Effects of Near-Infrared Low-Level Laser Irradiation on Microcirculation

Yasuyo Maegawa, MD,[1] Toshiyuki Itoh, MD, PhD,[2]* Toyoshi Hosokawa, MD, PhD,[1] Kazuhiro Yaegashi, MD, PhD,[2] and Mayumi Nishi, MD, PhD,[3]

[1]*Department of Anesthesiology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan*
[2]*Department of Physiology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan*
[3]*Department of Anatomy, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto, Japan*

Background and Objective: Recently, there has been an increase in the clinical application of low-level laser irradiation (LLLI) in various fields. The present study was conducted to explore the effects of LLLI on microcirculation.

Study Design/Material and Methods: We investigated the effects of LLLI on rat mesenteric microcirculation in vivo, and on cytosolic calcium concentration ($[Ca^{2+}]i$) in rat vascular smooth muscle cells (VSMCs) in vitro.

Results: LLLI caused potent dilation in the laser-irradiated arteriole, which led to marked increases in the arteriolar blood flow. The changes were partly attenuated in the initial phase by the superfusion of 15 μM L-NAME, but they were not affected by local denervation. Furthermore, LLLI caused a power-dependent decrease in $[Ca^{2+}]i$ in VSMCs.

Conclusion: The circulatory changes observed seemed to be mediated largely by LLLI-induced reduction of $[Ca^{2+}]i$ in VSMCs, in addition to the involvement of NO in the initial phase. Lasers Surg. Med. 27:427–437, 2000. © 2000 Wiley-Liss, Inc.

Key words: cytosolic calcium concentration; nitric oxide; photo-induced vasodilation; vascular smooth muscle

INTRODUCTION

Light exerts various effects on living beings. The clinical efficacy of low-level laser irradiation (LLLI) is widely accepted [1], and recently, there has been an increase in its use for the treatment of chronic pain [1–3] and to promote injury repair [4]. These therapeutic effects were first reported by Mester et al. [5] who showed that Ar laser or He-Ne laser irradiation promoted the healing of wounds by increasing the production of collagen, leukocytes, and angiogenesis. Other studies suggested that LLLI would exhibit some beneficial effects on the nervous system [6–8], the synthesis of fibroblasts [9], and the immune system [10].

Vascular reactions during LLLI are also postulated as one of the possible mechanisms responsible for the above-noted clinical effects, because blood flow is an important determinant of wound healing and relief of pain. For example, Furchgott et al. [11] showed that light in the wavelength range of 310–440 nm from a nonlaser source reduced vascular smooth muscle tone in vitro. Also, Gal et al. [12] showed that low-level laser irradiation reversed histamine-induced spasms in the coronary artery of atherosclerotic microswine. However, it is not clear so far whether these reactions occur specifically in the aorta and large arteries; in particular, microcirculatory changes during LLLI have not been well understood. Furthermore, the mechanisms of the above photo-

induced vascular relaxation have not been studied in depth, with the exception of a few reports [13,14], suggesting that light-induced vascular relaxation would be mediated by a rapid increase of intracellular cGMP.

In preliminary experiments, we found that LLLI induced a rapid arteriolar vasodilation in rat mesentery, which led us to a closer examination of the effects of LLLI on microcirculation. The aims of the present study were (1) to investigate in greater depth the time course of mesenteric arteriolar vasodilation and the consequent increase in microcirculatory blood flow after LLLI in vivo, concomitantly assessing the involvement of endothelial and neural factors in the laser-induced vasodilation; and (2) to examine the intracellular events of smooth muscle cells subjected to LLLI in vitro. In regard to (1), we compared the effects of local LLLI on intact, chemically treated, and surgically denervated rat mesenteric microvascular beds. For the latter, we conducted fluorometric studies on the changes in intracellular Ca^{2+} after LLLI in cultured vascular smooth muscle cells.

MATERIALS AND METHODS

Animal Preparations and Experimental Protocol

Male Wistar rats, each weighting 300 to 350g, were anesthetized with sodium pentobarbiturate (50 mg/kg, s.c.) and the right carotid artery was cannulated for continuous monitoring of arterial blood pressure and heart rate (P23XL, Gould, Oxnard, CA). Tracheotomy and endotracheal cannulation were performed, and the rats were under spontaneous respiration throughout the entire protocol.

To assess the involvement of nitrogen oxide (NO) and vasomotor nerves in the vasodilatory effect of LLLI, the rats were randomly assigned to one of the following experimental groups: *Control group* (n = 7), with no laser irradiation; *LLLI group* (n = 8), for which laser irradiation was only performed on intact mesenteric arterioles; *LLLI+denervation (DNRV) group* (n = 8), for which laser irradiation was performed on surgically denervated mesenteric arterioles; and *LLLI+L-NAME group* (n = 7), for which laser irradiation was performed on the mesenteric arterioles superfused with L-nitro L-arginine methylester (L-NAME). In the LLLI + DNRV group, the mesenteric microvascular bed of the rats was surgically denervated 2 weeks before the LLLI study.

Briefly, the animals were anesthetized with sodium pentobarbiturate (50 mg/kg, i.p.) and a transverse abdominal incision was made. Arterial denervation was achieved by removal of the celiac-superior mesenteric ganglia, dissection of the main postganglionic nerve trunk from the superior mesenteric artery, and topical application of phenol on the dissected artery [15,16]. After being subjected to the LLLI experiment, the rats in this group were killed and the local denervation was confirmed by immunohistochemistry by using protein gene product 9.5 (PGP-9.5), which allowed specific detection of nerve fibers [15].

In the L-NAME group, the mesentery was superfused with phosphate-buffered saline solution (PBS) (pH = 7.4, 285 mOsm, 37°C) containing 15 µM L-NAME (Sigma, St. Louis, MO) at a flow rate of 3 ml/min. It is known that this dose of L-NAME inhibits the total activity of nitric oxide synthase in mesentery by 88% [17].

Experimental System and Measurement of Microvascular Blood Flow

A schematic of the experimental system is shown in Figure 1. Briefly, it consisted of a transmission microscope, a temperature-controlled experimental chamber for observation of mesenteric microcirculation [18], a video recording system, and an optical velocimetry system [19]. After an abdominal midline incision was made, the rat intestinal mesentery was exteriorized and spread out in the chamber filled with PBS. The rat abdomen was connected to the chamber by means of an adaptor so that a tight seal was provided, and then, in turn, the chamber was placed on the microscope stage (inset A in Fig. 1). The temperature of the medium in the chamber and the body temperature were maintained at 37°C throughout the entire protocol, unless otherwise stated.

Mesenteric microcirculation was viewed through a glass window made in the chamber. The light source of the microscope (mercury-arc lamp; HBO 200W, Osram, Germany) was heavily filtered with an interference green filter to minimize temperature elevation in the tissue and also to improve the contrast of the blood flow images. Before LLLI and microcirculatory measurements, the microvasculature was examined carefully by using a ×4 objective lens, and an appropriate mesenteric field containing an arteriole ~20 µm in diameter was selected for observation. After the selection, video recordings of the selected arteriole and the measurements of arteriolar red cell velocity (V_{RBC}) were performed to obtain the base-
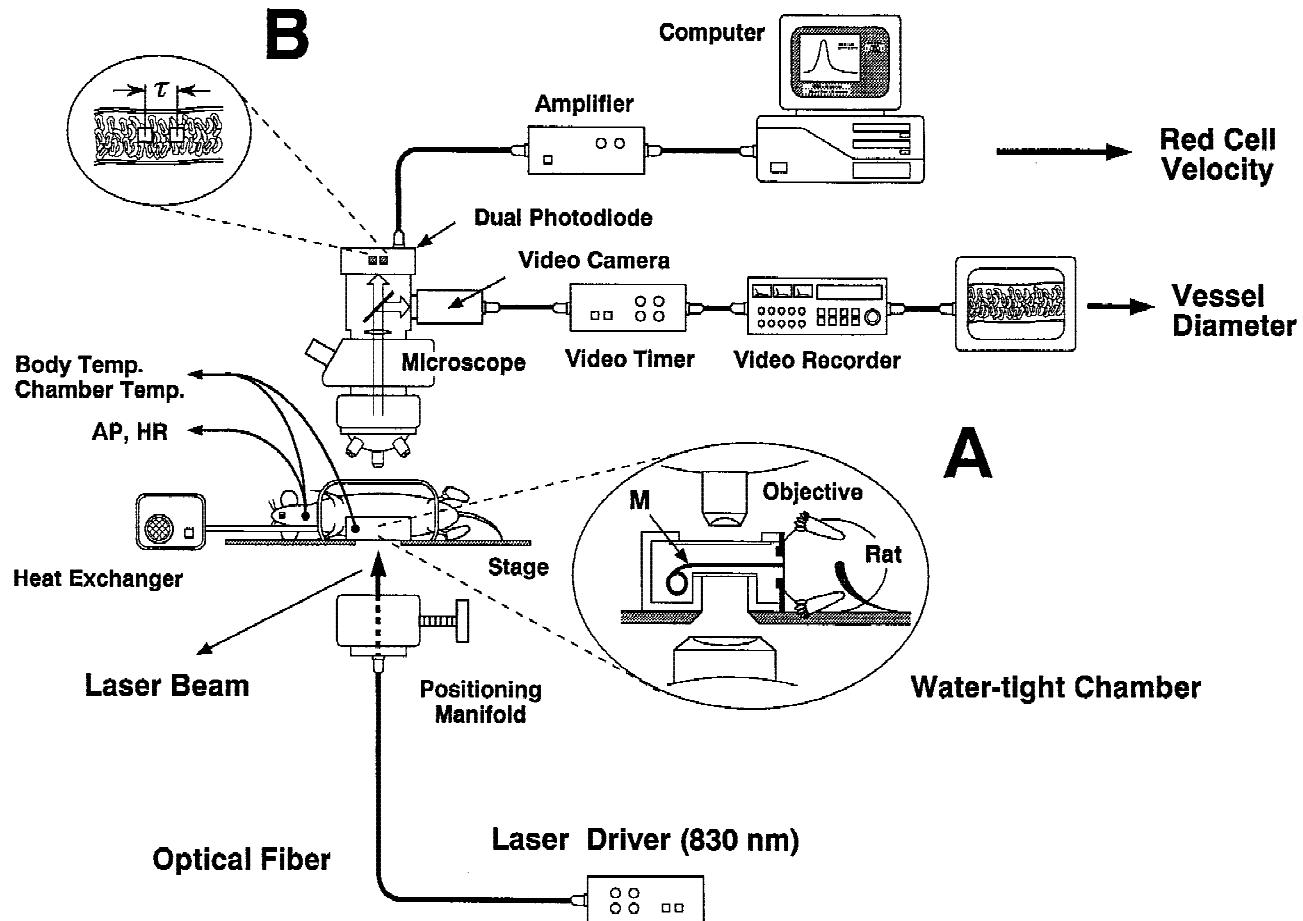
Fig. 1. Diagram of the experimental system. The intestinal mesentery of rat (**inset A,** arrow M) was exteriorized into an experimental chamber. The microscopic image of the vasculature at the velocity measurement site was divided by a half-prism into two light beams, which were simultaneously projected onto a video camera and a velocimeter. The low-level laser (λ = 830 nm) was irradiated locally on the mesenteric area by using a laser driver by means of an optical fiber, which generated a thin (1-mm core diameter of output) laser beam. The optical fiber was supported by a fine-positioning manifold. Red cell velocity was determined digitally by dual-slit photometric technique (**inset B**). Concomitantly, the image of the microvessel was recorded by the video recording system to measure the inner diameter through a digital image processor. The output of the thermometers was A/D converted together with that of the pressure transducer amplifiers, and stored in a microcomputer.

line value by using a ×10 objective lens (Plan 10 LWD, NA 0.25, Nikon, Tokyo, Japan), and then the mercury-arc lamp was switched to the laser irradiation by means of an optical fiber.

The LLLI was performed locally on the selected mesenteric area by using a laser driver (Panalas-1000A, Matsushita, Osaka, Japan) for 5 minutes by means of the optical fiber. This driver generated a very thin (1-mm output core diameter) circular laser beam with a wavelength of 830 nm. In the experimental protocol, the power density of the projected beam was set at 30 mW/fiber-core (equivalent to 38.2 mW/mm^2), unless otherwise stated. The power density of the LLLI was expressed with the mW/mm^2 unit.

The microscopic image of the vasculature was divided by a half-prism into two light beams, one of which was simultaneously projected onto the velocimeter for measurement of V_{RBC} and the other onto a video camera (C2400-77, Hamamatsu Photonics, Shizuoka, Japan) for measurement of the arteriolar diameter. In the velocimeter, two small sensor windows were placed along the axis of a magnified red cell flow image (inset B in Fig. 1) and photometric signals acquired from these windows were transferred to a desktop microcomputer (PC9801-FA, NEC, Tokyo, Japan) by means of an A/D converter. V_{RBC} was determined digitally by computing the delay time between these two signals (τ) (dual-slit photometric technique) [20,21]. The videotape was subsequently played back through a digital image processor

(ARGUS 10, Hamamatsu Photonics), and the inner diameter of the arteriole (D) at the V_{RBC} measurement site was determined. D and V_{RBC} were measured before and after (from 1 to 30 minutes) the LLLI. The arteriolar blood flow (Q) was calculated from these values as follows:

$$Q = \pi \times (D/2)^2 \times V_{RBC}/1.6,$$

where the factor 1.6 was used to convert the V_{RBC} measured along the centerline of the blood stream to mean red cell velocity averaged over the entire diameter [21].

Thermal Effects of LLLI

In this study, we applied a laser with a near-infrared (NIR) wavelength of 830 nm. Because an NIR laser irradiation of tissue might cause temperature elevation and consequently alter blood flow, the thermal effects of LLLI on the mesenteric microcirculation were also assessed. A thin thermocouple (BAT-10, Physitemp, Clifton, NJ) was placed in contact with the mesentery, positioning the tip in a close vicinity to a mesenteric arteriole but out of the area affected by the irradiation beam, and the changes in local tissue temperature were measured together with those in the arteriolar blood flow during the laser irradiation with graded power densities (0, 6.4, and 38.2 mW/mm^2). Subsequently, the bulk temperature in the experimental chamber was modified, simulating local temperature elevation during the LLLI, and the resultant changes in the microcirculatory blood flow were examined.

Effects of LLLI on Vascular Smooth Muscle Cell In Vitro

Vascular smooth muscle cells (VSMCs) were obtained from thoracic aortas of 10-week male Wistar rats as described by Kanaide et al. [22]. Briefly, aortic *tunica media* was dispersed into dissociated single cells by incubation with 1 mg/ml collagenase and 0.5 mg/ml elastase (Worginton, Lakewood, NJ) and the cells were seeded on a glass bottom chamber (Mat Tek, Ashland, MA) in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO). After the formation of a confluent monolayer, the cultured cells were used for the experiment. To confirm that the cultured cells were VSMCs, immunocytochemical localization of smooth muscle specific α actin was carried out with monoclonal antibody ASM-1 (Progen, Heidelberg, Germany) [23]. High viability (>95%) of the primary culture cells was maintained throughout the experiments, which was confirmed by the trypan blue exclusion test [22].

Measurement of [Ca^{2+}]i was performed by using a calcium-sensitive fluorescence dye, fluo 3 (Dojin, Kumamoto, Japan). The cells were labeled with fluo 3 by incubation with 5 μM acetoxymethyl ester of fluo 3 (fluo 3-AM) for 60 minutes at 37°C, and then washed with normal physiological salt solution (normal PSS) at 25°C to remove extracellular dye. Normal PSS contained the following constituents (in mM): NaCl, 135; KCl, 5; CaCl$_2$, 1; MgCl$_2$, 1; glucose, 5.5; and HEPES, 10. The cells were incubated in normal PSS for at least 30 minutes before the initiation of image recording. Bathing solution was exchanged continuously throughout the experiment while maintaining the temperature at 25°C. The cells were viewed through a confocal fluorescence microscope (model BX50, Olympus, Tokyo, Japan), by using a ×20 water-immersion objective. The dye was photoactivated with a 488-nm wavelength, and it emitted 530-nm fluorescence.

After the recording of the control image, laser irradiation (continuous wave, Ga-Al-As laser, 830 nm) was performed on the VSMCs for 3 minutes with graded intensities of 0, 2.5, 5, and 10 mW/mm^2. After the irradiation, a time series of images were recorded to evaluate the changes in [Ca^{2+}]i from 1 to 10 minutes. Identical experiments were performed with the Ca^{2+}-free PSS bathing solution, which was prepared in the same manner as the normal PSS except that 2 mM EGTA was added instead of 1 mM CaCl$_2$.

Analysis of the acquired confocal images was performed with a commercial software package (Flow View, Olympus, Tokyo, Japan). The intensity of the fluo 3 fluorescence was not converted to [Ca^{2+}]i, due to the nonratiometric property of fluo 3 [24]. The mean intensity of the fluo 3 fluorescence in the selected area after stimulation was divided by the mean intensity of the fluo 3 fluorescence in the same area before LLLI, after subtraction of background fluorescence [24] and this relative fluorescence intensity was used as an indicator for [Ca^{2+}]i.

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA) with Scheffe's

TABLE 1. Effects of Low-Level Laser Irradiation (LLLI) on Systemic Circulation in Four Experimental Groups

	Before LLLI	Time after LLLI	
		1 min	10 min
Mean arterial pressure (mmHg)			
Control (n = 7)	118 ± 6.7	114 ± 7.1	116 ± 8.8
LLLI (n = 8)	114 ± 5.1	115 ± 6.1	109 ± 5.1
LLLI+L–NAME (n = 7)	129 ± 7.5	127 ± 5.7	122 ± 5.8
LLLI+DNRV (n = 8)	112 ± 5.4	110 ± 7.1	110 ± 6.1
Heart rate (beats/min)			
Control (n = 7)	368 ± 19	372 ± 21	385 ± 11
LLLI (n = 8)	363 ± 17	365 ± 17	368 ± 18
LLLI+L–NAME (n = 7)	350 ± 18	349 ± 22	356 ± 17
LLLI+DNRV (n = 8)	359 ± 17	363 ± 12	364 ± 14

*DNRV, denervation. Values are mean ± SEM. See text for details of the four groups. LLLI exerted no significant effects on the circulatory parameters.

TABLE 2. Microcirculatory Parameters Before Low-Level Laser Irradiation (LLLI) in Four Experimental Groups

	Vessel diameter (μm)	Red cell velocity (mm/sec)	Blood flow ($\times 10^{-3}$ mm^3/sec)
Control (n = 7)	19.7 ± 1.9	10.0 ± 1.4	1.91 ± 0.24
LLLI (n = 8)	20.0 ± 1.5	9.6 ± 0.7	1.88 ± 0.33
LLLI+L–NAME (n = 7)	19.5 ± 0.9	9.8 ± 1.2	1.83 ± 0.23
LLLI+DNRV (n = 8)	20.2 ± 0.5	9.5 ± 1.2	1.91 ± 0.26

*DNRV, denervation. Values are mean ± SEM. See text for details of the four groups. No significant differences were detected among the four groups.

method. Statistical significance was tested at the $P < 0.05$ level.

RESULTS

Effects of LLLI on In Vivo Microcirculation

LLLI did not affect systemic circulation (mean arterial pressure and heart rate), as shown in Table 1. The microcirculatory variables measured in the control (preirradiation) state are summarized in Table 2. Because the microcirculatory parameters measured were heterogeneous, these variables were analyzed and presented as the relative percentage changes from the control state.

The arteriole showed a considerable dilation shortly after the LLLI, as typically presented in Figure 2. The arteriolar diameter (D) showed a rapid increase at ~1 minute after LLLI and further increased to ~120% of the preirradiation value (Fig. 3A, open circles). The occurrence of an increase in red cell velocity (V_{RBC}) was retarded slightly, but V_{RBC} showed a significant increase by ~5 minutes after LLLI, reaching ~130% of the preirradiation value at the final state (Fig. 3B, open circles). As a consequence of these changes, the blood flow (Q) showed a rapid initial increase even at ~1 minute after LLLI (increase in the *early phase*), and continued to increase till 30 minutes after LLLI (increase in the *delayed phase*), finally leveling off at ~200% of the preirradiation value (Fig. 3C, open circles). In several preparations, observations were continued for an extended period up to 120 minutes. In most of these preparations, however, the blood flow that leveled off at 25–30 minutes did not show any further increase throughout the extended period.

In the LLLI+L-NAME group (Fig. 3, triangles), the rapid increases in D, V_{RBC}, Q in the early phase were attenuated. In particular, increases in V_{RBC} were highly attenuated and significant changes in this variable were not detected until 10 minutes after LLLI. However, in the delayed phase, these microcirculatory parameters reached the same level as those observed in the LLLI group by 10 minutes. This result suggests that nitric oxide would be involved in the increase in Q in the early phase.

In the LLLT + DNRV group (Fig. 3, squares), microcirculatory responses were similar to those observed in the LLLI group in both the early and the delayed phases, which indicated that the local denervation did not affect the increase in blood flow after LLLI.

The changes in the microcirculatory blood flow after graded LLLI are summarized in Figure 4. When the laser power was set at 6.4 mW/mm^2, no significant elevation in the local tissue temperature occurred and the microcirculatory blood flow did not show any significant changes. When irradiated with the power density of 38.2 mW/mm^2, the maximal temperature elevations detected were 0.8–1.0°C. Thus, it seemed possible that the 38.2 mW/mm^2-irradiation used in most of our in vivo protocols would cause microcirculatory changes due to a direct heat effect. However, in the absence of LLLI, no significant arteriolar vasodilation was detected even if the bulk temperature in the chamber was raised by 1°C by injecting warmed PBS medium into the chamber (data not shown). From these results, the involvement of macrothermal effects due to LLLI in the observed circulatory changes should be ruled out under the present experimental conditions.
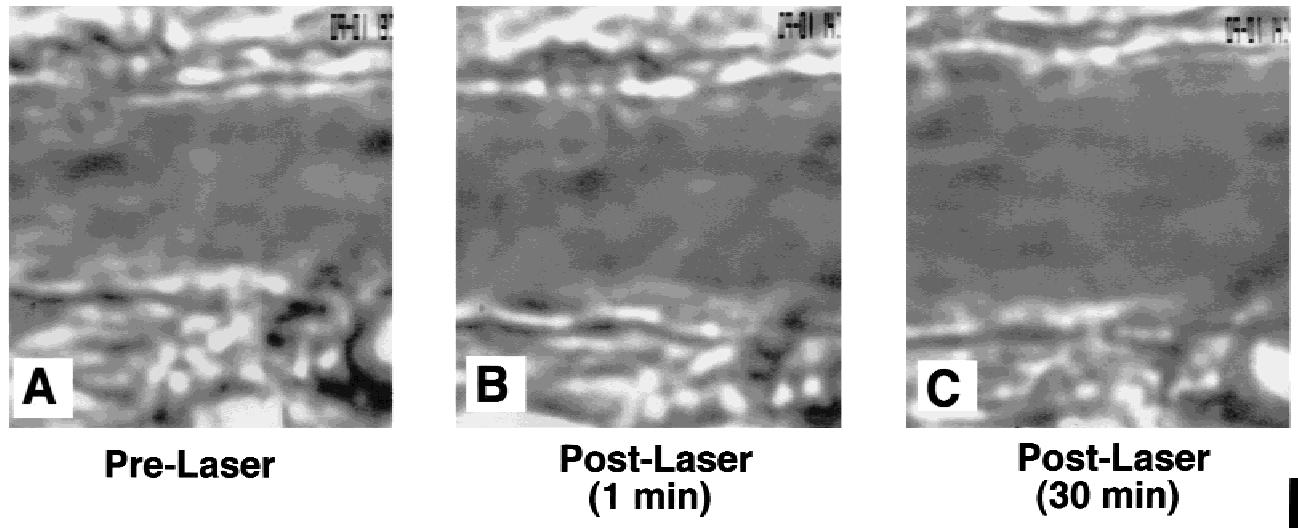
The possible involvement of microthermal ef-

Fig. 2. Photomicrographs showing typical arteriole responses to low-level laser irradiation (LLLI). The images were recorded before (**A**), 1 minute after (**B**), and 30 minutes after (**C**) the LLLI. Arteriolar dilation occurred shortly after the laser irradiation (B) and reached a maximum at ~30 minutes after the LLLI (C). Scale bars = 10 μm.

fects due to LLLI was also assessed by another spot-LLLI protocol, as follows. In this experiment, the system used for the laser irradiation, shown in Figure 1, was slightly modified, inserting a ×4 objective lens between the experimental chamber and the tip of optical fiber. This modification made it possible to further collimate the laser beam emitted from the fiber as a very thin (~8 μm in diameter) circular spot. Figure 5 shows a typical image of a spot LLLI projected on a single arteriole, with the identical power density of 38.2 mW/mm² used in the other in vivo protocols. In these additional experiments, the spot LLLI caused similar vasodilation shortly (1 minute) after the irradiation (9.7 ± 1.5%, n = 6, $P < 0.01$), although the thermal effect due to LLLI was minimized because the local heat possibly generated on the microvessel was not only absorbed by the surrounding medium but also washed out by the blood flow. Thus, it seemed unlikely that the observed microcirculatory changes were mainly attributable to microthermal effects due to LLLI.

Effects of LLLI on VSMCs In Vitro

LLLI induced a significant decrease in the fluorescence intensity (Fig. 6) in a power-dependent manner (Fig. 7A, filled circles, open triangles, squares). To confirm that the decrease in fluorescence intensity was not an artifact due to dye leakage caused by LLLI, a cell-free solution of 5 μM fluo 3 was subjected to 10 mW/mm² LLLI

(Fig. 7A, open circles). The resultant changes in the fluorescence intensity were not significantly different from those without LLLI. Furthermore, without the LLLI, cytosolic fluorescence intensity in VSMCs loaded with fluo 3 did not change significantly in normal PSS containing 1.0 mM Ca²⁺ throughout the observation period (Fig. 7A, filled triangles). These findings indicated that the decrease in fluorescence intensity after LLLI (see below) was not due to the so-called photo-bleaching effect of the dye by repeated 488-nm photoactivation, but the decrease in [Ca²⁺]i.

When the VSMCs were exposed to Ca²⁺-free PSS containing 2 mM EGTA, the fluorescence intensity decreased to reach a steady state level within 6 minutes, and it remained at this level during and after LLLI at a power level of 10 mW/mm² (Fig. 7B, filled triangles).

DISCUSSION

Improved circulation due to low-level laser irradiation is considered to be one of the possible mechanisms of the clinical effectiveness of LLLI for the treatment of pain or to promote the healing of wounds [1–4]. In regard to circulatory responses to LLLI, studies have been conducted on larger vessels such as, for example, the aorta [25]. To date, to the best of our knowledge, no studies have been performed in vivo on microcirculatory changes during LLLI. Our experimental system,
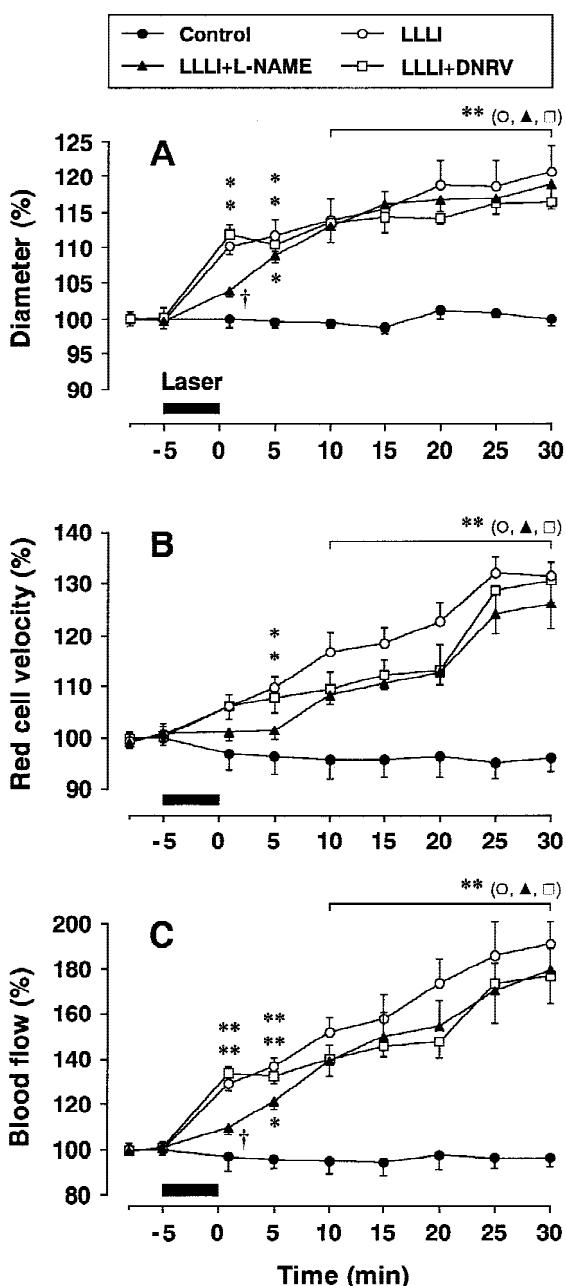
Fig. 3. Relative changes in arteriolar diameter (D), red cell velocity (V_{RBC}), and arteriolar blood flow (Q) after low-level laser irradiation (LLLI, 38.2 mW/mm²) in four experimental groups. See text for details of the four groups. Increases in D, V_{RBC}, and Q occurred at ~1–5 minutes after the laser irradiation (the early phase) and further increased, finally leveling off at ~200% of the preirradiation value. Note that those changes in $V_{RBC}$the early phase were highly attenuated in the LLLI+L-NAME group (triangles). Data are mean ± SEM. Single asterisks indicate $P < 0.05$ and double asterisks indicate $P < 0.01$ for Control vs. other three groups. Daggers indicate $P < 0.05$ for LLLI group vs. LLLI+L-NAME group or LLLI + DNRV group.
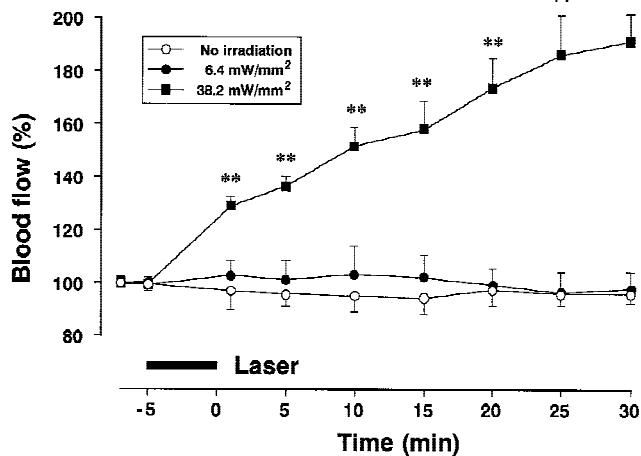

Fig. 4. Contrasted changes in arteriolar blood flow after laser irradiation with graded power densities (open circles, no irradiation; filled circles, 6.4 mW/mm²; squares, 38.2 mW/mm²). The LLLI administration period is indicated by a bar on the abscissa. No significant changes in blood flow occurred when the laser power was set at 6.4 mW/mm². Note that the apparent increase in blood flow at 30 minutes in this protocol is not statistically significant. Data are presented as the relative change from the control state (mean ± SEM). Double asterisks indicate $P < 0.01$ for no irradiation group vs. other three groups.

equipped with a watertight chamber with well-defined temperature control, has allowed us to perform in vivo observation and detailed evaluation of microcirculatory changes in rat mesentery caused by LLLI, together with an assessment of the involvement of NO and vasomotor nerves in these changes. Furthermore, we have studied the effect of LLLI on $[Ca^{2+}]i$ in cultured smooth muscle cells in vitro, which has provided us with some insights into the mechanisms of LLLI-induced vasodilation in microvascular beds.

Our results showed that LLLI is capable of inducing potent arteriolar vasodilation and a consequent increase of blood flow in mesenteric microcirculation. This increase in blood flow (Q) consisted of two phases; the first increase occurred shortly after the laser irradiation, and the increase of Q in this stage was ~20% (early phase). The second additional increase occurred approximately 20 minutes after the irradiation and, as a whole, the total increase in Q reached ~200% of the preirradiation level (delayed phase).

In some animals, we made additional observations of microcirculatory changes for up to 120 minutes after the irradiation. Throughout this extended period, the dilated arteriole exhibited no significant recovery or constriction. These changes in blood flow were not abolished by sur-
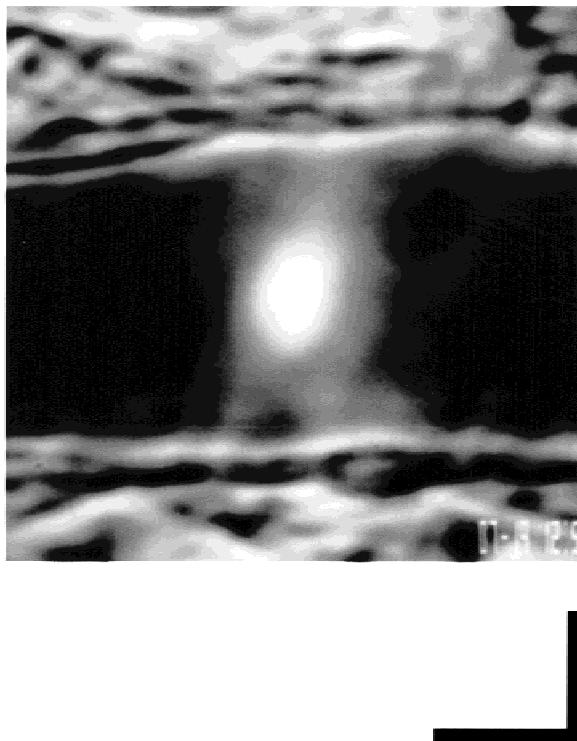
Fig. 5. Photomicrograph showing a thin circular spot of a laser beam (~8 μm in diameter, shown as a bright area surrounded by a gray-toned halo) projected on a single arteriole. Despite the very limited local irradiation, the spot LLLI still caused arteriolar dilation by 9.7 ± 1.5% (n = 6, $P < 0.01$). In this protocol, the experimental setup shown in Figure 1 was slightly modified, inserting a ×4 objective lens between the experimental chamber and the tip of the optical fiber. By this modification, the laser beam emitted from the fiber was further collimated as a thin circular spot. Scale bars = 10 μm.

gical, local denervation. However, in the mesentery treated by L-NAME, the occurrence of these changes was retarded and no significant increase in Q was detected until ~10 minutes after the irradiation.

The sympathetic vasomotor nerve plays a key role in the control mechanisms of arteriolar constriction [8]. Furthermore, it is known that LLLI attenuates neural conduction in the dorsal root of sensory nerves [6,7]. Thus, it is reasonable to hypothesize that the attenuation of the vasomotor nerve activity by the laser would be involved in the LLLI-induced arteriolar vasodilation. As noted above, however, local denervation of the superior mesenteric artery did not affect the vasodilation that occurred in laser-irradiated mesenteric arteriole. Thus, it seems unlikely that the arteriolar dilation observed in this study was caused by the attenuation of vasomotor nerve due to LLLI. From a clinical viewpoint, however, this

apparently negative finding would support the effectiveness of LLLI therapy in patients with neurologically impaired blood vessels [26] (patients with diabetes mellitus, for example).

As another mechanism of LLLI-induced vasodilation, we assessed the involvement of NO in the microvascular responses to LLLI. In the LLLI+L-NAME group, as noted above, rapid increases in D, V_{RBC}, and Q in the early phase were abolished, although they reached similar levels as those observed in the LLLI group by minutes after the irradiation. Furchgott et al. [11] stated that the relaxation of vascular smooth muscles after photo-irradiation highly resembles the vasodilation caused by certain nitrogen compounds such as nitroglycerin. Furthermore, Karlsson et al. [13] found that, in bovine mesenteric circulation, a close relationship exists between the photo-induced arterial relaxation and the cGMP content in the arterial wall. Because NO is known to up-regulate the production of cGMP [27], these previous studies and our present observations are consistent with each other and strongly suggest that NO plays some role in the vasodilation and the increase in blood flow observed in the early phase after the laser irradiation.

In this study, we applied a laser with a near-infrared (NIR) wavelength of 830 nm. Because an NIR beam is capable of producing heat in irradiated tissues, it seemed possible that direct macrothermal or microthermal effects of laser were involved in the microvascular responses observed after LLLI. This possibility of macrothermal effects was first assessed by measuring the changes in local temperature in the laser-irradiated area. When irradiated at 38.2 mW/mm^2, the measured maximal elevation in the local tissue temperature was 0.8–1.0°C. However, in the absence of LLLI, no significant arteriolar vasodilation was detected even when the bulk temperature of the PBS medium surrounding the mesentery was raised to the same temperature range. The possibility of microthermal effects at the tissue level was further assessed by performing spot laser irradiation on single isolated arterioles with an identical power density of 38.2 mW/mm^2 (Fig. 5). Such spot LLLI caused marked vasodilations similar to those shown in Figure 3. In these experiments, the thermal effect of the laser was minimized, because the local heat possibly produced by LLLI on the spot-lit microvessel was not only absorbed by the surrounding PBS medium but also washed out by the blood flow.
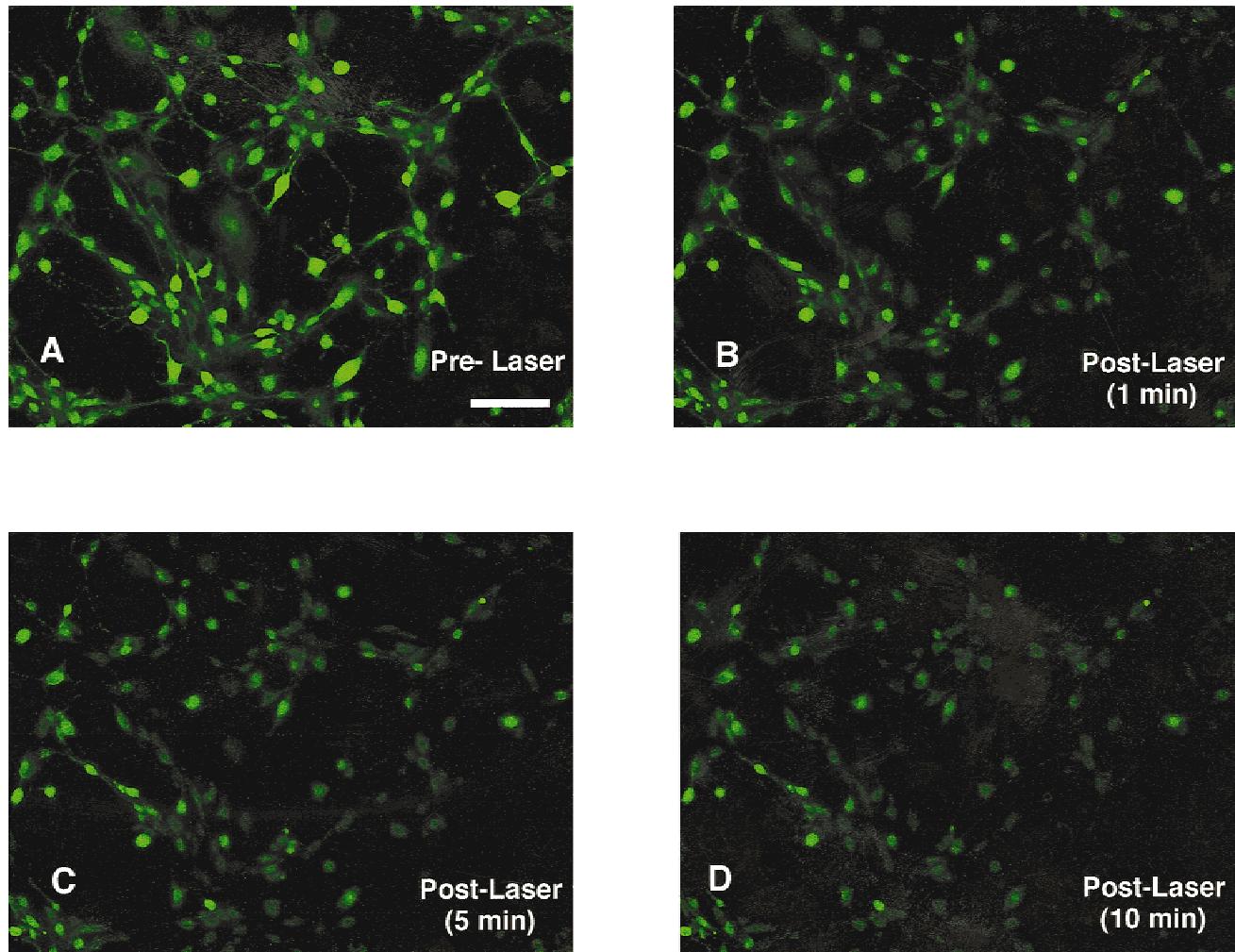
In clinical practice, LLLI treatments by us-

Fig. 6. Microfluorographs showing changes in fluorescence emitted from rat vascular smooth muscle cells (VSMCs) loaded with fluo 3. The micrographs were recorded before (**A**), 1 minute after (**B**), 5 minutes after (**C**), and 10 minutes after (**D**) the laser irradiation (λ = 830 nm, 10 mW/mm^2 for 3 minutes). The decrease in the emitted fluorescence indicates that the laser irradiation reduces intracellular Ca^{2+} concentration in VSMCs.

ing an NIR laser beam is expected in part to exert thermal effects on patients but, from the two assessments noted above, the involvement of heat due to LLLI in the observed circulatory changes seemed unlikely under the present experimental conditions. However, the possible existence of highly microscopic thermal effects manifested at, for example, macromolecular levels should not be ruled out, because such molecular effects by means of light absorption could cause changes in various enzymatic functions and lead to the bulk effects observed at the tissue level. These photochemical aspects deserve deeper investigations in the future.

Based on the assessment of these various factors, we next studied the involvement of vas-cular smooth muscles in the circulatory changes observed after LLLI. For this purpose, we examined the effect of LLLI on [Ca^{2+}]i in rat aortic vascular smooth muscle cells (VSMCs) in primary culture. This was done in vitro, because the inherent experimental limitations of in vivo studies did not allow us to differentiate in vivo changes in VSMCs from those in endothelial cells. LLLI induced a decrease of [Ca^{2+}]i in normal PSS containing 1 mM Ca^{2+} in a power-dependent manner (Fig. 7), which indicated that LLLI exerts a direct effect on VSMCs. In the absence of extracellular Ca^{2+}, LLLI did not reduce [Ca^{2+}]i in VSMCs (Fig. 7B), suggesting that the laser-induced reduction of [Ca^{2+}]i was attributable to increased Ca^{2+} extrusion by means of the sarcolemma, not to any
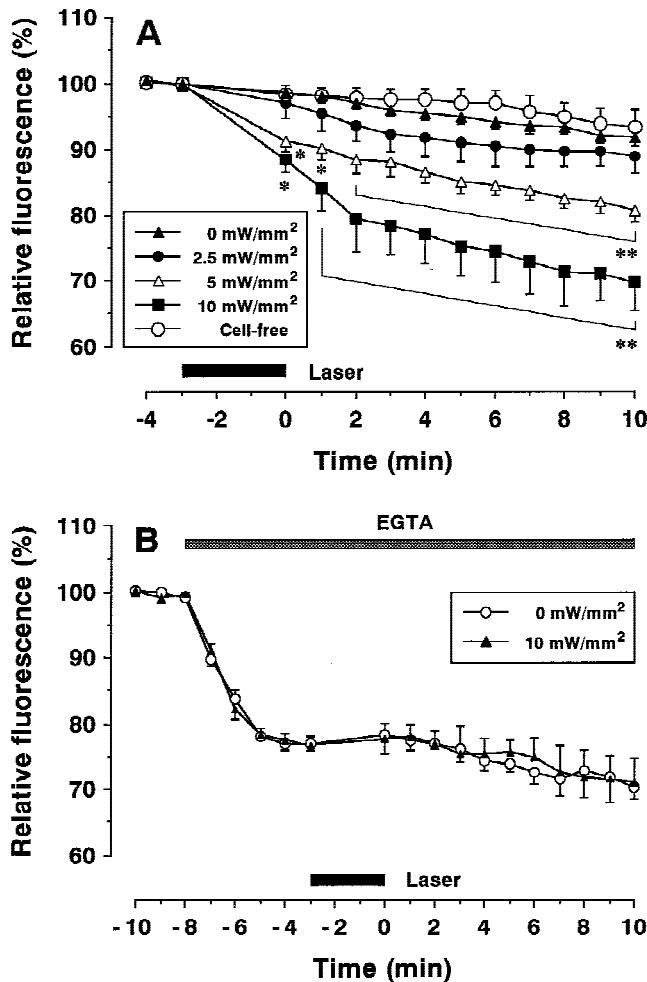
Fig. 7. Effects of low-level laser irradiation (LLLI, $\lambda = 830$ nm, 10 mW/mm^2) on fluorescence emitted from rat vascular smooth muscle cells (VSMCs) loaded with fluo 3. Data are presented as the relative change from the control state (mean $\pm$ SEM, n = 10). **A:** Dependence of the fluorescence intensity emitted from fluo 3-loaded VSMCs on the power density of irradiated laser. Open circles denote the fluorescence intensity emitted from a cell-free solution of 5 μM fluo 3 after LLLI (10 mW/mm^2). Single asterisks indicate $P < 0.05$ and double asterisks indicate $P < 0.01$ for no irradiation group vs. other three groups. **B:** Effects of the removal of extracellular Ca^{2+} from the suspension medium on the laser-induced reduction in fluorescence emitted from fluo 3-loaded VSMCs. Removal of extracellular Ca^{2+} from the medium exerted no significant influence on the changes presented by open circles and filled triangles.

acceleration of Ca^{2+} sequestration into the Ca^{2+} storage site in VSMCs. The possibility of increased Ca^{2+} extrusion in laser-irradiated smooth muscle has been pointed out by Nasu et al. [28]. By using histochemical techniques, they showed that Ca^{2+}-dependent ATPase was localized in endothelial cells and smooth muscle cells of rat saphenous arteries and that the activity of Ca^{2+}-dependent ATPase was increased by NIR laser irradiation without temperature elevation [28]. The power density of the laser applied was not provided in their report but, because activation of Ca^{2+}-dependent ATPase leads to a reduction in $[Ca^{2+}]i$, it seems highly likely that the decrease in $[Ca^{2+}]i$ in the VSMCs observed in the present study was caused by means of similar mechanisms.

As another possible mechanism leading to the dilation of smooth muscle cells, changes in membrane potential might be worthy of further interest. Shimoyama et al. [8] studied the effect of LLLI on the resting membrane potential of rat superior ganglion cells, and showed that LLLI induced hyperpolarization of the ganglion cells. Thus, assuming that ganglion cells and smooth muscle cells exhibit a similar electrical response to LLLI, it might be possible that a hyperpolarization mechanism was somewhat involved in the vasodilation observed in the present study.

In analyzing phenomena related to light, the dependence on wavelength should be taken into consideration. In this study, we used a laser with an NIR wavelength (830 nm) for LLLI, because this wavelength range is widely used in pain clinics due to its useful property of deeper penetration into tissues [6]. By using a xenon arc and a grating monochrometer, Furchgott et al. [11] showed that the light-induced relaxation of smooth muscles in the aorta strip was rather potent in the ultraviolet (UV) range and had a spectral dependence on wavelength of the irradiated light; the relaxation spectrum they presented showed a dominant peak at the wavelength of 350 nm and, in the remaining range of visible and infrared light, the relaxation was minimal. In contrast, Gal et al. [12] showed that light-induced vasodilation could be caused by visible and infrared light. Although the contradiction between these results seems attributable to the difference in the power of the laser applied, the potent effects of UV light on the aortic relaxation shown by Furchgott et al. [11] would require deeper investigation for possible clinical application.

In conclusion, we have shown that laser irradiation at a wavelength of 830 nm is a potent dilator of the arteriole and consequently causes a marked increase in blood flow in the rat microvascular bed. Nitric oxide seems partly involved in the vasodilation that occurs in the early phase. However, extrapolating our in vitro findings in VSMCs to in vivo situations, the marked arteriolar dilation throughout the delayed phase seems

attributable to the LLLI-induced reduction of $[Ca^{2+}]i$ in microvascular smooth muscles.

REFERENCES

1. Walker J. Relief from chronic pain by low power laser irradiation. Neurosci Lett 1983;43:339–344.
2. Goldman JA, Chiapella J, Casey H, Bass N, Graham J, McClatchey W, Dronavalli RV, Brown R, Bennett WJ, Miller SB, Wilson CH, Pearson B, Haun C, Persinsk L, Huey H, Muckerheide M. Laser therapy of rheumatoid arthritis. Lasers Surg Med 1980;1:93–101.
3. Iijima K, Shimoyama N, Shimoyama M, Yamamoto T, Shimizu T, Mizuguchi T. Effect of repeated irradiation of low-power He-Ne laser in pain relief from postherpetic neuralgia. Clin J Pain 1989;5:271–274.
4. Basford JR. Low-level laser treatment of pain and wounds. Mayo Clin Proc 1986;61:671–675.
5. Mester E, Spiry T, Szende B, Tota JG. Effect of laser rays on wound healing. Am J Surg 1971;122:532–535.
6. Tsuchiya K, Kawatani M, Takeshige C, Sato T, Matsumoto I. Diode laser irradiation selectively diminishes slow component of axonal volleys to dorsal roots from the saphenous nerve in the rat. Neurosci Lett 1993;161:65–68.
7. Kasai S, Kono T, Yamamoto Y, Kotani H, Sakamoto T, Mito M. Effect of low-power laser irradiation on impulse conduction in anesthetized rabbits. J Clin Laser Med Surg 1996;14:107–109.
8. Shimoyama M, Fukida Y, Sjimoyama N, Iijima K, Mizuguchi T. Effect of He-Ne laser irradiation on synaptic transmission of the superior cervical sympathetic ganglion in the rat. J Clin Laser Med Surg 1992;10:337–342.
9. Yu W, Naim JO, Lanzafame RJ. The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. Photochem Photobiol 1994;59:167–170.
10. Ohta A, Abergel RP, Uitto J. Laser modulation of human immune system: inhibition of lymphocyte proliferation by a gallium-arsenide laser at low energy. Lasers Surg Med 1987;7:199–201.
11. Furchgott RF, Ehrreich SJ, Greenblatt E. The photoactivated relaxation of smooth muscle of rabbit aorta. J Gen Physiol 1961;44:499–519.
12. Gal D, Chokshi SK, Mosseri M, Clarke RH, Isner JM. Percutaneous delivery of low-level laser energy reverses histamine-induced spasm in atherosclerotic Yucatan microswine. Circulation 1992;85:756–768.
13. Karlsson JO, Axelsson KL, Andersson RGG. Effects of ultraviolet radiation on the tension and the cyclic GMP level of bovine mesenteric arteries. Life Sci 1984;34:1555–1563.
14. Karlsson JO, Axelsson KL, Elwing H, Andersson RGG. Action spectra of photoactivated cyclic GMP metabolism and relaxation in bovine mesenteric artery. J Cyclic Nucleotide Protein Phosphor Res 1986;22:155–166.
15. Liu DT, Reid MT, Bridges DC, Rush RA. Denervation, but not decentralization, reduces nerve growth factor content of the mesenteric artery. J Neurochem 1996;66:2295–2999.
16. Salim AS. Reserpine, vagal adrenergic activity and stress-induced acute gastric mucosal injury in the rat. J Physiol (Lond) 1987;392:363–376.
17. Kosaka H, Seiyama A, Terada N, Yoneyama H, Hirota S, Kitamura Y. Absence of mast cell involvement in leukocyte adhesion and emigration induced by inhibition of nitric oxide synthase. Lab Invest 1997;77:575–580.
18. Itoh T, Yaegashi K, Kosaka T, Kinoshita T, Morimoto T. A closed chamber for intravital microscopy of the rat mesentery under controlled oxygen tension and temperature. Jpn J Physiol 1993;43:847–854.
19. Itoh T, Yaegashi K, Kosaka T, Fukushima H, Morimoto T. A simple construction of dual-slit red cell velocimeter. Jpn J Physiol 1995;45:681–686.
20. Wayland H, Johnson PC. Erythrocyte velocity measurement in microvessels by a two-slit photometric method. J Appl Physiol 1967;22:333–337.
21. Baker M, Wayland H. On-line volume flow rate and velocity profile measurement for blood in microvessels. Microvasc Res 1974;7:131–143.
22. Kanaide H, Kobayashi S, Nishimura J, Hasegawa M, Shogakiuchi Y, Matsumoto T, Nakamura M. Quin2 microfluorometry and effects of verapamil and diltiazem on calcium release from rat aorta smooth muscle cells in primary culture. Circ Res 1988;63:16–26.
23. Munzenmaier DH, Greene AS. Angiotensin II mediates a sustained rise in nuclear and cytoplasmic calcium via multiple receptor subtypes. Am J Physiol 1995;269:H565–H570.
24. Ohata H, Ujike Y, Momose K. Confocal imaging analysis of ATP-induced Ca^{2+} response in individual endothelial cells of the artery in situ. Am J Physiol 1997;272:C1980–C1987.
25. Steg PG, Gal D, Rongion AJ, DeJesus ST, Clarke RH, Inser JM. Effect of argon laser irradiation on rabbit aortic smooth muscle. Cardiovasc Res 1988;22:747–753.
26. Beggs J, Johnson PC, Olafsen A, Watkins CJ. Innervation of the vasa nervorum: changes in human diabetics. J Neuropathol Exp Neurol 1992;51:612–629.
27. Kowaluk EA, Seth P, Fung HL. Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. J Pharmacol Exp Ther 1992;262:916–922.
28. Nasu F, Tomiyasu K, Inomata K, Calderhead HG. Cytochemical effects of GaAlAs diode laser radiation on rat saphenous artery calcium ion dependent adenosine triphosphatase activity. Laser Ther 1989;1:89–92.