Modulating nitric oxide levels in dorsal root ganglion neurons of rat with low-level laser therapy*

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Nitric oxide (NO) and nitric oxide synthase (NOS) have an important role in pain signaling transmission in animal models. Low-level laser therapy (LLLT) is known to have an analgesic effect, but the mechanism is unclear. The aim of the study is to investigate the influence of LLLT on NO release and NOS synthesis in dorsal root ganglion (DRG) neurons, in order to find whether LLLI can ameliorate pain through modulating NO production at the cellular level. The results show that in stress conditions, the laser irradiation at 658 nm can modulate NO production in DRG neurons with soma diameter of about 20 μm in a short time after illumination, and affect NOS synthesis in a dose-dependent manner. It is demonstrated that LLLT might treat pain by altering NO release directly and indirectly in DRG neurons.

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Low-level laser therapy (LLLT) has been known since the first laser was invented. It has been used in many areas, such as photorejuvenation[1], as well as modulating a broad range of disease, from wound healing[2] to brain damage[3]. The efficacy of LLLT in the treatment of pain has also been established, including orthodontic pain[4], chronic neck pain[5], arthritis[6, 7] and so on. But the mechanism of LLLT in analgesia is not fully understood. Some evidences suggest that the neural mechanisms are the basis of laser-induced pain relief[8-10]. Kudoh et al[9] found that the gallium aluminum arsenide diode laser irradiation at 830 nm could inhibit rat saphenous nerve sodium-potassium-adenosine triphosphate activity. In addition, Chow et al[10] found that the laser irradiation at 830 nm could induce the varicosity formation, reduce the mitochondrial membrane potential and block the fast axonal flow in rat dorsal root ganglion (DRG) neurons with small and medium diameter. These data suggest that LLLT may result in an inhibition by altering sodium-potassium-adenosine triphosphate activity or by blocking axonal flow to increase the nociceptive threshold. However, the evidences for neural mechanism of light-induced analgesic effect are lacked, and more studies are needed.

Nitric oxide (NO) is a ubiquitous intercellular free-radical gaseous messenger, which is biosynthesized from the amino acid L-arginine by a family of at least three isoenzymes termed as nitric oxide synthase (NOS), namely inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS)[11,12]. The activity of iNOS is not calcium dependent, while the activities of both eNOS and nNOS are calcium dependent. NO is involved in a variety of physical and pathophysiological processes, such as pain signaling transmission[11,13-15]. Some in vivo studies have suggested that LLLT might have an analgesic effect by modulating levels of NO[16-19]. But studies about the effect of LLLT on NO production in cells especially in DRG neurons are few. Regarding DRG neurons, they are the primary sensory neurons, and the cell bodies of nociceptors (peripheral nerve fibers) are located in the DRG for the body, which might explain the analgesic effect of laser irradiation. In this paper, we aim to investigate the effect of laser irradiation at 658 nm on NO release and NOS synthesis in DRG neurons.

Primary DRG neurons were obtained from healthy Sprague Dawley rats (8–12 weeks old) mainly according to the previous report[20]. After seeding into 35 mm culture dishes for 24 h, DRG neurons were used for LLLI. As shown in Fig.1, the laser beam is expanded by a lens and then reflected by a mirror to irradiate the cells in 35 mm culture dishes perpendicularly. The main parameters for LLLI are

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shown in Tab.1. Briefly, the light source for LLLI is 658 nm laser diode modules (Newport Corporation, Irvine, CA). Three laser energy densities on the irradiation surface of 2 J·cm^{-2}, 6 J·cm^{-2} and 16 J·cm^{-2} are chosen by adjusting the irradiation time as 3 min, 9 min and 24 min, respectively. The laser output power is 26.85 mW measured by a laser power meter (Coherent, Wilsonville, OR). The irradiation time is calculated according to the penetration rate measured by a laser power meter. The laser irradiation is performed in a dark room at room temperature.

After laser irradiation, NO in DRG neurons was monitored by confocal laser scanning microscopy combined with a specific fluorescence probe (DAF-2 DA) according to the previous study[21]. And the synthesis of NOS in DRG neurons after laser irradiation was detected using immunofluorescence staining[22], and then it was imaged by confocal laser scanning microscopy (Zeiss LSM 510, Germany)[20]. The primary antibody was rabbit anti-NOS 1 (R20) polyclonal antibody (1:100, Santa Cruz, sc: 648), and the second antibody was goat anti-rabbit-FITC IgG (1:400, Beijing Biosynthesis Biotechnology Cat No.bs-0295G-FITC). Both the fluorescence probes (DAF-2 DA and FITC) were excited with laser light at 488 nm from an argon laser, and their emission wavelengths were both set at 510–560 nm.

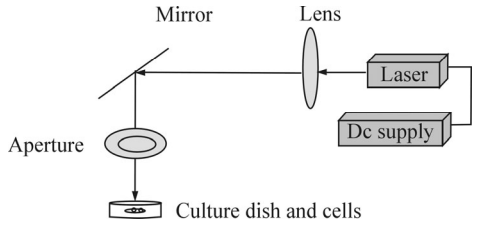


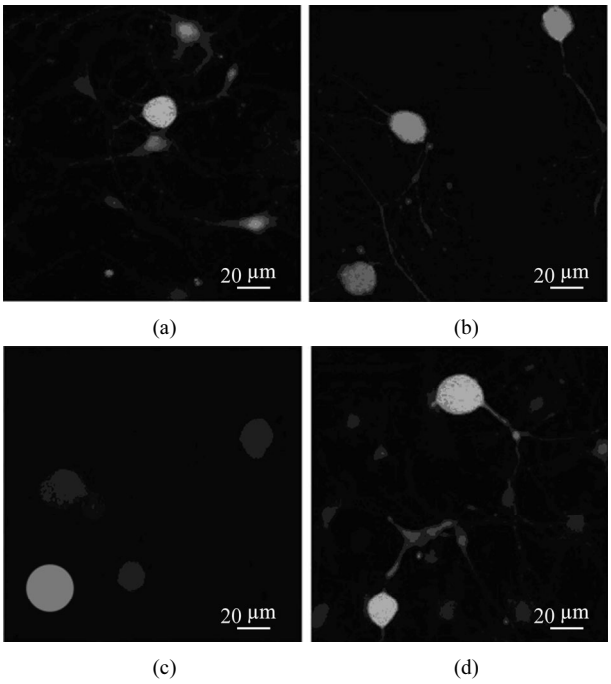
Fig.1 Schematic diagram of the optical setup for laser irradiation

Tab.1 Main parameters in laser irradiation

Parameter	Value		
Laser type (λ, nm)	658 nm		
Laser mode	Continuous wave		
Spot diameter (cm^2)	1.767		
Laser power (mW)	26.85		
Laser fluence (J·cm^{-2})	2	6	16
Irradiation time (min)	3	9	24
Irradiation treatment	Once a day		

DRG neurons express nNOS whose activity is in a Ca^{2+}-dependent manner. And it has been found from our previous studies that there was photobiostimulation in cells after laser irradiation only if cells are under stress conditions[23]. So in our present study, during irradiation, DRG neurons were incubated in Ca^{2+}-free Tyrode's solution with pH of 7.4, which contains 150 mmol/L NaCl, 5 mmol/L KCl, 10 μmol/L ethylene glycol tetraacetic acid (EGTA), 1 mmol/L $MgSO_4·7H_2O$, 10 mmol/L D-glucose and 10 mmol/L 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid (HEPES), to induce the stress conditions. Neurons were irradiated using 658 nm laser for 9 min with the corresponding laser energy density of 6 J·cm^{-2}. As shown in Fig.2, the fluorescence intensity of neurons incubated in Ca^{2+}-free Tyrode's solution changes significantly after laser irradiation (P<0.001), while there is little change of fluorescence intensity (P>0.05) in neurons incubated in Ca^{2+}-containing Tyrode's solution with pH of 7.4, which contains 150 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4·7H_2O$, 10 mmol/L D-glucose and 10 mmol/L HEPES. These results demonstrate that the laser irradiation can increase NO levels, and the phenomenon occurs only if cells are in stress conditions, which are in accordance with Ankri's findings[24]. We also find that the changes of NO release occur in DRG neurons whose soma diameter is about 20 μm. Passmorel[25] demonstrated that DRG neurons cultured in vitro especially with a soma diameter less than 30 μm shared characteristics with nociceptors. So these results suggest that NO in DRG neurons may be involved in irradiation-induced pain relieving.

Fig.3 shows that the NO release is elevated after 5 min illumination, and then it shrinks slowly after achieving a peak at 1 h. The increase of NO occurring immediately or shortly after irradiation may be associated with the direct release of NO by a photochemical mechanism. While the increase of NO occurring long after illumination, keeping for at least three hours in this study, may be proceeded by an increase of NOS activity resulting from illumination. Ankri et al[24] found that NO release increased after 1 h irradiation in endothelial cells, while evaluated immediately in sperm cell. All these results demonstrate that the mechanisms of illumination-induced NO production may be different in different cells. Another result also confirms that the NOS activity is involved in the light-induced NO release in DRG neurons.



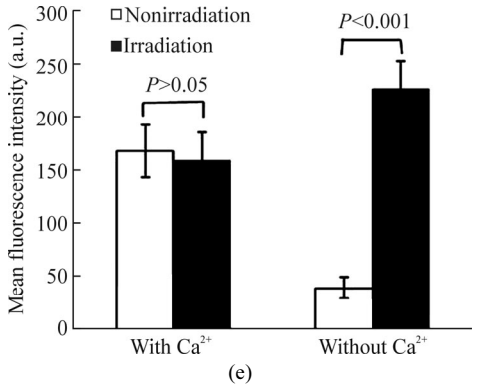


Fig.2 Images of neurons incubated in Ca^{2+}-containing Tyrode's solution (a) before and (b) after laser irradiation; Images of neurons incubated in Ca^{2+}-free Tyrode's solution (c) before and (d) after laser irradiation; (e) Mean fluorescence intensities of (a)–(d) (NO generation was induced by laser irradiation in DRG neurons in stress conditions.)

To investigate the effect of L-NAME, which is an inhibitor of NOS, on NO release in rat DRG neurons, neurons were incubated for 24 h at 37 °C with and without 300 μmol/L L-NAME, and stained with NO fluorescence dye of DAF-2DA for 45 min at 37 °C. Then the neurons were irradiated at 658 nm for 9 min at room temperature with and without 300 μmol/L L-NAME, and incubated for 20 min at room temperature. NO fluorescence was imaged using confocal microscopy, and then the NO concentrations were quantified using Image J software. The results represent n=15 neurons from three different culture dishes. As shown in Fig.4, the incubation of L-NAME does not significantly change the mean fluorescence intensity of neurons without laser irradiation (P>0.05), while the mean fluorescence intensity of neurons incubated with L-NAME (300 μmol/L) after laser irradiation is decreased significantly (P<0.05). These results demonstrate that the elevated NO production is coupled to the activation of NOS.

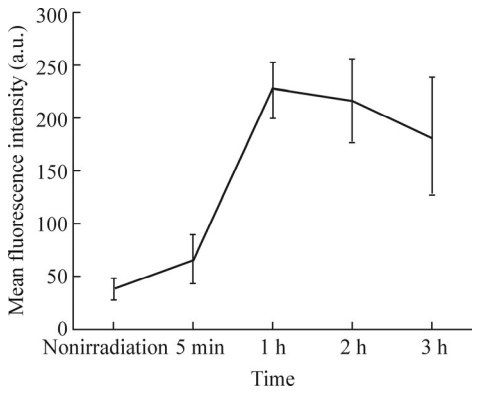


Fig.3 NO dynamics after irradiation with 658 nm laser

At last, the effect of laser irradiation at 658 nm with three different energy densities of 2 J·cm^{-2}, 6 J·cm^{-2} and 16 J·cm^{-2} on NOS production is investigated in order to detect the potential of laser irradiation in NO release indirectly. As shown in Fig.5, the neurons treated with 6 J·cm^{-2} irradiation evidently produce more NOS compared with the control group without irradiation (P<0.001) and those with 2 J·cm^{-2} irradiation (P>0.05), while 16 J·cm^{-2} irradiation inhibits the NOS production (P<0.05). This phenomenon may result from the biological characteristics of light, which is that the little-dose irradiation with enough time presents positive curative effect, while the high-dose irradiation with enough time shows negative effect.

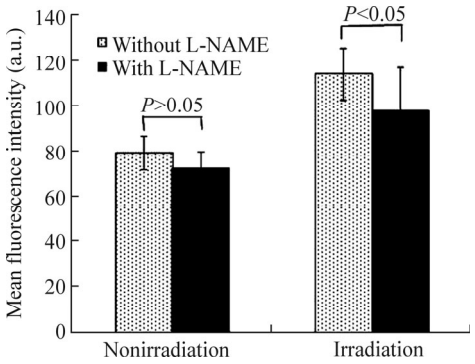


Fig.4 The effect of L-NAME on NO release in rat DRG neurons before and after irradiation

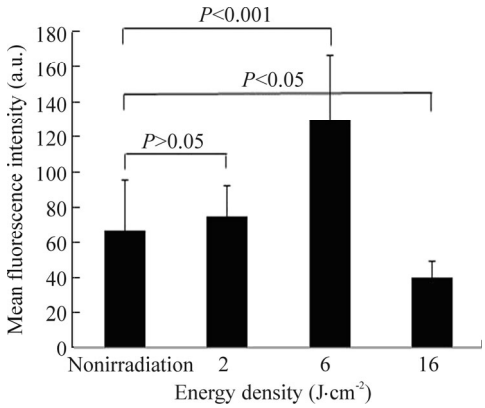


Fig.5 NOS concentration determined by immunofluorescence staining after irradiation with 658 nm laser in a dose-dependent manner

In conclusion, the irradiation with 658 nm laser shows positive effect directly or indirectly on NO release in DRG neurons, especially in DRG neurons with soma diameter of about 20 μm, and the NOS synthesis is in a dose-dependent manner. Both the photochemical mechanism and the NOS activity are involved in the illumination induced NO release. This study may partly explain the effects of laser irradiation on pain in the animal models.

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