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Influence of Low-energy Laser Irradiation on Nitric Oxide Expression in Vascular Endothelial Cells

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Anti-inflammatory action of low-energy laser has been reported and widely applied for clinical dental treatment. However, its action mechanism is unclear. In this study, focusing on the dynamics of nitric oxide (NO), we investigated the anti-inflammatory effect of low-energy laser on vascular endothelial cells.

Vascular endothelial cells were cultured and irradiated with low-energy laser. Cells were divided into those cultured in media containing (LPS additive group) and not containing LPS (control group), and each group was irradiated with laser. The amount of synthesized NO was quantitated 24 hours later using a nitrate/nitrite measurement kit, and expression of NO synthases (NOSs) was observed using immunostaining.

The NO quantitation, indicated that the synthesized amount decreased in the irradiated LPS additive group. On immunostaining, inducible NOS (i-NOS) expression reduced in the LPS additive group. These findings suggested that i-NOSs were involved in the low-energy laser-induced decrease in NO synthesis.

It was suggested that the anti-inflammatory effect of low-energy laser irradiation might arise from decreased NO synthesis in the inflammation.

Key words : low-energy laser, nitric oxide, vascular endothelial cells, inflammation

Introduction

Cell-activating action, apoptosis-inhibitory effect, and anti-inflammatory action of low-energy laser were reported and clinically applied widely for promotion of wound healing and bone calcification, anti-inflammatory treatment for arthrosis of the temporomandibular joint and periodontal treatment, and pain relief after implant surgery[1~4]. However, the action mechanisms are unclear, and elucidation of the mechanisms of the low-energy laser actions on the body is urgently needed.

It has recently been reported that cell activity observed after low-energy laser irradiation is mediated by a free radical, reactive oxygen species (ROS), clarifying a part of the biological action mechanism of low-energy laser[5,6]. However, it has not yet been clarified what type of synthetic and scavenging enzymes act in cells in response to laser irradiation, and how free radicals are controlled.

Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS). There are 3 isoforms of NOS (n-NOS, i-NOS, and e-NOS) with different expressions and actions[7], but the type of NOS expressed after low-energy laser irradiation or how nitric oxide changes have not

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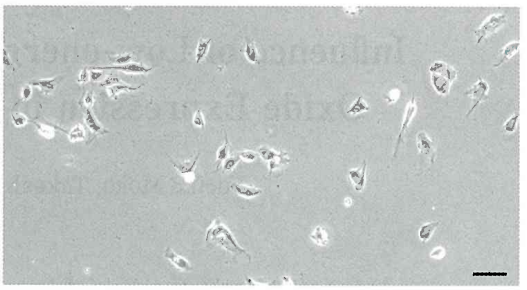
yet been clarified. Moreover, changes in nitric oxide after low-energy laser irradiation in inflammation have not been clarified.

In this study, focusing on the dynamics of nitric oxide, we investigated the anti-inflammatory effect of low-energy laser on vascular endothelial cells in vitro.

Materials and Methods

1. Cell culture

For the cells, vascular endothelial cells of the human umbilical vein (Cryo HUVEC Pooled) (EIDIA Co., Ltd., Tokyo) were used. For the medium, growth medium for vascular endothelial cells (EGM-2 : hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, FBS, hEGF, and GA-1000) (EIDIA Co., Ltd., Tokyo) was used. Cells were cultured for 3 days at 37℃ in 5% CO_2 at 100% humidity following the standard method. For passage, 0.0025% trypsin-containing 0.01% EDTA solution (EIDIA Co., Ltd., Tokyo) was used (Fig. 1).



(bar = 50 μm)

Fig. 1 Cells
Vascular endothelial cells (HUVEC, Sanko Junyaku Co., Ltd.) were cultured in basic medium for vascular endothelial cells (EBM-2) combined with an additive factor kit at 37℃ in 5% CO_2.

2. Laser equipment

For the laser equipment, a semiconductor surgical laser system for research and development (Yoshida Dental MFG, Tokyo) was used (Fig. 2). The specifications were : wavelength, 810±20 nm ; and output, 0.5-5.0 W (continuous irradiation mode : 0.5-3.0 W). For the irradiation mode, a semiconductor surgical laser system capable of setting continuous, repeat pulse, and single pulse irradiation modes was used.

Fig. 2 Laser equipment
For the laser equipment, a semiconductor surgical laser system for research and development (Yoshida Dental MFG, Tokyo) was used. Its output can be set at 0.5-3.0 W.

3. Conditions of laser irradiation

The optimum laser irradiation conditions for the cell growth rate were set : output- irradiation time, 0.5 W-2 seconds (irradiation distance: 3.06 cm, irradiation field : 0.31cm², power density : 0.26J/cm² ; continuous irradiation mode), and defocused irradiation was applied (Fig. 3).

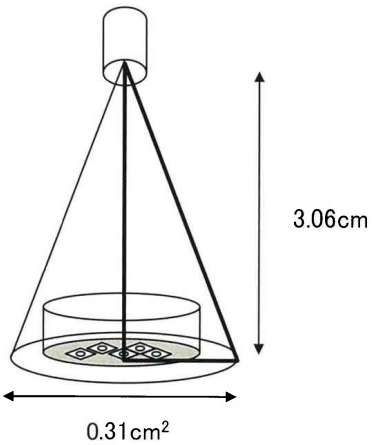


Fig. 3 Laser irradiation conditions
The laser irradiation conditions were set at 0.5 W-2 sec (energy density : 3.2 J/cm²) at which vascular endothelial cell growth is promoted based on the results of the preliminary experiment.

4. Time course of experiment

Cells were seeded at a density appropriate for each experiment. For the medium, EGM-2 with

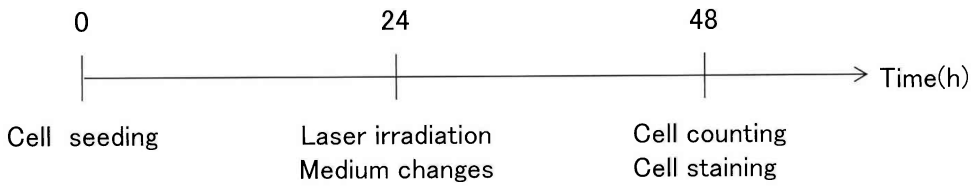


Fig. 4 Time course of experiment
Medium was changed after 24-hour culture, and at the same time, cells were irradiated with laser once under the optimum conditions. The measurement and staining of cells were performed 24 hours after laser irradiation.

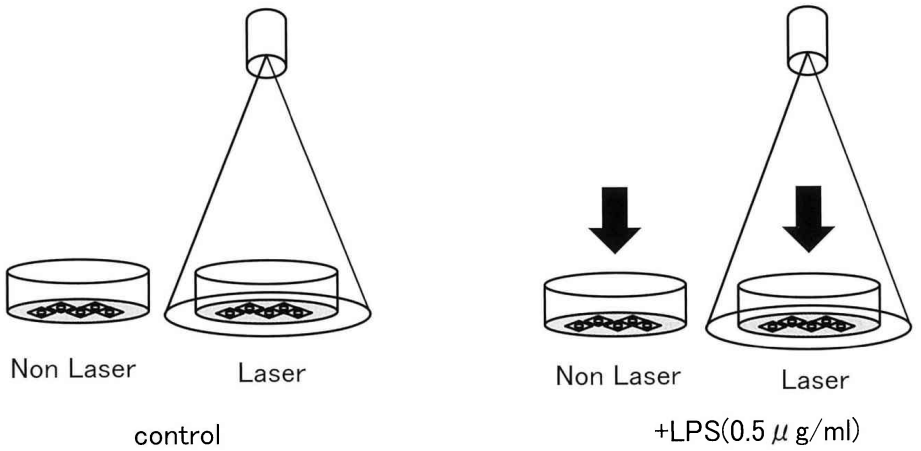


Fig. 5 Experimental methods
Cells were seeded and cultured in EGM-2 medium with (LPS additive group) or without (control group) the addition of 0.5 μg/mL lipopolysaccharide, and the media were changed after 24-hour culture. At the same time, cells were irradiated with laser once under the optimum conditions, and the non-irradiated (Non Laser) and irradiated (Laser) groups were compared.

and without the addition of 0.5 μg/mL lipopolysaccharides from Escherichia coli 005 : B5 (Sigma-Aldrich Japan, Tokyo) (LPS additive and control groups, respectively) were used and changed after 24 hours. At the same time, cells were irradiated with laser once under the optimum conditions. The measurement and staining of cells were performed 24 hours after irradiation (Figs. 4 and 5).

5. Changes in the cell growth rate after low-energy laser irradiation

Cells were seeded in 96-well plates at $5x10^3$ cells/well. After 24-hour culture, the medium was changed to that with (LPS additive group) or without (control group) LPS, and the cells were irradiated with laser. Changes in the cell growth rate at 24 hours after laser irradiation were measured using the Cell counting kit-8 (Dojindo, Kumamoto) and compared between the irradiated (Laser) and non-irradiated (Non laser) groups.

6. Changes in the amount of nitric oxide (NO) synthesis after low-energy laser irradiation

Cells were seeded in 6-well plates at $1.2x10^5$ cells/well, medium was changed after 24-hour culture, and the cells were irradiated with laser. Mammalian Protein Extraction Buffer (GE Healthcare Life Sciences, UK) was added to the cells 24 hours after laser irradiation to prepare samples. The samples were processed following the attached instruction manual, and nitric oxide contained in the cells was quantitated using

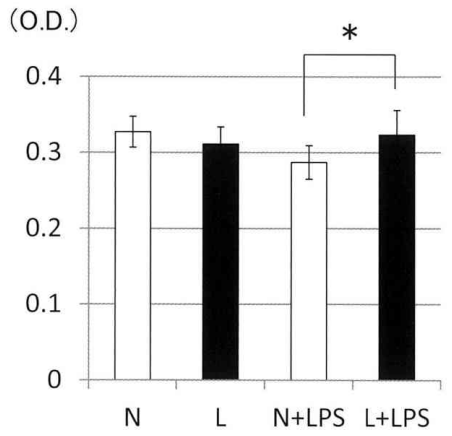


Fig. 6 Changes in the cell growth rate after low-energy laser irradiation
No significant difference was noted in the control group, but an about 13% increase was noted in the irradiated group compared with that in the non-irradiated group in the LPS additive group.
*: U-test, P<0.05 Mean±SD (n=8)

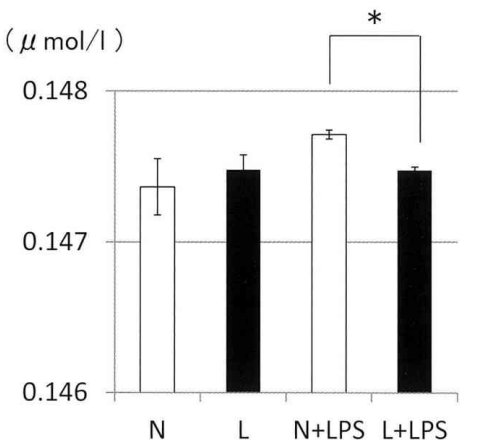


Fig. 7 Changes in the amount of nitric oxide (NO) synthesis after low-energy laser irradiation
No significant change was noted in either the non-irradiated or irradiated group in the control group. In the LPS additive group, a 1.6% decrease was noted in the irradiated group.
*: U-test , P<0.05 Mean±SD (n=8)

the NO_2/NO_3 Assay kit-CII (Colorimetric)-Griess Reagent Kit-(Dojindo, Kumamoto).

7. Changes in nitric oxide synthase (NOS) expression after low-energy laser irradiation

Cells were seeded at $5x10^3$ cells/well in 10-well Teflon-lined slide glasses (Thermo Fisher Scientific Inc., USA), medium was changed after 24-hour culture, and the cells were irradiated with laser. At 24 hours after irradiation, the cells were fixed in 4% paraldehyde-phosphate buffer (Wako Pure Chemical Industries, Ltd., Osaka). Immunohistological staining was performed using rabbit anti-n-NOS (Zymed laboratories Inc., USA), rabbit anti i-NOS (Zymed laboratories Inc., USA), and mouse anti-e-NOS (Life Technologies Japan Ltd., Tokyo) for the primary antibodies, the VECTASTAIN ABC kit (Vector Laboratories, USA) for the secondary antibody, and a DAB substrate kit (Nitirei, Tokyo) for color development, and expression of each NOS isoform was investigated.

8. Statistical analysis

The data were presented as the mean± standard error. The Mann-Whitney U-test with Bonferroni's correction was performed after the Kruskal Wallis H-test, and the non-irradiated and irradiated groups were compared in the control and LPS additive groups. A significance level of 5% or lower was regarded as significant.

Results

1. Changes in the cell growth rate after low-energy laser irradiation

No significant difference was noted in the control group, but the rate increased by 13% in the irradiated group compared with that in the non-irradiated group in the LPS additive group (Fig. 6).

2. Changes in the amount of nitric oxide (NO) synthesis after low-energy laser irradiation

No significant change was noted in either the non-irradiated or irradiated group in the control group, but a 1.6% decrease was noted in the irradiated group in the LPS additive group (Fig. 7).

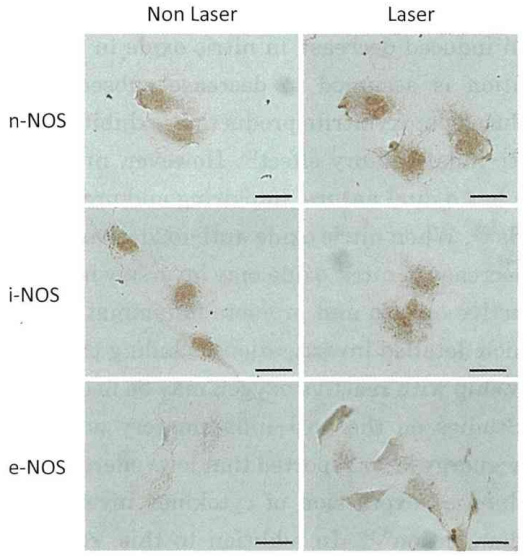


Fig. 8 Changes in nitric oxide synthase (NOS) expression after low-energy laser irradiation (control group)
n-NOS and e-NOS expressions were slightly enhanced in the irradiated group.

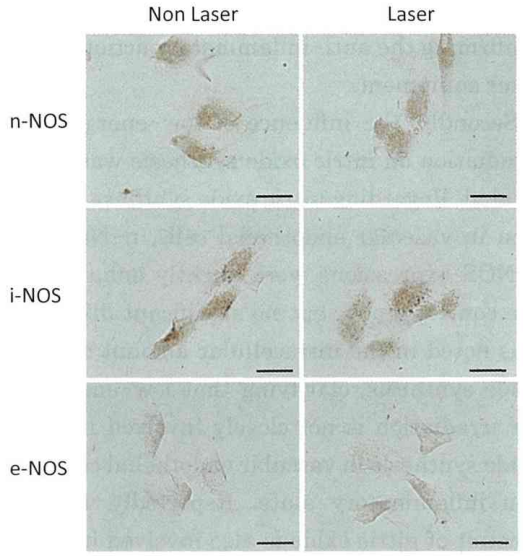


Fig. 9 Changes in nitric oxide synthase (NOS) expression after low-energy laser irradiation (LPS additive group)
i-NOS and e-NOS expressions decreased in the irradiated group.

Table 1 Changes in nitric oxide synthase (NOS) expression after low-energy laser irradiation

	control		+LPS	
	Non Laser	Laser	Non Laser	Laser
n-NOS	+	++	++	++
i-NOS	+	+	+++	++
e-NOS	+	++	++	+

Regarding NOS expression in vascular endothelial cells after laser irradiation, n-NOS and e-NOS expressions were slightly enhanced in the control group, whereas i-NOS and e-NOS expressions decreased in the LPS additive group.
+ : positive reaction

3. Changes in nitric oxide synthase (NOS) expression after low-energy laser irradiation

n-NOS, i-NOS, and e-NOS were expressed in the vascular endothelial cells, and n-NOS and e-NOS expressions were slightly enhanced after laser irradiation in the control group (Fig. 8, Table 1). In contrast, i-NOS and e-NOS expressions decreased in the LPS-added and irradiated group (Fig. 9, Table 1).

Discussion

Nitric oxide acts on vascular endothelial cells as an inflammatory mediator[7]. Although the anti-inflammatory action of laser is known, its association with nitric oxide has not been sufficiently clarified. In this study, we investigated how the amount of synthesized nitric oxide changes in vascular endothelial cells after low-energy laser irradiation and the type of and changes in NOS expression.

Firstly, the anti-inflammatory action of this laser equipment on vascular endothelial cells was investigated using the cell growth rate as an index as a precondition of this study. Laser irradiation promoted cell growth in the LPS additive group. It is considered that the anti-inflammatory effect of low-energy laser is exhibited through inhibition of inflammatory cytokines, anti-apoptosis effect, and activation of the cell function[1,8]. Cell growth was promoted

under the irradiation conditions of this study, confirming the anti-inflammatory action of this laser equipment.

Secondly, the influence of low-energy laser irradiation on nitric oxide synthesis was investigated. Regarding nitric oxide synthase expression in vascular endothelial cells, n-NOS and e-NOS expressions were slightly enhanced in the control group, but no significant difference was noted in the intracellular amount of nitric oxide synthesis, clarifying that low-energy laser irradiation is not closely involved in nitric oxide synthesis in vascular endothelial cells in a non-inflammatory state. Reportedly, a trace amount of nitric oxide is also involved in maintenance of the vascular function of vascular endothelial cells, and nitric oxide is involved in signaling of vascular endothelial cell growth factor (VEGF) in vascularization[9,10]. Since low-energy laser irradiation was reported to enhance e-NOS expression and promote vascularization[11,12], a slight increase in nitric oxide synthase observed in our study was assumed to be involved in cell activation and promotion of vascularization by low-energy laser.

Thirdly, the influence of low-energy laser irradiation on nitric oxide synthesis in inflammation was investigated. Regarding nitric oxide synthase expression in vascular endothelial cells after laser irradiation, i-NOS and e-NOS expressions decreased in the LPS additive group, and nitric oxide synthesis also decreased. In a study on the association between nitric oxide and low-energy laser in inflammation, nitric oxide synthesis was promoted in macrophages after low-energy laser irradiation[13], and a decrease in i-NOS in a human osteosarcoma-derived cell line (MG63) has been reported[14]. i-NOS transiently produces a large amount of nitric oxide compared with the other NOS isoforms, and reacts with reactive oxygen, through which peroxynitrite is produced and exhibits strong cytotoxicity[7]. A low-energy laser irradiation-induced decrease in nitric oxide in inflammation is assumed to decrease subsequently induced peroxynitrite production, exhibiting the anti-inflammatory effect[15]. However, nitric oxide has a dual nature, impairing and protecting cells[7,16]. When nitric oxide anti-oxidatively acts, a decrease in nitric oxide may inversely increase reactive oxygen and promote inflammation, for which detailed investigation including the relationship with reactive oxygen may be necessary.

Studies on the anti-inflammatory action of low-energy laser reported that low-energy laser influences expression of cytokines involved in inflammation[17,18]. In addition to this, recently, several studies reported that low-energy laser exhibits the anti-inflammatory action by decreasing oxidative stress[19,20]. It is now being clarified that laser serves as the initial signal of cell activity and changes dynamics of free radicals, influencing cell activity involved in inflammation.

Regarding the anti-inflammatory action of laser observed in our study, it is also possible that a laser irradiation-induced decrease in nitric oxide not only reduced peroxynitrite production but also acted on cells as an intracellular signaling molecule and changed some inflammatory factor produced in cells, resulting in the anti-inflammatory action, for which further investigation is necessary.

Based on the above investigation using nitric oxide as an index, it was suggested that low-energy laser irradiation in an inflammatory state exhibits the anti-inflammatory effect by mainly reducing i-NOS expression in vascular endothelial cells and decreasing nitric oxide synthesis.

To clinically apply the findings of this study, further investigation may be necessary, such as associations with reactive oxygen and inflammatory substances, irradiation conditions, and

investigation using experimental animals in consideration of contradiction of free radical dynamics between different experimental systems[21~23] and variation of its expression due to differences in the laser wavelength[24,25].

Conclusion

1. Low-energy laser irradiation promoted vascular endothelial cell growth in the LPS additive group.

2. Low-energy laser irradiation decreased nitric oxide synthesis in the LPS additive group.

3. Regarding the effect of low-energy laser irradiation on nitric oxide synthase expression in vascular endothelial cells, n-NOS and e-NOS were expressed in the control group, and n-NOS, i-NOS, and e-NOS were expressed in the LPS additive group, suggesting their involvement in nitric oxide synthesis.

4. Low-energy laser irradiation reduced i-NOS and e-NOS expressions in vascular endothelial cells in the LPS additive group.

C.O.I

The authors indicated no conflicts of interest.

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