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ABSTRACT

Discoveries are rapidly being made in multiple laboratories that shed "light" on the fundamental molecular and cellular mechanisms underlying the use of low level light therapy (LLLT) in vitro, in animal models and in clinical practice. Increases in cellular levels of respiration, in cytochrome c oxidase activity, in ATP levels and in cyclic AMP have been found. Increased expression of reactive oxygen species and release of nitric oxide have also been shown. In order for these molecular changes to have a major effect on cell behavior, it is likely that various transcription factors will be activated, possibly via different signal transduction pathways. In this report we compare and contrast the effects of LLLT in vitro on murine embryonic fibroblasts, primary cortical neurons, cardiomyocytes and bone-marrow derived dendritic cells. We also examined two human cell lines, HeLa cancer cells and HaCaT keratinocytes. The effects of 810-nm near-infra-red light delivered at low and high fluences were addressed. Reactive oxygen species generation, transcription factor activation and ATP increases are reported. The data has led to the hypothesis that cells with a high level of mitochondrial activity (mitochondrial membrane potential) have a higher response to light than cells with low mitochondrial activity.

Keywords: biostimulation, low level laser therapy, mitochondria, cytochrome c oxidase, reactive oxygen species, nuclear factor kappa B, ATP

1. Introduction

Although low level light therapy (LLLT) has been known and increasingly widely practiced for over forty years, it is still regarded with some skepticism by laymen and medical professionals alike, and has not reached acceptance by mainstream medicine. The single most important reason for this lack of acceptance is likely to be the inability of most practitioners of LLLT to satisfactorily explain how it works on a molecular, cellular and tissue level. There is a need for more fundamental research on identifying photoacceptor molecules, elucidating cell and signaling pathways that are engaged after cells absorb visible photons. Furthermore it is necessary to investigating

relationships between the optical parameters of the light such as wavelength, total delivered energy, rate at which energy is delivered, coherence, polarization state and pulse structure.

Many of the molecular and cellular events that happen after cellular chromophores absorb light photons of the correct wavelength (generally red or near-infrared) are beginning to be understood. It is generally believed that photons are absorbed in mitochondria by constituents of the respiratory chain, and in particular by cytochrome c oxidase. ATP is increased together with mitochondrial membrane potential, reactive oxygen species are increased and changes in cellular calcium are observed. However_ many of these light-mediated biochemical and biological changes have been reported in extremely diverse types of cells. At the very basic levels light has been studied in mammalian, bacterial, fungal, plant and even insect cells. In mammalian cells light-mediated changes have been reported in diverse types of cancer cell lines (HeLa, melanoma etc) and in various non malignant cells that have more or less characteristics of so-called "normal" cells. These normal cells encompass various immortalized cell lines such as 3T3 fibroblasts, cells transformed with SV40 large T antigen or other approaches and finally, the most relevant cells for medical application, primary cell isolates. The primary cell isolates can be derived from different organs (whether from humans or from laboratory animals such as mice and rats) that may in fact actually receive light during clinical LLLT

In this report we begin the Herculean task of comparing the light-mediated cellular responses in different cell types with the goal of drawing mechanistic conclusions that could be usefully applied in rationally selecting diseases and patients in that would receive clinical LLLT.
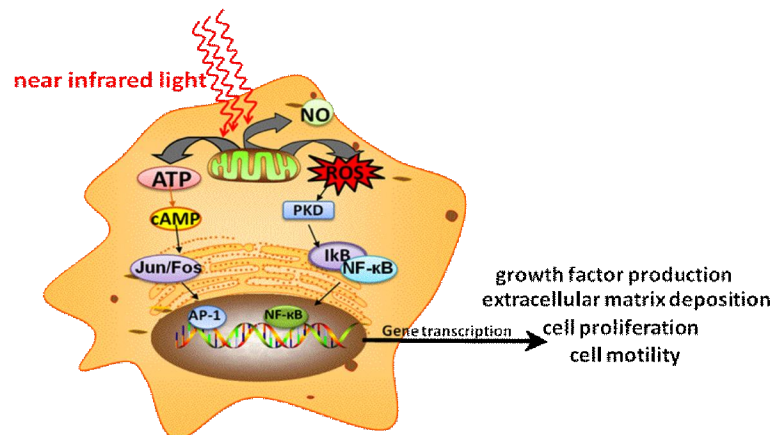
Figure 1. Schematic representation of the main molecular and cellular processes involved in LLLT

2. Materials and methods

2.1 Cell isolation from mice

All animal procedures were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital and met the guidelines of the National Institutes of Health.

2.1.1 Primary Mouse Embryonic Fibroblasts

Pregnant Female C57BL/6N mice (age, 8-10 weeks) were sacrificed at 16 days post-coitus. Embryos were isolated in cold phosphate-buffered saline and then incubated in 2ml of trypsin (0.05%) for 10 min at 37 °C with intermittent agitation. Embryos were disrupted by pipetting and then added to at least a 3_ volume of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated into 75cm^2

flasks. For all the experiments, cells were grown to at most 80% confluence before seeding to 96 well plates and only the cells between passage 3 and passage 8 were employed.

2.1.2   Primary Mouse Cortical Neurons

Pregnant female C57BL/6 mice (age, 8-10 weeks) were sacrificed at 16 days post-conceptiom. The cortical lobes of 6-10 embryonic brains were separated from subcortical structures in calcium-magnesium-free, Hank's balanced salt solution containing 10 mM HEPES buffer (Invitrogen, Carlsbad, CA). After removal of meninges, brain tissue was placed into calcium-magnesium-free, Hank's balanced salt solution containing 10 mM HEPES buffer, triturated briefly with pipetting, and incubated at 37°C for 10 min with 2.5% trypsin (Invitrogen) and deoxyribonuclease I (Type IV). Cells were then further dissociated mechanically and centrifuged at 1000 rpm, at 4°C for 5 min. The pellet was resuspended in Neurobasal Media (NBM; supplemented with glutamate, glutamine, antibiotic-antimycotic solution, and B27 supplement). Cells were plated at a density of approximately 30,000 cells/well on poly-D-lysine (Sigma, St. Louis, MO)-coated cell culture plastic or sterile glass coverslips. The plating and maintenance media consisted of Neurobasal plus B27 supplement (NB27; Invitrogen/Gibco) with or without 25_M beta-mercaptoethanol (Invitrogen), used as described below. This media formulation inhibits the outgrowth of glia resulting in a neuronal population that is >95% pure. See Brewer et al. [1] for complete composition information for Neurobasal media and B27 supplement. Cells were cultured up to 28 days at 37°C in a humidified atmosphere of 5% CO_2/95% air, changing media every 2-4 days or as required for the experiments described below.

2.1.3   Mouse bone-marrow derived dendritic cells.

Femurs from mice were dissected, and muscle and tissue were removed. Cleaned bones were washed twice with Hank's Buffer Salt Solution, and placed into culture media composed of RPMI-1640 (Gibco) with 1% penicillin–streptomycin (Cellgro), 0.1% Beta-mercaptoethanol (Invitrogen), with 10% heat inactivated fetal bovine serum (Invitrogen) (murine DC media). Bones were cut and bone marrow was flushed with at least 5 mL of media. The bone marrow suspension was strained with a 70 mm cell strainer (Becton Dickinson), cells collected by centrifugation at 1500 rpm for 5 min, and erythrocytes were lysed with ammonium chloride (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Bone marrow cell suspension was resuspended at $1.5x10^5$ / ml in murine DC media with 20 ng/ mL GM-CSF (Sigma). Cells were plated at 3 ml / well in six well plates (Corning), and incubated at 37°C with 95% relative humidity and 5% CO_2. On day 3, cells were fed by exchanging half of the media with fresh murine DC media. Purity and yield of CD3 and CD4 positive cells were inspected by flow cytometry. In addition, cell death was quantified by propidium iodide by flow cytometry. To assure the consistency of cell population, plates with over 85% purity and less than 10% cell death were selected for experiments.

2.1.4   Mouse cardiomyocyte cell line

HL-1 cells were a cardiac muscle cell line, first established and characterized the cell line by Dr W. C. Claycomb [2]. Briefly, cells were grown as a monolayer (37 °C, 5% CO_2) in culture flasks. HL-1 cells were maintained in DMEM supplemented with 10% foetal bovine serum, 4 mM L-glutamine, 100 _M noradrenaline (from 10 mM stock solution in L-ascorbic acid 30 mM) and 1 _ antibiotic/antimycotic solution (standard medium). The medium was changed every 24–48 h.

2.1.6   Human HeLa cancer cells

A human cervical cancer cell line, HeLa[2] was obtained from ATCC (Manassas, VA). The cells were cultured in DMEM medium with L-glutamine and $NaHCO_3$ supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) (Sigma, St. Louis, MO) at 37 °C in 5% CO_2-humidified atmosphere in 75 cm^2 flasks (Falcon,

Invitrogen, Carlsbad, CA). When the cells reached 80% confluence, they were washed with phosphate-buffered saline (PBS) and harvested with 2 mL of 0.25% trypsin-EDTA solution (Sigma). Cells were then centrifuged and counted in trypan blue to ensure viability.

2.1.7 HaCaT human keratinocyte cell line

The human spontaneously immortalized non-tumorigenic keratinocyte cell line HaCaT [3] was obtained from Dr. J Ulrichova (Institute of Medical Chemistry and Biochemistry, Tufts University School of Medicine, Boston MA) and maintained in DMEM medium (Sigma Chemical Co., St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Lab., Logan, UT), 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma), in plastic disposable tissue culture flasks at 37° C in a 5% CO_2/95% air incubator. When the cells reached 80% confluence, they were washed with phosphate-buffered saline (PBS) and harvested with 2 mL of 0.25% trypsin-EDTA solution (Sigma). Cell viability was routinely assayed by standard trypan blue staining.

2.2 Laser irradiation

The in vitro experiments were conducted with a diode laser (Photothera Inc., Carioca, CA), which emits 810nm near infrared radiation. 1 mW to 30 mW powers was generated for 7 seconds to 5 minutes to deliver different energy densities, including 0.3, 3 and 30 J/cm^2. 3000 cells were seeded into each well of 96 well plates the night before the experiment. Additional overnight incubation with 0.5% FBS DMEM might be needed for certain assays.

2.3 ATP assay

After irradiation, at the various time points, cells in each well were first lysed with 50µL cell lysis buffer and the plate was placed on shaker for 2 minutes to ensure completely release of ATP. 5µL were then transferred to 96 well plates with black wall and clear bottom for BCA assay for protein concentration measurement, while the rest cell lysates were transferred to 96 well plates with white wall and clear bottom for ATP measurement. 100 µL of Cell-Titer Glo Assay (Promega, Madison, WI) was added into each sample and wait about 5 minutes for stabilizing the luminescence signal. The luminescence was measure by a luminometer. Reading time was set to be 10 seconds when the signal is significantly stronger than background noise, and each sample was read twice.

2.4 Analyses of ROS production

Cells plated in glass-bottom dishes were incubated in 37° C incubator overnight, washed three to four times by PBS, and then changed the medium to 0.2% FBS DMEM for at least 12 hour incubation before the experiments. After experiments, add 10_M 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Inc, Invitrogen) into the medium and waited for 30 minutes. The CM-H_2DCFDA fluorescence was observed in Zeiss Axiovert 100 TV Microscope with FITC filter. H_2O_2 induced ROS fluorescence was used to determine the exposure time and gamma parameters for all the samples. MitoSOX Red (Molecular Probes, Inc, Invitrogen). Dissolve in 13.2 µl DMSO for 5 mM(1,000X) stock just before (<15 min) the experiment. Prepare in the dark and cover with a foil. Add MitoSOX Red into warmed PBS at final concentration of 5 µM and incubate cells for 30 min at 37 °C. Keep the cells covered with the foil to prevent light exposure. The MitoSOX Red fluorescence was observed in Zeiss Axiovert 100 TV Microscope a 580 ± 30-nm emission filter.
We also applied flow cytometer to analyze CM-H_2DCFDA fluorescence. Cells were incubated with 10µM CM-H_2DCFDA at 37°C for 30mins. The cells were collected, washed with PBS twice, and analyzed by BD FACSCalibur System flow cytometer using Flow Jo software for quantitative analyses. To test the effect of NIR on cellular ROS, irradiation was performed immediately before the cells were collected for analysis.

2.5 Intracellular NO production

DAF-FM is essentially non-fluorescent until it is nitrosylated by products of oxidation of NO, resulting in DAF-FM triazole which exhibits about a 160-fold greater fluorescence quantum efficiency [4].

Incubation of cells D-PBS supplemented with 2%FBS with 10 µM DAF-FM. The NO donors *S*-nitroso-*N*-acetylpenicillamine (SNAP) 100 µM was added to the cells as positive control. Microscopy and fluorescent imaging The image-capture system consisted of a Zeiss Axiovert 100M microscope equipped with a 500/20 excitation, 535/30 emission filter set (Filter set 46, Carl Zeiss GmbH, Germany). All experiments were carried out at 25°C.

2.6 Statistical Analysis

All chemiluminescence readings were normalized to total protein measured by Bradford's technique (BCA, Pierce Biotechnology Inc.). All assays were performed in duplicate, and each sample was read twice and took the average. Then we used Excel software to perform Single-Factor ANOVA to evaluate the statistical significance of experimental results ($p < 0.05$).

3. RESULTS

3.1 Laser induces different ATP level in different cell types

To test if different types of cells have different response to LLLT, we first irradiated the cells with 5 different fluences and measured ATP levels over time. First of all there were significant differences in the baseline ATP levels in the different cell types. The order of baseline ATP was HeLa > HaCaT ≈ MEF >> HL1 >> DC (Figure 2). We observed that there was no significant difference in ATP between 0 03 J/cm^2 and 0.3 J/cm^2 in any cell type. Energy densities of 3 J/cm^2 and 30J/cm^2 induced significant ATP increases in three of the cell types (HeLa, HaCaT and HL-1). Interestingly the biggest percentage increase in ATP was in the cardiomyocyte cell line that is presumed to have a lot of mitochondrial activity. We observed that the highest ATP increase in all five different cell types showed up with 3 J/cm^2 illumination and decreased somewhat at 30J/cm^2. Although fibroblasts had a high initial level the increase with light was modest. DC did not respond.
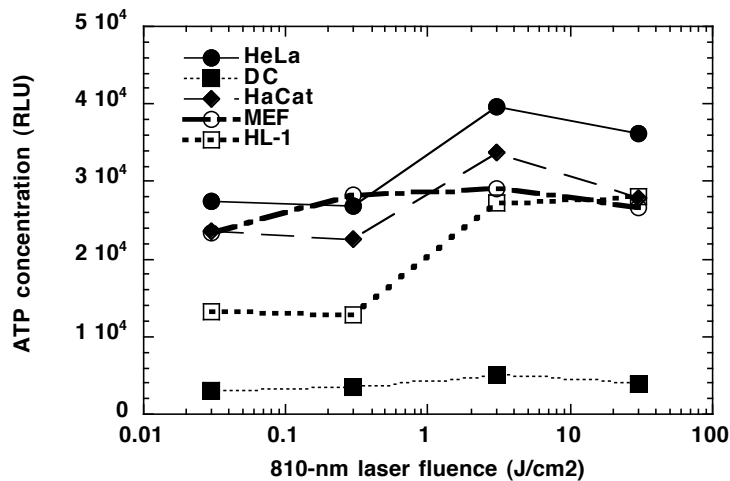

Figure 2 Laser-induced increase in ATP synthesis in different cell types.

3.2 Laser activates transcription factor NF-kB in MEF

We first applied 0.5 µg/ml Lipopolysaccharide (Sigma-Aldrich) to confirm luciferase expression responded to NF-kB activation in Luc MEF. The results started showing luciferase response in 6 hours and increased approximately 5 fold in 10 hours (Figure 3B). We observed NF-kB luciferase response with 0.3 J/cm^2 was first detectable as early as 1 hour post-light, increased at 6 hours and the luminescence reached a peak at 10 hours before returning to the base line at 24 hours. As a control for ensuring the luminescence was really due to NF-kB mediated transcription of the luciferase enzyme we used the broad-spectrum protein synthesis inhibitor cycloheximide (Fig 3B). This addition reduced the baseline luminescence by approximately 50% and totally abrogated the light-mediated increase in luminescence.
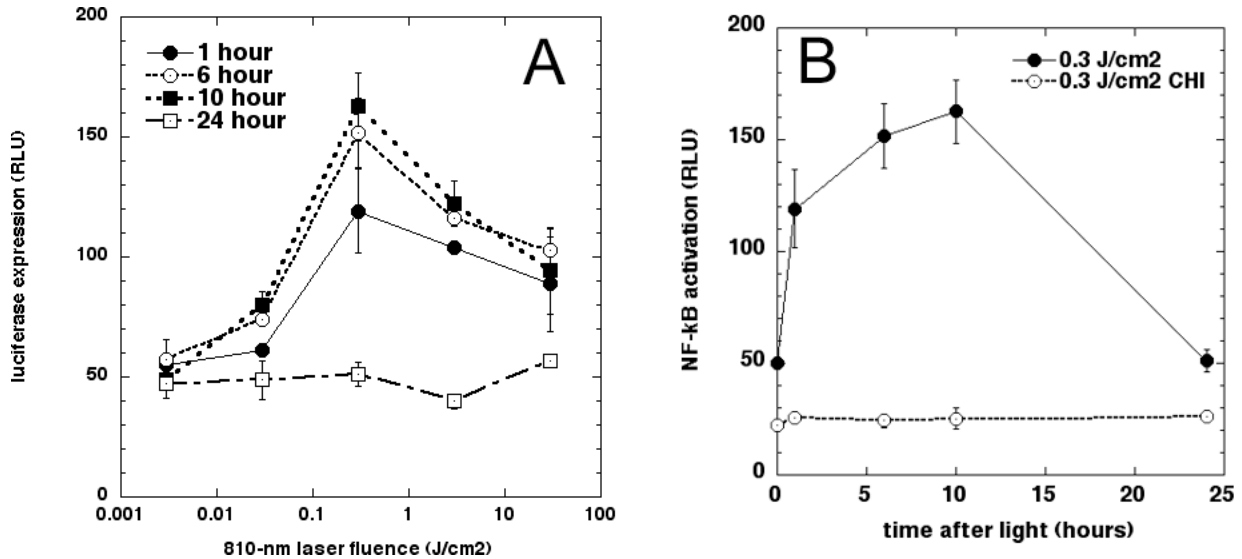
Figure 3. Dose response of NF-kB activation in Luc MEF cells after 810-nm laser. B. Time course of NF-kB activation in Luc MEF cells after 0.3 J.cm^2 of 810-nm laser.

3.3 Laser increases ROS production in MEFs and primary cortical neurons

The hypothesized theory states that low level laser upregulates the activity of cytochrome c oxidase and generates more reactive oxygen species (ROS). Furthermore, ROS level is highly regulated by intracellular superoxide dismutase (SOD), which converts ROS to hydrogen peroxide. Increase in hydrogen peroxide concentration should be detectable after laser irradiation if ROS participates in the laser induced NF-kB activation pathway. We used DCFDA to detect hydrogen peroxide concentration in various power densities. The microscopy images, comparing with the dark control, showed that laser irradiated MEF cells emitted stronger green fluorescence (Figure 4-A-D). MitoSOX red is selectively targeted to mitochondria and is able to compete efficiently with superoxide dismutase (SOD) for superoxide. This validates the use of the MitoSOX Red probe as a measurement of mitochondrial superoxide. Figure 4-E-H shows the results of superoxide production.
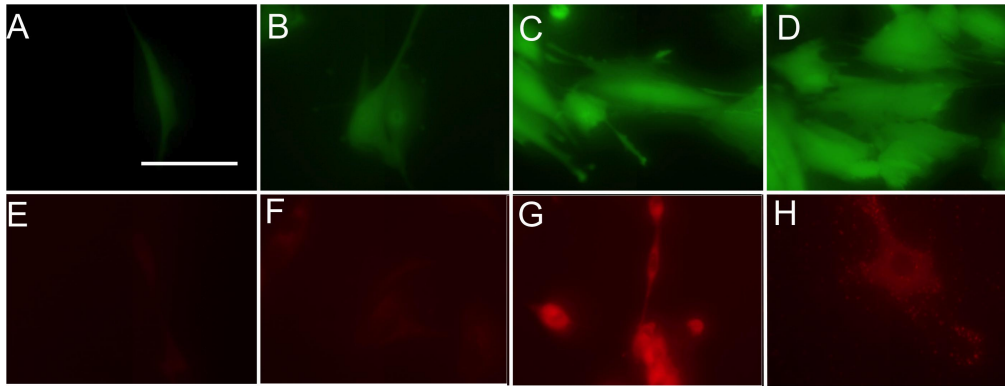
Figure 4. Top Row. MEFs containing CMDCDHF treated with 810-nm laser and A, dark; B, 0.3 J/cm^2; C, -3 J/cm^2; D, 30 J/cm^2. Bottom row MEFS containing MitoSOX red and treated 810-nm laser and E, dark; F, 0.3 J/cm^2; G, -3 J/cm^2; H, 30 J/cm^2

Laser produces ROS in cortical neurons in medium without beta-mercaptoethanol. Having demonstrated that cortical neurons can grow in the absence of B27 antioxidants after first passage, we wanted to compare the difference between ROS levels with and without antioxidants. Neurobasal plus B27 and beta-mercaptoethanol protected cortical neurons against glutamate toxicity when compared to other media formulation [5]. Indeed, we demonstrated that laser cannot stimulate cortical neurons to generate ROS with beta-mercaptoethanol in the media (Fig 5A). In the absence of beta-mercaptoethanol however there was a robust ROS generation at all fluences tested (Fig 5A) which endured for 1 hour post-laser (Fig 5B).
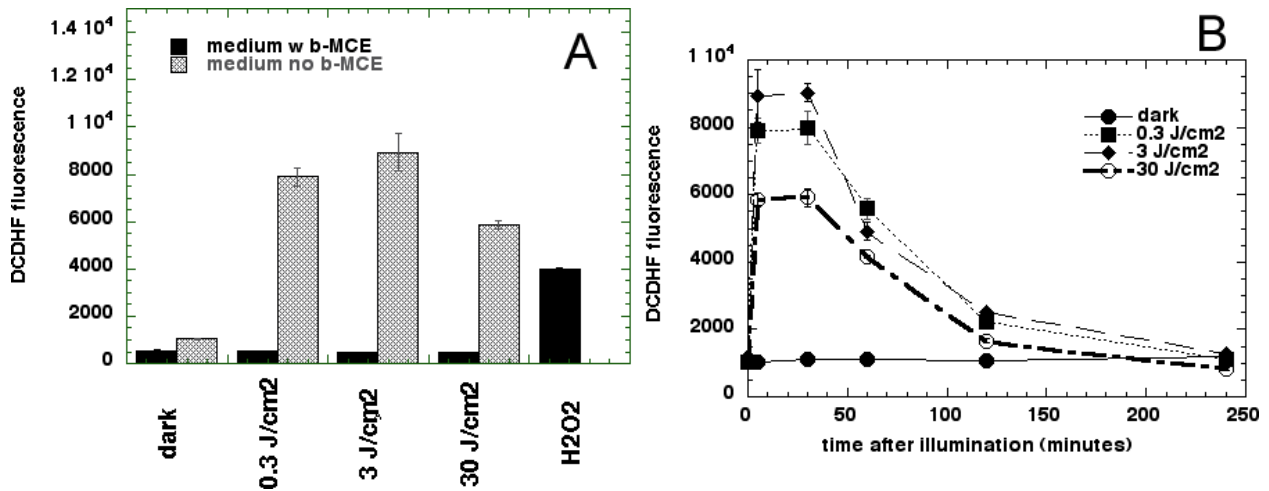

Figure 5. A. DCDHF fluorescence measured by FACS from mouse primary cortical neurons treated with 810-nm laser in neurobasal medium with or without the addition of beta-mercaptoethanol. B. Time course of DCDHF fluorescence in mouse primary cortical neurons in neurobasal medium without beta-mercaptoethanol and treated with 8110-nm laser.

3.4 Laser increases intracellular NO in cardiomyocytes

810-nm laser treatment increased the intracellular production of nitric oxide in the cardiomyocyte cell line HL1 as demonstrated by the fluorescent probe DAF 30 minutes in a light-dose dependent manner after exposure to fluences between 0 and 10 J/cm^2.
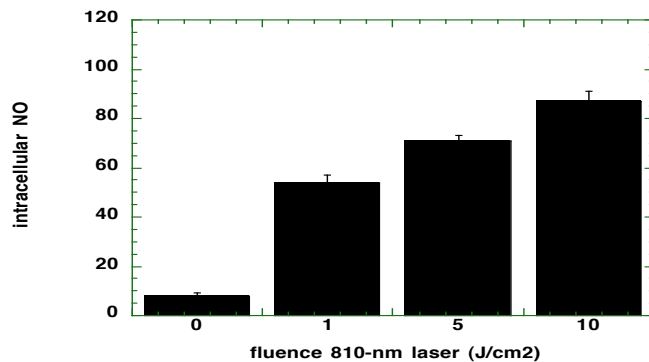
Figure 6. Cardiomyocytes were treated with 810-nm laser and NO assayed by 30 mins later.

4. Discussion

This goal of current study is to demonstrate different responses to LLLT within different cells.

LLLT has been shown to enhance cell proliferation in vitro in several cell systems: fibroblasts [6, 7, 8], keratinocytes [9], endothelial cells [5], and lymphocytes [11, 12] . In vitro study by Luciana show that LLLT with administrated fluence of 2 J/cm^2 resulted in increased proliferation rate on fibroblasts grown in a nutritional deficit setting (5% serum), while LLLT had low or no effect on fibroblasts grown under ideal growth condition (10% serum). Using the same fluence, a shorter application time of LLLT gave a higher increase in cellular proliferation rate [6], however, the mechanism by which LLLT effects cellular proliferation is still under discussion.
Several pieces of evidence suggest that mitochondria are responsible for the cellular response to red visible and NIR light. The most popular system to study is the effects of HeNe laser illumination of mitochondria isolated from rat liver, in which Increased proton electrochemical potential and ATP synthesis was found [7]. Increased RNA and protein synthesis was demonstrated after 5 J/cm^2 [8]. Irradiation of mitochondria with light at other wavelengths such as 660 nm [9], 650 nm and 725 nm [10] also showed increases in oxygen consumption, membrane potential, and enhanced synthesis of NADH and ATP. Irradiation with light at 633 nm increased the mitochondrial membrane potential and proton gradient, and increased the rate of ADP/ATP exchange [11], as well as RNA and protein synthesis in the mitochondria. It is also believed that mitochondria are the primary targets when the whole cells are irradiated with light at 630, 632.8 or 820 nm [19, 20, 21].

Karu *et al.* proposed a novel mitochondrial signaling pathway in mammalian cells initiated by red and near-IR light in vitro in 2004 [12]. Recently, Schroeder *et al.* discovered that IR light could initiate a cellular signaling response in normal human skin fibroblasts. All these data support the proposed model that mitochondria can communicate with other cellular organelles via specific signaling mechanisms [13]. Numerous reports indicate that light could regulate gene expression via mitochondrial mechanisms. For example, Hu *et al.* [14] showed that He-Ne laser illumination increased mitochondrial membrane potential together with ATP synthesis in melanoma cells. They also found upregulation of cytochrome c oxidase activity, increased phosphorylation of Jun N-terminal kinase (JNK) and later, activated activator protein-1 (AP-1), which led to increased cell proliferation.

2-mercaptoethanol (BME) is considered to be a survival factor for cultured cortical neurons in many protocols [15, 16] Since many studies that look at primary neuronal cultures are involved with oxidative stress and potential protection by anti-oxidants, it was considered problematic to have BME a known thiol-containing anti-oxidant

molecule present throughout the experiment. Perry et al showed [17] that the BME is only critical to neuronal survival during the first 24 hours after explantation. Therefore we compared light-mediated production of ROS in cortical neutrons in the presence and absence of BME and found a huge difference, in that virtually no ROS were produced by light in presence of BME, while there was robust generation in its absence. Further studies are underway to define the relevance of this observation.

There is growing interest in the production of nitric oxide (NO) in cells after LLLT. However traditional assays for the main NO break-down product, nitrite (Griess reaction) or assays for NO directly (selective electrodes) are limited in sensitivity. There is a good assay for intracellular NO consisting of DAF-FM which is nitrosylated by products of oxidation of NO, resulting in DAF-FM triazole which exhibits about a 160-fold greater fluorescence quantum efficiency [4]. Using this assay we detected increased intracellular NO in the cardiomyocyte cell line HL-1. Further studies are underway to explore the generalizability of this observation to other cell types.

Our present hypothesis is that the response of cells to light is determined by (among other factors) their mitochondrial number, activity and membrane potential. Huang et al [18] used tetramethylrhodamine methyl ester to compare the mitochondria of six different cell types. They found that the mean mitochondrial membrane potential (MMP) differed significantly between cell types, but that the cell area or size also differed, and that a more accurate comparison was to calculate the integrated MMP over the cell area. Although fibroblasts had a high measured value of mean MMP, when integrated over the whole cell the value was actually the lowest of the cell types tested because fibroblasts had a much greater area. Neuronal cells had higher mean MMP values in the cell bodies compared to the growth cones. Further work is necessary to determine if mean MMP or mean MMP per cell area correlated with measures of cellular response to light. However evidence is building that cell types with more overall mitochondrial activity (for instance cortical neurons and cardiomyocytes) do in fact respond well to light.

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