石黒　一美　㊞

五十嵐（武内）寛子　㊞

沼部　幸博　㊞

Introduction

Since Maiman invented the dental laser in 1960 [1], a variety of dental lasers have been applied in dental treatments, including the neodymium-doped yttrium aluminum garnet (Nd:YAG) laser. The Nd:YAG laser wavelength is in the near-infrared region of 1064 nm and shows negligible water absorption. This enables the laser light to reach deep into tissues without being affected by the moisture present in the tissue, making the Nd:YAG laser a tissue-transmission-type laser device. Moreover, light from the Nd:YAG laser is readily and selectively absorbed by black pigments such as hemoglobin and melanin [2, 3] .

The various types of laser therapies include high-level laser therapy (HLLT), which is based on a reaction that destroys living tissues by heat and light from the high level laser, and low-level laser light therapy (LLLT), which is designed to stimulate a biological tissue using heat and light from a low-level laser [4, 5]. HLLT involving the use of a Nd:YAG laser is accompanied by tissue destruction, such as the ablation and coagulation of periodontal tissues. HLLT can be used for incision of gingival tissue [6], ablation of diseased granulation tissue [7-11], sterilization of periodontal pockets by irradiation, cutting an inflamed area in the inner pocket wall during flap surgery [12-17], removal of gingival melanin pigmentation utilizing pigmentation selectivity [18], and removal of subgingival calculus [19]. In contrast, LLLT using the Nd:YAG laser irradiates extensive areas of periodontal tissues to stimulate cells via photochemical action. Examples of LLLT include pain relief [20], improving and promoting blood flow [21], promoting tissue regeneration [22] , and post-operative wound healing [23]. Thus, the Nd:YAG laser has different effects on the irradiated tissue depending on the radiation output and conditions such as time and distance. Various therapies utilizing these properties and their effects have been previously reported [24].

Periodontal treatment with LLLT can be used to promote post-operative wound care of gingival tissue. It has been reported that wound healing of damaged gingival tissue proceeds from the overlapping stages of hemostasis, an inflammatory phase, proliferation phase, and tissue regeneration phase. During the inflammatory phase, platelets are triggered by wound formation and aggregate to close the wound; a variety of cell growth factors are released from the platelets, followed by infiltration of the wound by neutrophils, macrophages, and lymphocytes. In the subsequent proliferation phase, cell growth factors stimulate the proliferation of epithelial cells, fibroblasts, and endothelial cells in the blood vessel, and these cells migrate to the appropriate sites. As a result, re-epithelialization and formation of granulation tissues occur. During the regeneration phase, normal tissues are formed and replaced with scar tissue [25, 26].

Nd:YAG laser irradiation in periodontal tissues is often used to target gingival tissues. In addition, because the Nd:YAG laser can reach deep into the tissues, human gingival fibroblasts are exposed to strong laser light. This is thought to be significantly related to the post-operative wound healing process of the gingival tissues. Human gingival fibroblasts produce numerous cell growth factors thought to play important roles in tissue regeneration, periodontal tissue homeostasis, and wound

healing [27]. Major cell growth factors, particularly those involved in the wound healing process, regulate a variety of cellular functions such as cell proliferation, differentiation, migration, morphogenesis, and death [28]. The involved factors include basic fibroblast growth factor (FGF-2) and transforming growth factor β1 (TGF-β1) [29].

FGF-2 is among the 22 members of the human FGF family and has a molecular weight of 17.4 kDa. FGF-2 can be isolated from a nerve tissue, the pituitary gland, the adrenal cortex, or placenta [30]. In the similar manner to FGF-2, TGF-β1, has various functions and is involved in promoting cell proliferation and cell migration, angiogenesis, regulating differentiation potency, morphogenesis, and promoting extracellular matrix production [31,32]. It has been also reported that FGF-2 promotes the proliferation of fibroblasts [33], osteoblasts [34], cementoblasts [35], and endothelial cells [36], which constitute periodontal tissues.

TGF-β1, a member of the TGF-β superfamily, is a polypeptide dimer with a molecular weight of 12.5 kDa [37]. Depending on types of target cells or cell culture conditions, the involvement of TGF-β1 in both cell proliferation and cell suppression has been reported [38] . In addition, TGF-β1 is predicted to be involved in cellular differentiation, enhanced synthesis of extracellular matrices such as collagen, vascularization, and the modulation of granulation tissue formation [39-41].

Heat shock protein 47 (HSP47) is a stress protein thought to be present in the endoplasmic reticulum of cells. HSP47 is a molecular chaperone involved in regulating the three-dimensional structure of collagen during biosynthesis [42-45]. In addition, previous studies reported the disappearance of HSP47 expression under excessive heat stress conditions in cells [46,47]. Thus, because the Nd:YAG laser can reach deep into the tissue without being absorbed by water in the tissue, it is necessary to evaluate the thermal effects of the laser light on the tissue.

Although LLLT using the Nd:YAG laser has been reported to affect the wound healing process of human periodontal tissues, few studies have been conducted on a cellular level. Therefore, in order to study the effects of the Nd:YAG laser on the wound healing process in human periodontal tissue on the cellular level, we examined important wound healing processes such as cell proliferation, cell growth factor production, and cell migration capability, as well as the thermal effects during the wound healing process. The main aim of this study was to explore the effects of LLLT using Nd:YAG laser irradiation of Gin-1 on cell proliferation, cell migration ability, cell growth factor expression, FGF-2 and TGF-β1, and HSP47.

Materials and Methods

1) Cell culture

This study used a human gingival fibroblast cell line (Gin-1) (Normal Gingival, DS Pharma Biomedical, Osaka, Japan). The cells were cultured in Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 (Ham) (DMEM / F-12, Life Technologies, Tokyo, Japan) containing 10% fetal bovine serum （ fetal bovine serum sterile filtered, Moregate, Queensland, Australia) 50 U/ml Penicillin-Streptomycin (Penicillin-Streptomycin Liquid, Life Technologies, Tokyo, Japan), and 50μg/ml Amphotericin B (Fungizone , Life Technologies, Tokyo, Japan), at 37℃ in the presence of

5% CO_2. Cell cultures used in all the experiments were between passages 4 and 7.

2) Laser irradiation

The irradiation was performed using a Nd:YAG laser neocure 7200 (neocure 7200, SHOUFU, Kyoto, Japan) (Fig.1, A). The laser beams were vertical to the cell level from fiber chip. The diameter of fiber chip was 320μm. The cells were irradiated from a distance of 20 mm at 0.5 W (5 pps, 100 mJ), 1 W (5 pps, 200 mJ), or 2 W (5 pps, 400 mJ) for 30 s. In control cultures, only the medium was changed but the cells were not irradiated. In this study, the original stent was made for 96-well plates and can uniformly irradiate the bottom of the plate (Gikousya, Kanagawa, Japan) (Fig.1, B, C).

3) Cell proliferation assay

Fig. 2A describes the time course of cell proliferation assay. To evaluate Gin-1 cell proliferation, the

Figure 1.

A) This Nd:YAG laser equipment was used in this study (neocure 7200, Shofu, Japan)
B) Photograph of the hand-piece and stent. This stent was made for a 96-well plate and can uniformly irradiate the bottom of the plate.
C) Internal design of the stent. The distance from the fiber to bottom of the plate is 20 mm.

cells were seeded at a density of 1.0×10^3 cells/well in the 96-well culture plates. The Gin-1 cells were allowed to grow for 1, 3, and 5 d. WST-8 (Cell Counting Kit-8, DOJINDO Laboratories, Kumamoto, Japan) was used as the modified MTT assay to evaluate cell proliferation at each time point, by adding the WST-8 solution (100μl/ml of cell culture medium). After incubation at 37°C for 2 h, the optical density of the solution was measured using a microplate reader (Bench mark Plus, Bio-Rad Laboratories, Tokyo, Japan) at a wavelength of 450 nm (n = 7). The relative cell proliferation activity was expressed as the ratio of the proliferative activity of the irradiated cells to that of the control cells.

4) Enzyme-linked immunosorbent assay (ELISA assay)

Fig. 2B shows the time course of the ELISA assay. The amounts of FGF-2 and TGF-β1 were determined by using Human FGF basic Immunoassay kit (Quantikine ELISA Human FGF basic Immunoassay, R&D system, Minnesota, USA), Human TGF-β1 Immunoassay kit (Quantikine ELISA Human TGF-β1 Immunoassay, R&D system, Minnesota, USA).

To evaluate the amount of FGF-2 and TGF-β1 secretions of Gin-1 in the culture supernatant, cells were seeded at a density of 2.5×10^3 cells/well in the 96-well culture plates. After 24 h of cell seeding, laser irradiation was performed. After laser irradiation, the Gin-1 was allowed to grow for 1, 2, and 3 d. The cell culture supernatants were then collected at each time point. The absorbance was measured at 450 nm by using a microplate reader (Bench mark Plus, Bio-Rad Laboratories, Tokyo, Japan), and the concentrations of FGF-2 and TGF-β1 contained in the samples were calculated (n = 7).

5) Cell migration assay

Fig. 2C describes the time course of this assay. A scratch wound healing assay was performed to assess the effect of LLLT on Gin-1 migration in response to an injury. This assay was performed with a modification as described in the study by Igarashi et al.[48] The Gin-1 was seeded on a 60 mm dish and allowed to grow to confluence. Next a scratch wound was made across the diameter of the dish by using the end of a 200μl pipette tip, and the scraped cells were removed by washing once with Hanks' balanced salt solution (Hanks' balanced salt solution, Life Technologies, Tokyo, Japan). The cells were maintained in the culture medium as control or laser irradiations. For each well, images were taken at 0, 5, and 24 h, after the injury, and the area of the cells that migrated into the wound spaces was calculated by Image J (n = 5).

6) Cell cytotoxicity LDH assay

Fig. 2D shows the time course of this assay. To evaluate the cell damage of Gin-1, cells were seeded at a density of 1.0×10^3 cells/well in the 96-well culture plates. Cells were allowed to grow for 3 h, 1 d, and 3 d. A Cytotoxicity LDH assay kit (Cytotoxicity LDH assay kit-WST, DOJINDO Laboratories, Kumamoto, Japan) was used to evaluate the cell damage at each time point, by adding the solution. After incubation, the optical density of the solution was measured using a microplate reader (Corona grating microplate reader, Hitachi High Technologies, Tokyo, Japan) at a wavelength of 490 nm (n = 7).

7) Immunofluorescent staining

Fig. 2E describes the time course of immunofluorescent staining. The Gin-1 was seeded on an 8-well chamber slide (Falcon 8 well culture slide, CORNING, New York, USA) and maintained in culture until they reached subconfluence and were laser irradiated. After 3 h incubation, the cells were washed with a phosphate buffered saline (PBS) (Phosphate Buffered Saline, TAKARA BIO, Shiga, Japan) and fixed with a 4% paraformaldehyde phosphate buffer solution (Paraformaldehyde phosphate buffer solution , Wako, Osaka, Japan) for 15 min at room temperature. The cells were then permeabilized with 99% methanol for 5 min at -20℃, and washed with PBS. After blocking with 5% goat serum in PBS for 1 h, the cells were incubated overnight with anti HSP47 (Anti-Hsp47 antibody, abcam, Cambridge, UK) at a dilution of 1:1000, and then washed with PBS. Next, the cells were incubated for 1 h by using Alexa Fluor 488-conjugated goat anti-mouse (Alexa Fluor 488-conjugated goat anti-mouse, Invitrogen, California, USA) at a dilution of 1:1000. The cells were then washed with PBS and counterstained with a Vecta shield mounting medium with DAPI (Vecta shield mounting medium with DAPI, Vector Laboratolies, Burlingame, USA). The slides were examined under a confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

8) Statistical analysis

All values are expressed as mean±standard deviation except for the HSP47 expression. Analysis of

variance was used for all group comparisons, followed by Dunnett's test to compare the difference between each group and control. A probability value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS (v17.0; SPSS Inc., USA).

Results

1) Cell proliferation assay

The results of the WST-8 cell proliferation assay are shown in Fig. 3. Gin-1 proliferation was examined 1, 3, and 5 d after irradiation by using WST-8. Compared with that in the non-irradiated control, cell proliferation was significantly higher in the groups exposed to 100, 200, and 400 mJ of radiation after 3 d, and 200, 400 mJ on 5 d.

2) ELISA assay

(1) FGF-2 quantification

The amount of FGF-2 secreted by Gin-1 was quantified in culture supernatants, as shown in Fig. 4. After 1 and 2 d, the amount of FGF-2 secreted by all laser-irradiated groups was significantly higher than that secreted by the control group ($p < 0.05$). At 3 d, only the 100 mJ group showed significantly higher FGF-2 secretion than the control group ($p < 0.05$).

(2) TGF-β1 quantification

The amount of TGF-β1 secreted by Gin-1 was quantified in culture supernatants as shown in Fig. 5. After 1 and 2 d, the 200 mJ group secreted significantly higher amount of TGF-β1 than the non-irradiated cells (control group) ($p < 0.05$). At 3 d, the 100 mJ and 200 mJ groups showed significantly higher TGF-β1 secretion than the control group did ($p < 0.05$).

3) Cell migration assay

The results of the cell migration assay are shown in Fig. 6 and 7. After 5 h, only the 200 mJ group showed significantly higher rate of scratch closure than the control group did ($p < 0.05$). At 24 h both the 200 and 400 mJ groups, but not the 100 mJ group, showed a significantly higher rate of scratch closure than the control group did ($p < 0.05$).

4) Cell cytotoxicity LDH assay

The results of the lactate dehydrogenase (LDH) cytotoxicity assay are shown in Fig. 8. No significant change in LDH level was observed in any of the irradiated groups.

5) Expression of HSP47 in cultured Gin-1

HSP47 expression was detected by immnofluorescence staining, as shown in Fig. 9. Both the control group and the laser-irradiated groups of cultured Gin-1 showed positive staining for HSP47. HSP47 expression was detected in the cytoplasm, with homogenous staining around the nucleus.

Discussion

Nd:YAG laser is being applied in a variety of periodontal treatments. Favorable results have been reported from the use of the laser in periodontal treatments, which utilize the characteristics of the laser beam such as deep tissue attainment and pigmentation selectivity [49].

Reports on the effectiveness of the Nd:YAG laser in the treatment of wounds on gingival tissues with LLLT have been made in the past [24]. However, very little has been reported on the basic research at cellular level thus far. This study was conducted in order to explore the cellular-level impact of the Nd:YAG laser when used for the treatment of human gingival tissues.

This study examined the effects of low-level Nd:YAG laser irradiation for Gin-1, as well as the presence of cell damage arising from the laser irradiation conditions adopted by the study. Cell damages were determined based on the manifestation of LDH and HSP47, with focus on cell proliferation, production of fibroblast growth factors, FGF-2 and TGF-β1, which are essential for wound healing, and elements of cell migration.

In this study, the laser irradiation conditions were 0.5 W (100 mJ, 5 pps), 1.0 W (200 mJ, 5 pps), and 2.0 W (400 mJ, 5 pps). Laser irradiation output (W) is determined by a combination of pulse energy (mJ) and pulse rate (pps). In order to study the effects of LLLT with Nd:YAG laser irradiation, we set the pulse rate to the minimum output (5 pps) for suppressing the thermal accumulation of the laser beam within the cells as much as possible and the pulse energy (mJ) to 100, 200, and 400 mJ for analyzing the effects of photochemical action.

In the analysis of the effects of Nd:YAG laser irradiation on the cells, errors in angles and distances of laser irradiation are considered to be a cause of variations in the experimental data. Figure 1-B, C show the stent that was used to uniformly distribute the Nd:YAG laser beams while irradiating cells in this study. The laser beams were uniformly irradiated over the cells in a laser-irradiated group using this stent. Errors with laser irradiation angles and distances are presumed to be potential factors that cause variances in experimental data when conducting examinations on the irradiation of the Nd:YAG laser beams on cells. Therefore, we set the radiation distance to 20 mm as a distance at which the whole cells on the bottom of a well of the 96-well plate were uniformly irradiated by the laser beam.

In a part of the report by Yoshihashi et al. [24], they stated that as a result of retrieval on the cell proliferation and the production of cell growth factors including TGF-β1, where the Nd:YAG laser radiation condition was 2.0 W (400 mJ, 5 pps) at a radiation distance of 20 mm for radiation time of 10 s, the significant difference was not observed when the radiation time was 10 s. Therefore, we set 30 s as a radiation condition in this study.

The results for the cell proliferation from the LLLT indicate that there is no significant statistical difference between the laser non-irradiated and the laser irradiated groups one day after laser irradiation. By the third day, however, significant statistical differences were confirmed between all the laser-irradiated and laser non-irradiated groups. By the fifth day, a statistically significant increase in the cell proliferation rate was confirmed in the 200 mJ and 400 mJ irradiated-groups. The

discrepancy between the irradiated and non-irradiated groups was particularly large on the third day (as opposed to the fifth day). Gkogkos et al. [50] reported that the Nd:YAG laser beam irradiations were performed on human gingival fibroblasts under the following laser-parameters: a pulse energy of 50 mJ and a pulse rate of 10 Hz with an output of 0.5 W, and the duration of 20 or 40 s. No difference in terms of cell proliferation was confirmed between the laser non-irradiated and irradiated groups 24 h after irradiation; however, after 72 h, a progress in the cell proliferation was confirmed in the laser-irradiated groups. Gkogkos et al. [50], used a stent to perform laser beam irradiations for their study. The results of Gkogkos et al. coincided with the results on the cell proliferation obtained from this study [50]. The report published by Morimoto et al .[51] indicated that the cell proliferation effects due to laser irradiation varied depending on the cell cycle. They claimed that this meant that cell proliferation is promoted when laser beam is irradiated during a proliferation phase when cells are particularly active with proliferation and division. However, no cell-proliferation-promoting effects could be confirmed when the laser beam was irradiated during a static phase, when the cells are in a dormant state in terms of proliferation and division. A cell proliferation curve can be plotted to determine the proliferation cycle of the cells by calculating the number of cells over time, as new cells are cultured. Cell proliferation curves vary depending on the types of cells and conditions under which they are cultured. However, they are generally categorized into delay phase, exponential growth phase, stationary phase, and death phase. In this study, a cell proliferation curve for Gin-1 was plotted in advance and used for this study. Gin-1, in the exponential growth phase, in which the cells actively repeated proliferation and division, was then produced for laser beam irradiation. This resulted in a significant increase in the cell proliferation rate three days after the laser beam irradiation in comparison with that of the fifth day. This resulted due to the promotion of the cell proliferation arising from the effects of the LLLT on the Gin-1, which was in the exponential growth phase, during which cells actively proliferated and divided. The cell proliferation rate on the fifth day was presumed to have declined in comparison with that of the third day because the cell proliferation and division had by then reached the stationary phase.

The wound healing process for gums is categorized into inflammatory phase, proliferation phase, and tissue regeneration phase. Platelets coagulate when a wound is formed during the initial inflammatory phase. This triggers the closure of the wound, and releases and produces a variety of cell growth factors. Moreover, the proliferation of the epithelial cells, fibroblasts, and endothelial cells occurs in the blood vessels of the gums and migrate to the appropriate areas of the wound. This process is due to the release and production of cell growth factors during the tissue regeneration phase. As a result, re-epithelialization and formation of new granulation tissues occur. The complete healing of the wound occurs in the regeneration phase, which results in normal periodontal tissues [25, 26]. The gingival fibroblasts play an important role during the wound healing process of the gingival tissues [27]. To form the granulation tissues, the gingival fibroblasts that migrate to the wounded area produce extracellular matrices such as collagen and hyaluronic acid after cell proliferation. Gingival fibroblasts also produce cell growth factors such as FGF-2 and TGF-β1, which are essential cell proliferation factors. FGF-2 has been reported to be a cell growth factor that plays an important role in the wound

healing process, enhancing the proliferating effects of fibroblasts, vascularization, extracellular matrices, etc. [31, 32]. Clinical trials are currently under way to test the efficacy of FGF-2 in tissue regeneration therapies for periodontal treatments provided in Japan [52]. TGF-β1 has been reported to be deeply involved with the wound healing process through actions such as the promotion of collagen synthesis, vascularization, and by inhibiting the actions of enzymes that destroy tissues [39-41]. Pauw et al. [53] have reported that Emdogain promotes the production of the TGF-β1 in the fibroblasts derived from periodontal membranes and promotes the healing of the periodontal tissues. Usumez et al. [22] stated that fibroblasts play an important role in the wound healing process. This group explored the impact of LLLT on the wound healing process and reported that the promotion of the production of cell growth factors (FGF-2 and TGF-β1) through laser beam irradiation can potentially promote wound healing. This research led us to consider both FGF-2 and TGF-β1 as cell growth factors that play an important role in the wound healing process. Therefore, we decided to explore their production in the wound healing process at the cellular level after laser beam irradiation.

A significant statistical increase in the amount of production of FGF-2 was confirmed in all the laser-irradiated groups in comparison with the laser non-irradiated groups on the first and second day after laser irradiation. A significant statistical increase in the amount of production was confirmed with the 100 mJ irradiated group in comparison with the laser non-irradiated group on the third day. FGF-2 is reported to be produced in macrophages or damaged endothelial cells during the wound healing process of tissues and induce fibroblast proliferation [54], but the cell division/ proliferation are considered to also occur in the early phase during the wound healing process. We observed the significant increase in the FGF-2 production by Gin-1 in the relatively early phase, such as on the first and second days after laser irradiation, and the decrease in the production on the third day due to the effect of laser irradiation in this study. Thus, we considered that the result of cell proliferation after laser irradiation demonstrated the significant Gin-1 proliferation on and after the third day of irradiation also caused by the addition of the action of produced FGF-2.

As for TGF-β1, a significant statistical increase in the amount of production was confirmed with the 200 mJ irradiated group in comparison with the laser non-irradiated group on the first and second day after laser irradiation. On the third day a significant statistical increase in the amount of production was confirmed with 100 mJ and 200 mJ irradiated groups in comparison with the laser non-irradiated group. TGF-β1 plays various important roles in cell proliferation, cell migration ability, and synthesis of extracellular matrices by fibroblasts in the wound healing process, and TGF-β1 is considered being deeply involved in all the processes [54]. In addition, we consider that setting the laser irradiation time to 30 s in the current study based on the report by Yoshihashi et al.[24] resulted in the significant increase in the production of TGF-β1 in Gin-1 for the consecutive days.

Cell migration to the wounded area is important for the wound healing process, and it is one of the factors that are instrumental in determining whether subsequent healing of wound can occur. The cell migration assay presented by Igarashi et al. [48] was used for the determination of the cell migration capabilities. Although no significant difference in the cell migration was confirmed with the 100 mJ or 400 mJ irradiated groups in comparison with the laser non-irradiated group 5 h after laser beam

irradiation, a significant promotion of cell migration was indicated with the 200 mJ irradiated group. Although no significant difference in the cell migration was confirmed with the 100 mJ irradiated groups in comparison with laser non-irradiated group 24 h after laser beam irradiation, a significant promotion of cell migration was indicated in the 200 mJ and the 400 mJ irradiated groups. One of the apparent functions of FGF-2 and TGF-β1 (which are produced by gingival fibroblasts), is the promotion of cell migration. This study revealed that irradiating Gin-1 with the Nd:YAG laser beams promotes the production of FGF-2 and TGF-β1. Furthermore, we consider that LLLT also promotes the cell migration of Gin-1, driven by the autocrine and paracrine of FGF-2 and TGF-β1. Basso et al. [55] studied the cell migration capacity of Gin-1 by performing a low-level irradiation with InGaAsP laser beams on the gingival fibroblasts. They reported that cell migration was promoted, similar to the findings of the current study. Basso et al. [55] indicated, with regards to the promotion of the cell migration capability, that promoting the production of cell growth factors of the gingival fibroblasts using LLLT also led to the promotion of the cell migration capacity. This view is identical to the views of this study.

The impact of the Nd:YAG laser on irradiated tissue varies, depending on different irradiating conditions such as the irradiation output, time, and distance. When a laser beam is absorbed by the substance targeted for irradiation, heat is generated within the target. This is the thermal effect caused by the laser beam. On the other hand, there exists a non-thermal effect results when the heat is barely generated inside the substance due to the laser beam, and the substance is instead heated only on the surface. The factors that determine such effects on the targeted substances are the laser beam irradiating conditions [56]. The output boundary between the high-output laser that brings about irreversible reactions in tissues due to thermo-photo processes, and the low-output laser that bring about vitalization and activation of tissues by reversible reactions caused by the interaction between the heat and light is considered to be 0.5 W [57]. The laser beam outputs in the current study were 0.5, 1, and 2 W. Considerations conducted based on these laser beam outputs would lead to the irradiation conditions that cause irreversible reactions in tissues. However, the energy intensity increased over a longer irradiation time than with a low-output laser. Even with a high-output laser, a longer distance between the irradiating port of the laser beam and the target of irradiation would reduce the laser strength to one divided by the square root of the distance [24]. Furthermore, cell damage due to thermal impact and collapse of homeostasis are of concern, depending on the irradiating conditions when using the laser beam on gingival fibroblasts. As the Nd:YAG laser used in this study is not absorbed by water, the laser light exhibits a high degree of tissue permeability. An evaluation based on the manifestation of the LDH and the HSP47 was conducted in order to explore the presence of cell damage due to thermal impact under the Nd:YAG laser irradiation conditions set for this study.

LDH is a stable enzyme that exists in cytoplasms. This enzyme can be detected when cell membranes are damaged. It exists in a variety of cells and the detection of LDH is a method that is widely used to evaluate cell damage. Ogita et al. [58] also utilized LDH to evaluate the cell damage caused by heat effects as the low-output Er:YAG laser irradiation was performed. No significant statistical difference in the amount of LDH detection was confirmed between the laser non-irradiated

group and respective laser-irradiated groups. This was true for all measurement durations.

HSP47 exists within the endoplasmic membrane of cells and functions as a molecular chaperone for the proper construction of the protein structure collagen [42-45]. It is believed to manifest when cells function normally and have sustained homeostasis [46-47]. When tissues are irradiated with laser beams, the tissues that are in the vicinity of the laser beam irradiation port form layers comprised of carbonation, transpiration as well as vaporization, protein coagulation and protein modification [5]. These layers are all due to the effects from HLLT. Once the laser beam reaches down deeper into the tissue, however, the effects gradually diminish and a tissue activation layer is formed due to the LLLT [5]. It has been reported that no HSP47 is expression in cell layers that have coagulated and necrosed due to HLLT. However, damage to cells, collapse of cell organelles and cytoskeletons, as well as vacuolar degeneration of cytoplasms have been confirmed [59]. This means that as the manifestation of the HSP47 is inhibited when the thermal impact from laser beam irradiation is such that cellular degeneration occurs, the presence of HSP47 in the Gin-1 cytoplasms after laser beam irradiation had to be observed using a immunofluorescent assay. The search for HSP47 was performed 3 h after the laser beam irradiation, based on the findings provided by the report presented by Sajjadi et al. [60] These findings confirmed the expression of HSP47 2 h after laser beam irradiation and were the result of searching for a chronological manifestation of HSP47 using the Nd:YAG laser beam. There is also a report presented by Yoshihashi et al. [24], which indicates that the expression of HSP47 was observed 3 h after the laser beam irradiation was performed. This resulted from the observation of HSP47 in the Gin-1 cytoplasms of all groups. Yoshihashi et al. [24] performed the Nd:YAG laser beam irradiations on Gin-1 under the irradiation conditions of 80 mJ, 90 pps, 7.2 W, an irradiation duration of 10 s, as well as irradiation distance of 10 mm. Although there is some degree of variance in the irradiation conditions of the laser, and in spite of the fact that the irradiation output was greater and the laser intensity was greater than the irradiation conditions of the current study, the manifestation of the HSP47 was confirmed in the Gin-1 cytoplasms 3 h after irradiation. These results confirm the verification of the sustained homeostasis of Gin-1.

The manifestations of LDH and HSP47 were explored as indices for cell damage in order to determine the presence of the thermal impact from laser beam irradiation in this study. The results described above did not confirm any thermal impact from laser beam irradiation under the irradiation conditions of this study, which suggest that homeostasis was sustained. In order to conclude that there was no thermal impact however, we believe it would be necessary to conduct a quantitative evaluation of HSP47. It would likewise be beneficial to explore the manifestation of other heat shock proteins, and to conduct investigations on the genetic level.

Humans are subject to a variety of stimuli from the environment as well as from within and outside the body. The body is constantly responding to them in order to maintain biofunctions in a healthy state. In other words, homeostasis is being sustained. The stimuli on the tissue and cellular levels by the LLLT induce vitalization and the activation of cells. This plays a role in sustaining or recovering the homeostasis of the body. There is an optimum irradiation intensity with the LLLT, however. Morimoto et al. [51] reported that the energy density that produces optimal cell proliferation by laser

beam irradiation as well as activation effects was about 0.1 to 10 J/cm^2. On the other hand, Huang et al. [61] reported that excessive irradiation with an energy density that reaches 50 to 100 J/cm^2 is harmful to living organisms. Cell proliferation, production of cell growth factors, as well as cell migration were believed to have been promoted, while on the other hand no cell damage due to laser irradiation was confirmed. This is due to the fact that the irradiation conditions for this study were within the range of 0.1 to 10 J/cm^2.

Thus, our results showed the promotion of gingival fibroblast proliferation, promotion of the expression of FGF-2 or TGF-β1, and activation of cell migration ability by the low-level Nd:YAG laser irradiation, which suggests that the Nd:YAG laser irradiation conditions in this study allowed the promotion of cell activation by LLLT without damaging cells. In this study, favorable results are considered to have been obtained using conditions of 1.0 W (200 mJ, 5 pps) laser irradiation of Gin-1 cells. However, to carry out routine clinical application by determining the optimal irradiation conditions with the objective of gingival wound-healing promotion and cell-damage prevention, it is necessary to perform basic research and studies at the in vivo level. Such research should include an investigation of laser irradiation of periodontal tissue constituent cells other than gingival fibroblasts, the expression of other cell proliferation factors that are related to wound healing, and an analysis at the genetic level. It is hoped that the current study will offer some assistance in clarifying the mechanism of action for the promotion of healing wounds with LLLT using an Nd:YAG laser.

Conclusion

This study was conducted to examine the effects of low-level Nd:YAG laser irradiation for Gin-1, and for investigating for the presence of cellular damage arising from the specified laser irradiation conditions. This was done in order to explore the impact of the Nd:YAG laser on the healing effects of human gingival tissues at cellular level. Cellular damage was determined based on the manifestation of LDH and HSP47. The healing effects were examined with a focus on cell proliferation, production of fibroblast growth factors FGF-2 and TGF-β1, which are essential for wound healing, as well as elements of cell migration. The following knowledge was gained from this study.

1. The promotion of statistically significant cell proliferation was confirmed in all laser-irradiated groups by the third day. Cellular proliferation was confirmed in the 200 mJ and 400 mJ laser-irradiated groups by the fifth day in comparison with the laser non-irradiated group.
2. The promotion of a statistically significant manifestation of FGF-2 was confirmed in all laser-irradiated groups by the first and second day, while on the third day, the same was confirmed in the 100 mJ laser-irradiated group as compared with the laser non-irradiated group.
3. The promotion of a statistically significant manifestation of TGF-β1 was confirmed in the 200 mJ laser-irradiated group on the first and second day, while on the third day, the same was confirmed in the 100 mJ and 200 mJ laser-irradiated groups as compared with the laser non-irradiated group.
4. The promotion of statistically significant cellular migration was confirmed in the 200 mJ laser-irradiated group 5 h after irradiation, and in the 200 and 400 mJ laser-irradiated groups, 24 h after irradiation as compared with the laser non-irradiated group.

5. No significant statistical difference in the amount of the LDH manifestation was confirmed between the laser non-irradiated group and all laser-irradiated groups.
6. The expression of HSP47 was observed in the Gin-1 cytoplasms of all groups.

The cell proliferation was promoted, manifestation of FGF-2 and TGF-β1 was promoted, and the activation of the cell migration capability were observed for groups treated with LLLT using Nd:YAG lasers. These findings suggest a potential for the promotion of cellular activation. Furthermore, no cell damage was confirmed under the irradiation conditions used in this study.

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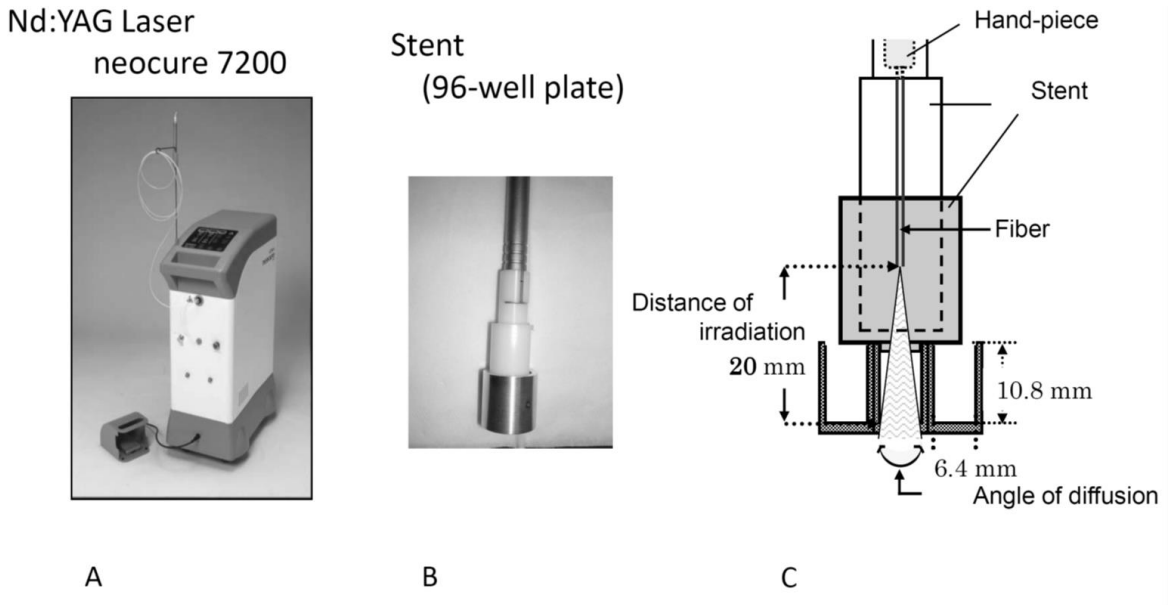
Nd:YAG Laser
neocure 7200
Stent
(96-well plate)
Hand-piece
Stent
Fiber
Distance of
irradiation
20 mm
10.8 mm
6.4 mm
Angle of diffusion
A
B
C

Figure 1.

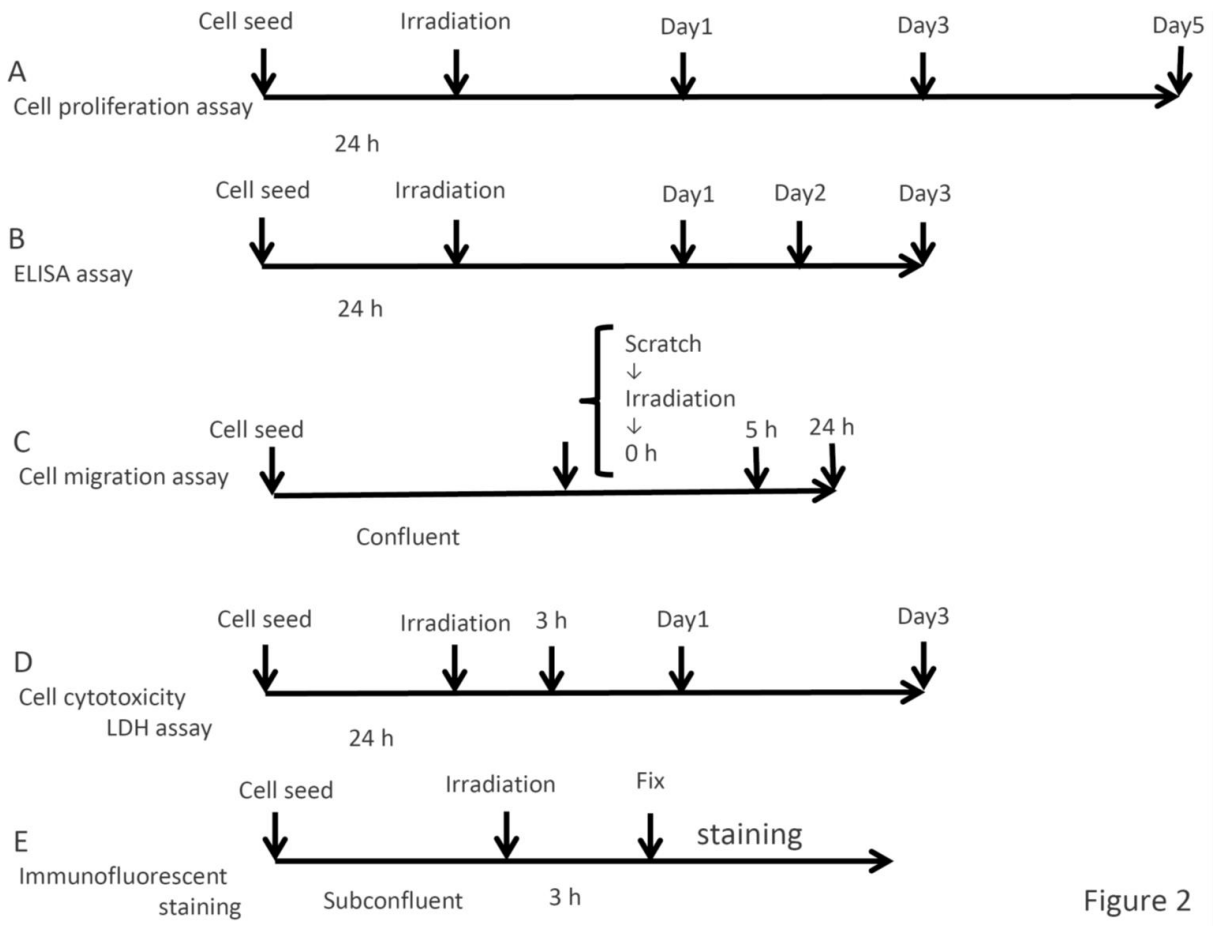

Figure 2.

Time course of the experimental design.

A) Cell proliferation assay: Cells were irradiated after 24 h pre-culture. Each time point (1, 3 and 5 d), for WST-8, the cell proliferation was measured.

B) ELISA assay: (FGF-2 and TGF-β1). Cells were irradiated after 24 h pre-culture. At each time point (1, 2 and 3 d), culture supernatants were harvested.

C) Cell migration assay: After pre-culture, that is, when cells reached confluence in the 60 mm dish, a scratch was made and irradiation was performed. Next, cell migration was immediately recorded for (0 h), 5 and 24 h.

D) Immunofluorescent staining: After 72 h pre-culture (subconfluence), cells were irradiated. 3 h after irradiation, cells were fixed and immunofluorescent staining was performed.

E) Cell cytotoxicity LDH assay: Cells were irradiated after 24 h pre-culture. At each time point (3 h, 1 d and 3 d), the cell cytotoxicity was measured.

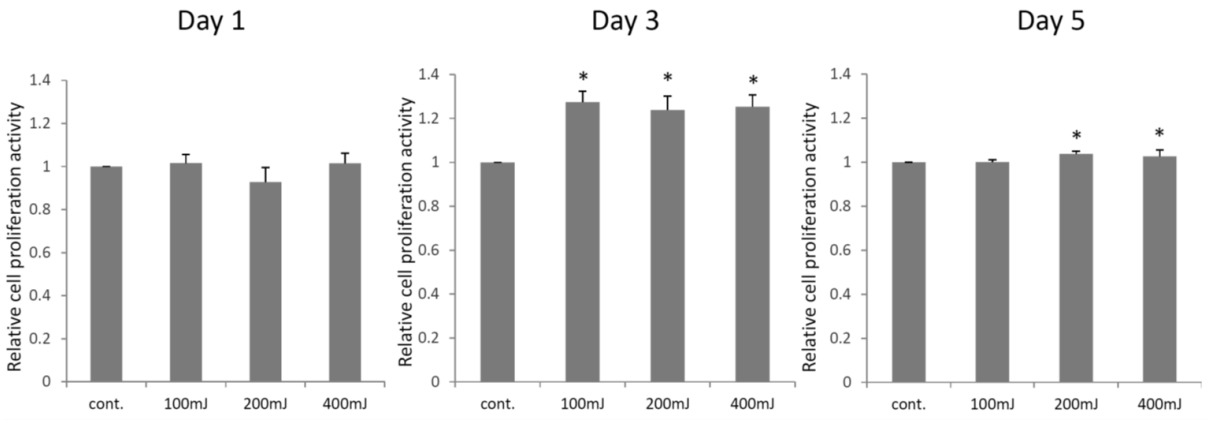

Figure 3.

Cell proliferative activity (1, 3 and 5 d) after low-level Nd:YAG laser irradiation.

Cell proliferation was examined 1, 3 and 5 d after irradiation by addition of WST-8. Compared with that in the control, cell proliferation was significantly higher in the groups exposed to 100, 200, and 400 mJ of radiation after 3 d, and in the 200,and 400 mJ group after 5 d. Data are presented as mean±SD. *p<0.05

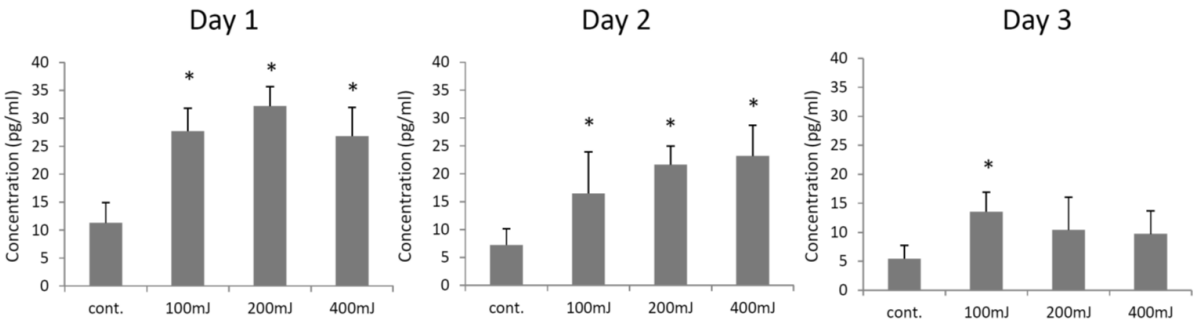

Figure 4.

FGF-2 secreted by Gin-1 was quantified in culture supernatants by ELISA.

After 1 and 2 d, the amount of FGF-2 secreted was significantly higher for all laser- irradiated groups than for the control group. At 3 d, 100 mJ group showed significantly higher FGF-2 secretion than the control group. Data are presented as mean±SD. *$p<0.05$

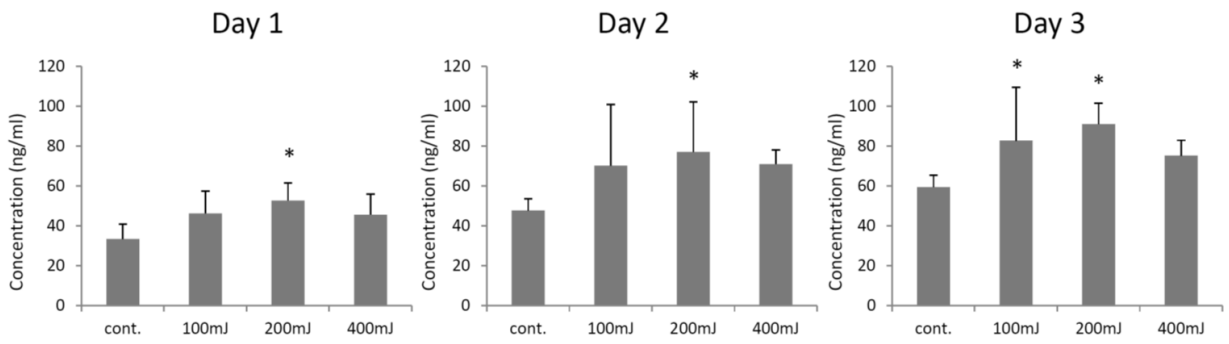

Figure 5.

TGF-β1 secreted by Gin-1 was quantified in culture supernatants by ELISA.

The amount of TGF-β1 secreted by the 200 mJ group was significantly higher than that secreted by the control group at both 1 and 2 d. The 100 and 200 mJ groups showed significantly higher TGF-β1 secretion than the control group did at 3 d. Data are presented as mean±SD. *p<0.05

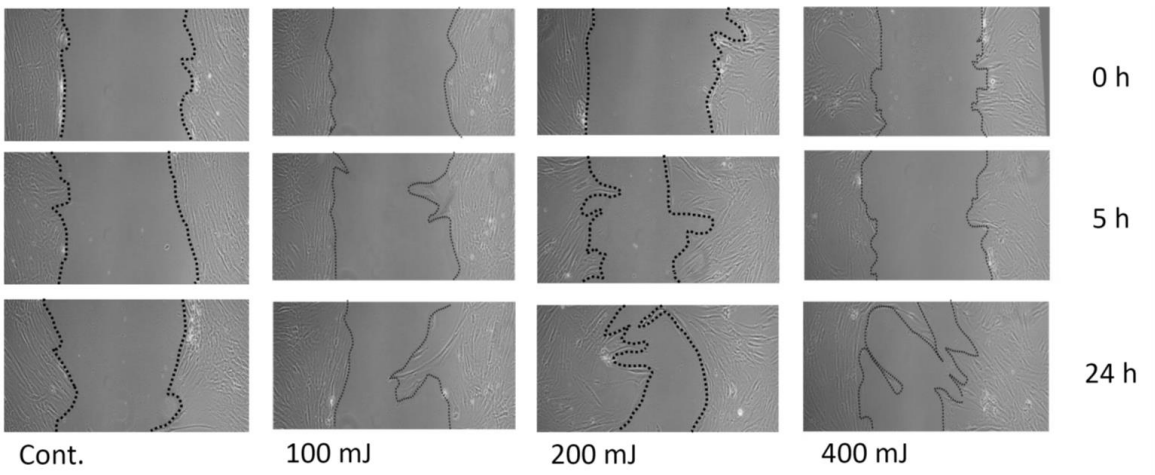

Figure 6.

Comparison of the migration activity of Gin-1 using a scratch wound healing assay.

The dashed lines delimit the initially wounded regions, at 0, 5 and 24 h after laser irradiation. At each time point, the extent of scratch closure by migration of cells into the wound space was measured.

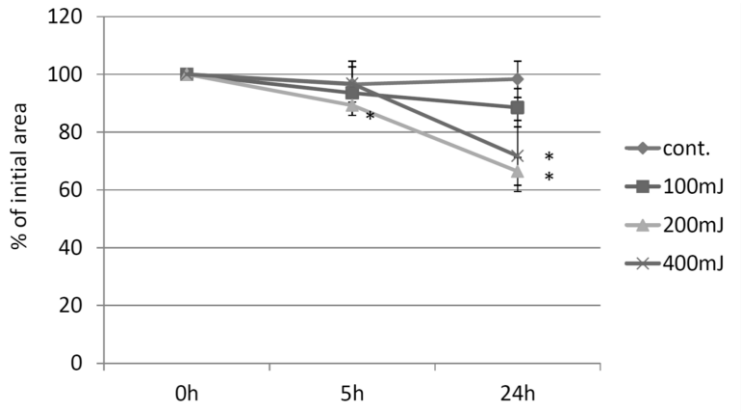

Figure 7.

Change in scratch-filling scores over time after irradiation.

Rate of scratch closure is expressed as a percentage of the initial wound area. Data are presented as mean±SD. *p<0.05

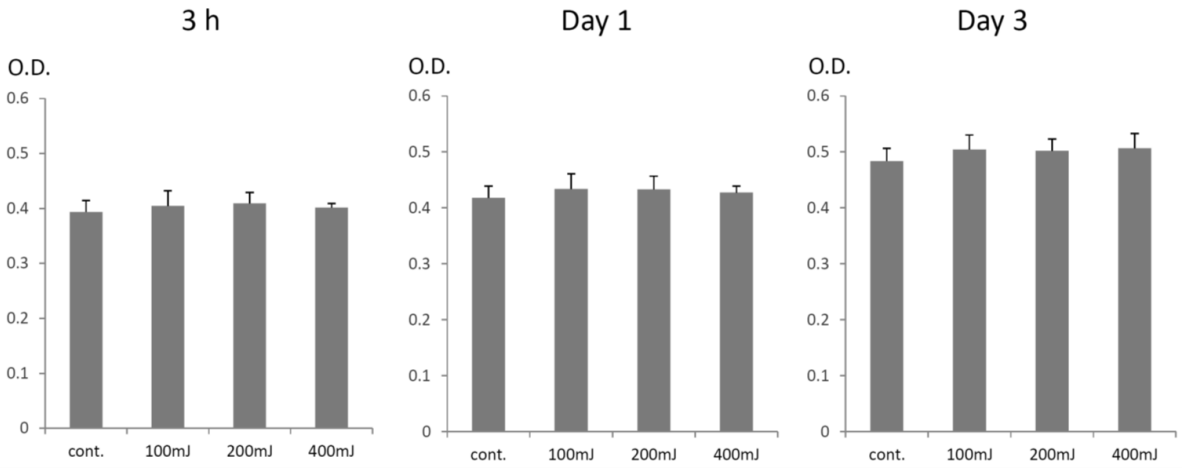

Figure 8.

Cell cytotoxicity LDH (3h, 1d and 3d) after low-level Nd:YAG laser irradiation.

No significant change was observed in the LDH level in any of the laser-irradiated groups, at any of the time points. Data are presented as mean±SD. *$p<0.05$

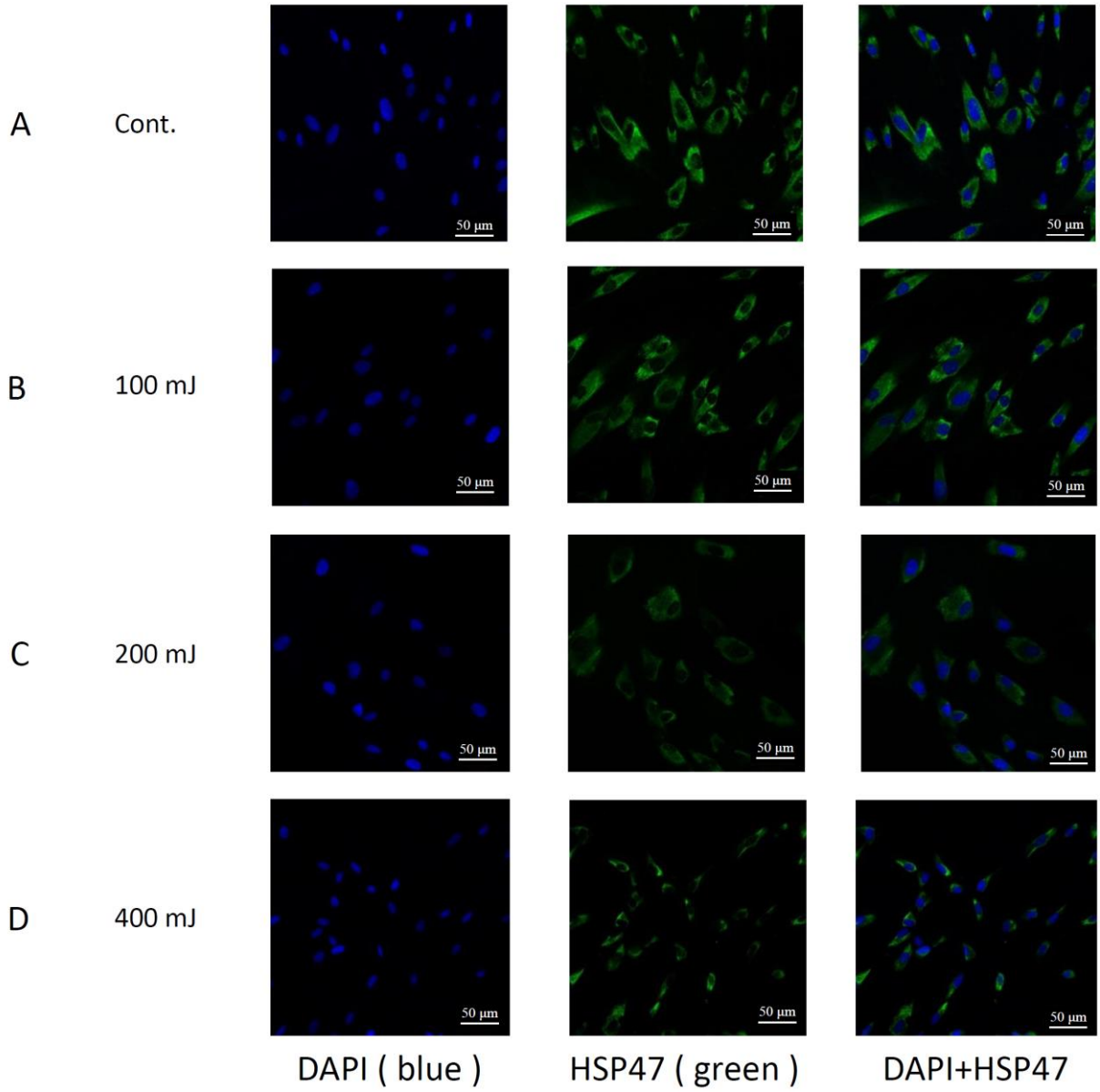

Figure 9.

Expression of HSP47 in cultured Gin-1.

Immunofluorescent staining for HSP47 (green) was performed using cultured Gin-1. The cells in all conditions were counterstained with DAPI (blue). Both the control group and the laser-irradiated groups of cultured Gin-1 showed positive staining for HSP47. HSP47 expression was detected in the cytoplasm, with homogenous staining around the nucleus. Scale bars = 50μm