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Biological Function of Low Reactive Level Laser Therapy (LLLT)

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Abstract

Low reactive level laser therapy (LLLT) and photobiomodulation are mainly focused on the activation of intracellular or extracellular photoabsorbable molecule (chromophore) and the initiation of cellular signaling using low power lasers and lights. Over the past 40 decades, a number of basic and clinical researches were reported that the laser therapy had the potential to improve wound healing and reduce pain and inflammation. In recent years, the term “LLLT” has become widely recognized. In this review, the mechanisms of action of LLLT at a cellular level are described. Finally, our recent research results that LLLT enhanced the cells differentiation are also described.

Keywords: low reactive level laser therapy (LLLT), intracellular chromophore, regulation of gene expression

1. Introduction

Low reactive level laser therapy (LLLT) is a form of medical treatment in which human tissue is irradiated with a low-powered laser (on the order of several 100 mW) to induce therapeutic changes. In an attempt to explore the carcinogenic potential of laser light, Mester et al. applied a low-powered ruby laser with a 694-nm wavelength to the shaved dorsal skin of mice [1]. Contrary to their expectations, the laser irradiation did not cause cancer, but instead improved hair growth. As the first study to document the biological effect of lasers, their findings became a springboard for subsequent LLLT research. Although light-based therapies had been used for a long time, and ultraviolet therapy has a history longer than a century [2], the work of Mester et al. was significant in demonstrating the effects of laser light, which has the unique characteristics of monochromaticity and coherence. Following subsequent experiments, Mester and colleagues reported in 1971 that low-level laser rays accelerated wound healing [3]. From this

time onward, experimental and clinical studies demonstrated many therapeutic effects of LLLT, including improvements in wound healing, collagen synthesis, cell proliferation, fracture repair, and local blood circulation, as well as suppression of inflammation and pain. These effects will be explained in more details in the sections to follow. The accumulated volume of clinical research suggests that LLLT has the potential to gain wide acceptance in clinical practice as a modality with few adverse effects. Today, however, that potential remains incompletely developed. What are the roadblocks to the clinical application of LLLT?

In their 1971 articles [3, 4], Mester et al. proposed irradiation of wounds with 5–25 mW helium-neon laser at an energy density (fluence) of 1–1.5 J/cm². Although many subsequent studies reported positive effects under these irradiation conditions, several studies did not demonstrate reproducibility. In addition, some scientists claimed that these positive effects were merely the result of laser-induced temperature increases, and others argued that outcomes differed significantly by study site and operator. These conflicting results and interpretations underscore the need to investigate and elucidate the therapeutic mechanisms of LLLT using an interdisciplinary approach involving molecular biology and other advanced sciences. To this end, we believe it is important to scrutinize published clinical studies of low-energy laser effects and to translate the clinical observations into molecular, cellular, and biological mechanisms. Although large volumes of *in vitro*, *in vivo*, and clinical articles on LLLT are published every year, this 50-year-old technique has not gained wide acceptance as a first-line option for treatment in clinical settings. A common problem described in review articles about the therapeutic use of lasers is that laser irradiation parameters vary considerably among operators, sites, and manufacturers. However, it is not technically feasible to apply particular uniform irradiation conditions to different types of patients or experimental animal species, because one must consider differences in the biological and physical conditions of the target organisms (cells) to which these lasers are administered.

In the following paragraphs, the typical biologic effects of LLLT are described, and then the cellular effects of LLLT that underlie its biological actions are discussed. Through our research, we have discovered (i) the presence of intracellular photoreceptors and physiological changes resulting from photoreception, (ii) postirradiation modifications in cellular signal transduction cascades, and (iii) postirradiation alterations in gene expression. These various effects do not occur in an isolated manner. Here, we focus on how these effects interact with each other to induce modifications in cellular functions. We also describe the typical results of several of our experiments involving different laser wavelengths, output levels, pulse lengths, irradiation times, and a variety of species and cell types.

2. Biologic effects of LLLT

2.1. Wound healing

A large number of studies have shown that LLLT accelerates wound healing, and we present some typical results here. Irradiation of cultured human keratinocytes with a 632-nm helium-neon laser elevated the interleukin-1 α and interleukin-8 mRNA levels, promoted

keratinocyte migration and proliferation, and accelerated wound repair [5]. In addition, *in vitro* studies of laser-irradiated cells revealed elevated levels of vascular endothelial growth factor (VEGF) [6] and transforming growth factor β (TGF β) expression [7]. These findings illustrate the laser-enhanced expression of many cytokines and growth factors in keratinocytes and fibroblasts, the key cellular mediators of the wound-healing process.

2.2. Antiinflammatory action

When mice with lipopolysaccharide-induced peritonitis were irradiated with a 904-nm gallium arsenide laser, inflammatory cell migration was inhibited [8]. In a rat model of carrageenan-induced pleuritis, a 660-nm indium-gallium-aluminum-phosphate laser suppressed the production of inflammatory cytokines and the migration of inflammatory cells [9]. A group of researchers led by Albertini are actively pursuing research on LLLT's antiinflammatory effects [10–30].

2.3. Bone growth and repair

LLLT accelerates osteoblast proliferation, bone formation [31], and bone repair [32]. Various groups have suggested the involvement of insulin-like growth factor 1 (IGF-1) [33], mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) [34], and bone morphogenetic protein (BMP)/Smad signaling cascades [35].

2.4. Neurologic effect

In addition to regeneration of damaged neurons [36–39], LLLT is effective in reducing pain. A rapidly growing body of literature has described the pain-relieving effect of LLLT. For more details, refer to the review article on this topic in those special journal issues.

2.5. Other effects

LLLT confers physiological effects on the articular cartilage [40] and muscle tissue [41–43]. In addition, LLLT confers aesthetic benefits, including its effects on hair growth/regrowth [44–48], acne treatment, [49, 50] and skin rejuvenation [51, 52].

3. Laser-induced cellular responses

In order to elucidate the biological mechanisms underlying effects of low-level lasers documented in experimental and clinical studies, one must consider the cellular responses to laser irradiation. In this section, we describe the intracellular photoreceptors and the cellular responses to laser light.

3.1. Intracellular photoreceptor

In photobiology, photoreception refers to the intracellular process whereby wavelength-specific photoreceptors absorb photon energy [53]. Photoreceptors are biomolecules that are

capable of absorbing photoenergy, either intrinsically or through a molecular component. The mitochondrial respiratory chain includes multiple photoreceptors, as described below.

3.1.1. Cytochrome *c* oxidase

The enzyme cytochrome *c* oxidase receives electrons from respiratory-chain substrates through the cytochrome pathway and transfers them to oxygen molecules. The photoabsorption spectra of cytochrome *c* oxidase in its various oxidation states are very close to the action spectra for various biological responses. Cytochrome *c* oxidase has been proposed as the endogenous photoreceptor in the visible to near-infrared region (above 600 nm) [54]. Scientists have conducted extensive research on the photobiomodulation by cytochrome *c* oxidase, particularly in neuronal cells. In a study of neurons functionally inactivated by tetrodotoxin, a voltage-dependent sodium channel blocker [55], near-infrared irradiation restored the activity of intoxicated cytochrome *c* oxidase by altering its redox state. In another study, laser irradiation of mitochondria increased cytochrome *c* oxidase activity, polarographically measured levels of oxygen uptake, and subsequent ATP production [56]. Many other *in vitro* and *in vivo* studies of laser-induced cell growth have reported changes in cytochrome *c* oxidase activity and ATP production following irradiation [57–65].

3.1.2. Porphyrin

Porphyrins are a group of macrocyclic organic compounds that contain four pyrrole subunits joined by methine bridges. These mostly green- or red-colored compounds have specific absorption spectra and emit red fluorescence. Naturally occurring porphyrins, including those found in the human body, often form complexes with an iron or magnesium ion coordinated to the four pyrrole nitrogen atoms. For example, iron protoporphyrin IX (PPIX) complexes (i.e., heme *b*) form the prosthetic groups of hemoglobin, catalase, and peroxidase. Mitochondrial cytochromes also contain iron-porphyrin groups (nonheme *b*). The PPIX absorption spectrum has five major peaks in the range of 400–650 nm, with peak height decreasing as the absorption wavelength increases. The excited triplet state of PPIX, formed by absorption of laser photons, generates reactive oxygen species by transferring energy to ground-state oxygen atoms. A mode of photodynamic therapy that exploits this feature has been developed for anticancer treatment. In this technique, patients are administered PPIX or its precursor, 5-aminolevulinic acid (ALA), and reactive oxygen species are generated with local laser irradiation to kill malignant cells or epithelial cells of vascular neoplasms. In addition to the tumoricidal effects of reactive oxygen species, photodynamic therapy also induces energy-demanding apoptotic process by maintaining intracellular ATP levels [66]. Furthermore, my colleagues and we also discovered changes in the functions of cells irradiated with lasers in the presence of low doses of PPIX [67]. As the intracellular photoacceptors, porphyrins mediate a wide variety of biochemical reactions through the production of reactive oxygen species following photoreception. We refer to the roles of reactive oxygen species in more details below.

3.1.3. Flavoproteins (*flavin proteins*)

Flavoproteins are a group of protein complexes containing a riboflavin prosthetic group (e.g., flavin adenine dinucleotide [FAD] or flavin mononucleotide [FMN]). Most flavoproteins function as flavin enzymes, which use iron, molybdenum, copper, manganese, and other heavy metal ions as cofactors. These proteins have major absorption peaks in the range of 350–500 nm. Flavoproteins mediate a wide array of biological processes, such as bioluminescence, quenching of oxidative stress-induced radicals, DNA repair, and apoptosis [68]. A large number of researchers, including the present author, have reported the roles of flavoproteins as intracellular photoacceptors [69–71].

3.1.4. Other groups of photoreceptors

In addition to the three major groups of photoreceptors explained above, there are other types of photoreceptors, including rhodopsin, bilirubin, melanin, pterin, vitamin B6, vitamin K, nicotinamide adenine dinucleotide (phosphate) hydrogen [NAD(P)H], urocanic acid, and tryptophan.

3.2. Laser-induced changes in signaling cascades

Many researchers believe that the photon energy captured by intracellular receptors leads to alterations in gene and protein expression through a series of processes that modify signaling cascades. However, little is known regarding how light-stimulated receptors transduce their signals to the nucleus, or how these signals mediate the expression of particular genes. We have studied the mechanisms underlying the promotion and suppression of stem-cell differentiation, with a focus on FAD-containing cryptochromes as cellular photoreceptors [70, 71]. Our research suggested that light-activated cryptochromes migrate into the nucleus, where they regulate the expression of proteins located downstream of the E-box sequence. As a matter of course, cell functions are regulated by an array of other factors, including reactive oxygen species. Therefore, We now describe the biochemical changes LLLT induces beyond the photoreceptor absorption of light energy, as reported in the literature.

3.2.1. Redox pathways

Several oxygen and nitrogen radicals have been proposed to transduce mitochondrial signals to the nucleus. Those species react with NAD, NADH, NADP, NADPH, glutathione, glutathione sulfide, thioredoxin, and thioredoxin sulphide [72]. The cell contains several endogenous sensors for these species (typically, superoxide dismutase [SOD]) [73]. Upon detection of reactive oxygen species, the cell activates self-defense pathways by altering its gene expression patterns [74]. If these self-defense mechanisms fail, the cell will undergo apoptosis. The levels of reactive oxygen species strictly determine the expression of proteins regulating cell proliferation, suggesting that oxygen radicals act as second messengers [75, 76]. Reactive oxygen species are considered to play key roles in the control of cellular functions [77]. Low-level laser beams with wavelengths around 630 nm generate oxygen radicals in exposed cells [78, 79]. We have also discovered significant increases in the levels of oxygen radicals in cells exposed

to 405-nm laser light [80]. Although the specific mechanism remains unknown, laser-induced intracellular generation of reactive oxygen species probably involves energy transfer from PPIX and other photoacceptors present in the cell. In addition, several groups have described cellular functions mediated by nitric oxide (NO), which is upregulated by laser irradiation, as well as by inducible nitric oxide synthase (iNOS) [79, 81–84]. The mechanism of laser-induced control of cellular functions is believed to hinge on the regulation of photoacceptor activity and the intracellular levels of reactive oxygen species.

3.2.2. *Transcription factors*

Several researchers have reported that the aforementioned redox pathways trigger changes in the expression of many transcription factors. Here, We do not go beyond a brief description of one of the best-characterized transcription factors, nuclear factor (NF)- κ B [85, 86]. Published articles on other transcription factors mediating a multitude of cell functions have made it clear that their expression levels are also modified upon exposure to laser irradiation. As a transcription factor, NF- κ B can simultaneously induce the expression of interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, tumor necrosis factor (TNF)- α , and other proinflammatory cytokines. It also controls the expression of apoptosis-related proteins, which play a critical role in tumor cell growth and immortalization. Several studies have shown that the aforementioned redox pathways trigger increases in NF- κ B levels. [85, 86] This mechanism is considered to account, at least in part, for the observation that low-level laser irradiation induces the expression of various cytokines.

3.2.3. *Circadian rhythm*

The circadian rhythm, a roughly 24-h cycle of cellular events, was probably acquired during the early stages of evolution, and is ubiquitous from unicellular organisms to mammals. Several mammalian clock genes work together to establish a stable oscillation of approximately 24 h. Circadian clock proteins, such as brain-muscle Arnt-like protein 2 (Bmal2), CLOCK (Clk), cryptochrome (Cry), and Period (Per), set the pace of the clock in almost all cell types (e.g., the timing of cell division and other cellular activities). Cry, a blue-light receptor in higher plants and *Drosophilidae* [87], utilizes as its chromophore the FAD coenzyme, which undergoes blue-light excitation. The intramolecular changes that occur in Cry upon photoreception remain unclear. Most photoreceptors identified so far undergo a conformational change to their apo state when their chromophore is photoisomerized, and the resultant structural change in the protein molecule triggers photoreceptor signaling. In the case of Cry, however, no photoisomerization takes place, because FAD is the chromophore. This observation led to the idea that light-excited FAD transfers electrons to a certain substrate. However, the validity of this theory has not been tested.

Bone metabolism (remodeling) is a continuous homeostatic process involving resorption of existing bone by osteoclasts and formation of new bone by osteoblasts. Fu et al. showed that circadian rhythms mediate bone formation [88], and Kawasaki et al. reported that the E-box motif, a circadian regulatory sequence, is involved in the osteoblast expression of MBP-4 [89]; these findings indicate that Cry proteins regulate various homeostatic and physiological events through E-box elements. We conducted research on the effects of lasers on

endocellular distribution and expression of Cry using 405-nm laser beams, which correspond to the absorption band of the Cry coenzyme FAD [70]. **Figure 1** presents the beam profile of the 405-nm laser used in the study (Panel A) and the changes in mouse marrow mesenchymal

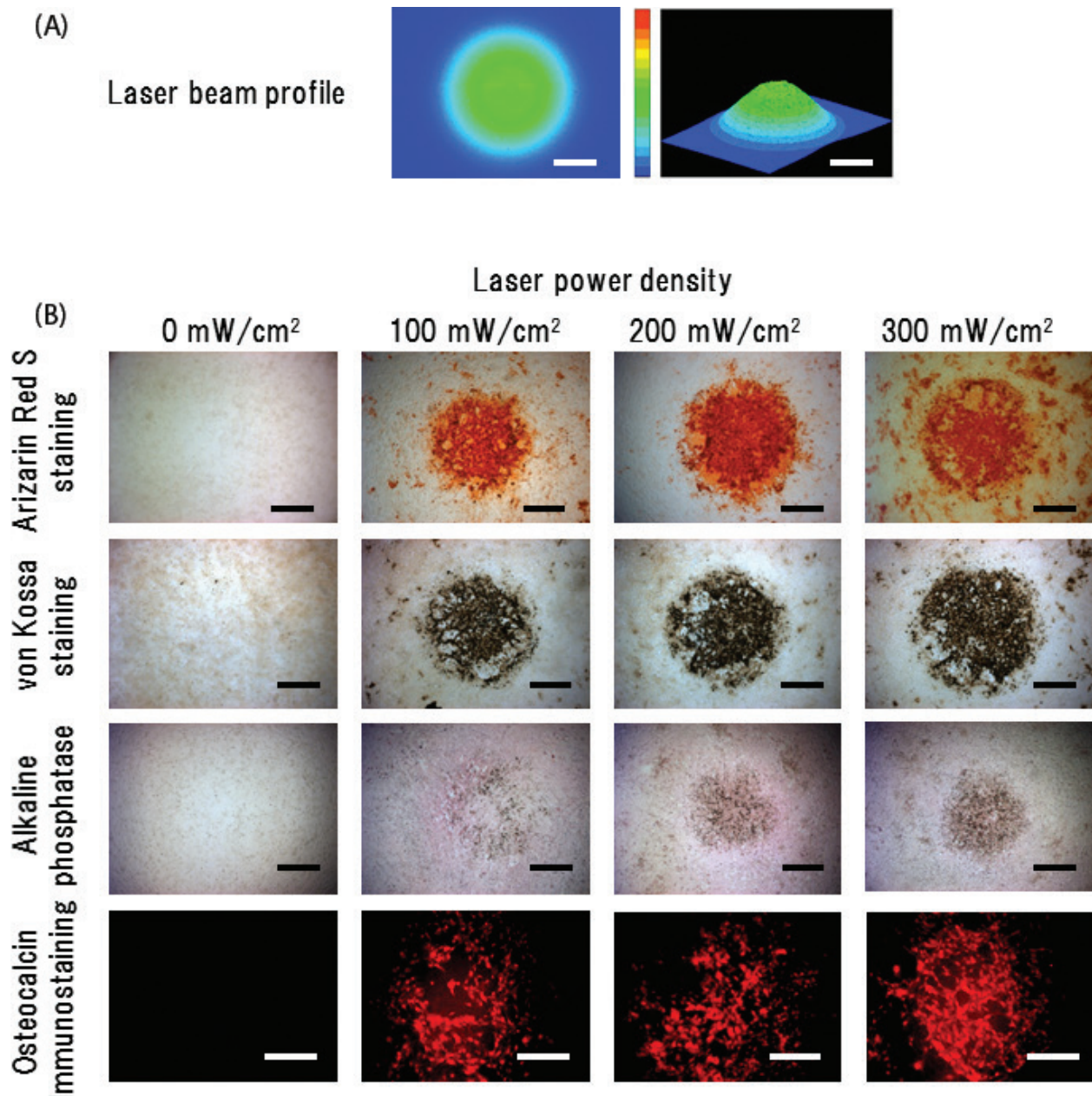


Figure 1. (A) The beam profile of the blue laser (wavelength; 405 nm, continuous wave). Mouse mesenchymal stromal cells were irradiated for 180 s at various laser power densities. Scale bars = 200. (B) Histochemical analysis of laser-irradiated mouse mesenchymal stromal cells. Calcium deposition of laser-irradiated mouse mesenchymal stromal cells was stained by Alizarin red-S (magnification: $\times 50$). At 5 days postirradiation, calcium deposition had increased around the cells in a dose-dependent manner. Calcium phosphate deposition was evaluated by von Kossa staining (magnification: $\times 50$). The area expressing alkaline phosphatase (ALP) activity was stained (magnification: $\times 50$). Laser-irradiated samples displayed immunopositive staining for osteocalcin, a marker of osteoblast differentiation (magnification: $\times 100$). Scale bars = 200 (for Alizarin red-S, von Kossa, and ALA staining) and 100 μm (for osteocalcin immunostaining). Adapted with permission from [70], copyright (2008).

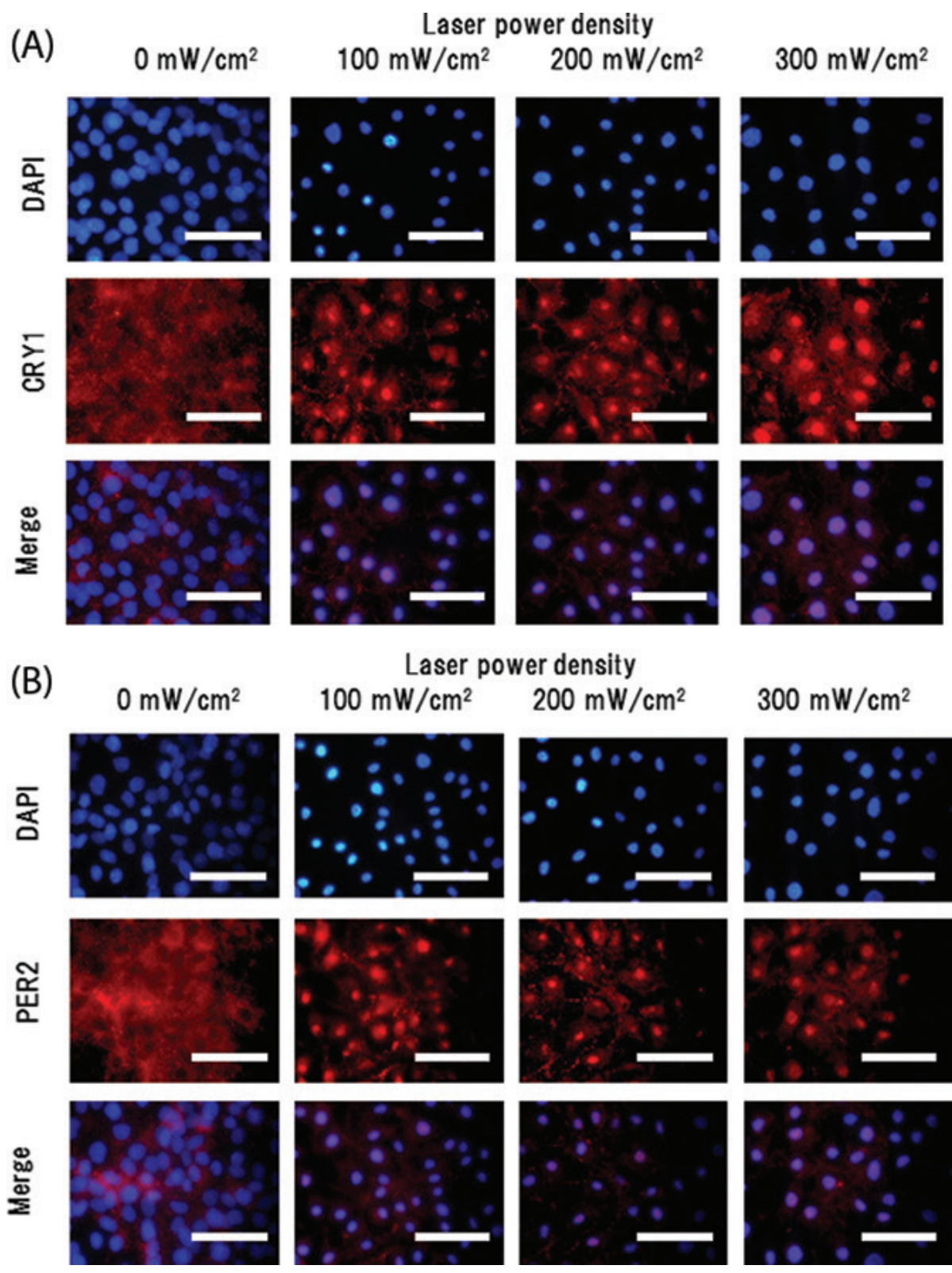


Figure 2. Intracellular location of mCRY1 (A) and mPER2 (B) proteins in mouse mesenchymal stromal cells 24 h after laser irradiation. Cells were double-labeled with DAPI (blue and upper panel) and mCRY1 or mPER2 (red and center panel). The lower panel provides a merged image. mCRY1 and mPER2 localized to the cytoplasm prior to laser irradiation. After laser irradiation, proteins translocated to the nucleus. Scale bars = 30 μm. Adapted with permission from [70], copyright (2008).

stromal cells irradiated for 3 min and then cultured for 5 days in osteoblast differentiation medium (Panel B). Alizarin red and von Kassa treatments, performed to detect calcium phosphate deposits, revealed that the stained cells were distributed in a circular area with a diameter similar to that of the laser beam. **Figure 1** also shows positive immunostaining results for alkaline phosphatase and osteocalcin, markers for osteoblast differentiation. These results confirmed that 405-nm laser irradiation accelerated osteoblast differentiation from mesenchymal stromal cells. In addition, the results of immunostaining for Cry1 and Per2 proteins are represented in **Figure 2**. Although Cry1 and Per2 were distributed across the cytosol in control cells, they were localized to the nucleus in cells exposed to 405-nm laser irradiation. Our results show that 405-nm laser beams promote the nuclear localization of Cry1 and mediate the expression of Cry1 and other proteins downstream of the E-box. We also reported that low-level laser irradiation suppressed the adipocyte differentiation of mesenchymal stromal cells [70], and accelerated their differentiation into chondrocytes [90].

4. Conclusions

Since the inception of life on earth, light has been one of the fundamental sources of biological energy. Today, researchers are conducting intensive basic and clinical research in the arena of laser medicine and photobiology, with the goal of developing new diagnostic and therapeutic modalities. Here, We described some of the latest advances in research on the cellular effects of irradiation with lasers and other forms of light. A great deal of future work will be required in order to broaden the applications of LLLT and achieve technical breakthroughs. In my past research, We found that living organisms and cells always respond to lasers and other forms of light in one way or another. The biological mechanisms underlying such responses significantly differ by the type of laser, target, and other experimental conditions. We must accumulate a systematic knowledge base by carefully analyzing the vast amount of experimental data currently available, as well as data collected in the future. We believe that light-based biomedical research will open new horizons for photodiagnosis, LLLT, and photodynamic therapy.

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