Mechanisms of Low Level Light Therapy.

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ABSTRACT

The use of low levels of visible or near infrared light for reducing pain, inflammation and edema, promoting healing of wounds, deeper tissues and nerves, and preventing tissue damage has been known for almost forty years since the invention of lasers. Originally thought to be a peculiar property of laser light (soft or cold lasers), the subject has now broadened to include photobiomodulation and photobiostimulation using non-coherent light. Despite many reports of positive findings from experiments conducted in vitro, in animal models and in randomized controlled clinical trials, LLLT remains controversial. This likely is due to two main reasons; firstly the biochemical mechanisms underlying the positive effects are incompletely understood, and secondly the complexity of rationally choosing amongst a large number of illumination parameters such as wavelength, fluence, power density, pulse structure and treatment timing has led to the publication of a number of negative studies as well as many positive ones. In particular a biphasic dose response has been frequently observed where low levels of light have a much better effect than higher levels. This introductory review will cover some of the proposed cellular chromophores responsible for the effect of visible light on mammalian cells, including cytochrome c oxidase (with absorption peaks in the near infrared) and photoactive porphyrins. Mitochondria are thought to be a likely site for the initial effects of light, leading to increased ATP production, modulation of reactive oxygen species and induction of transcription factors. These effects in turn lead to increased cell proliferation and migration (particularly by fibroblasts), modulation in levels of cytokines, growth factors and inflammatory mediators, and increased tissue oxygenation. The results of these biochemical and cellular changes in animals and patients include such benefits as increased healing in chronic wounds, improvements in sports injuries and carpal tunnel syndrome, pain reduction in arthritis and neuropathies, and amelioration of damage after heart attacks, stroke, nerve injury and retinal toxicity.

Keywords: biostimulation, low level laser therapy, wound healing, biomodulation, cold laser, action spectra

1. HISTORY

In 1967 a few years after the first working laser was invented, Endre Mester in Semmelweis University, Budapest, Hungary wanted to test if laser radiation might cause cancer in mice [1]. He shaved the dorsal hair, divided them into two groups and gave a laser treatment with a low powered ruby laser (694-nm) to one group. They did not get cancer and to his surprise the hair on the treated group grew back more quickly than the untreated group. This was the first demonstration of "laser biostimulation". Since then, medical treatment with coherent-light sources (lasers) or noncoherent light (light-emitting diodes, LEDs) has passed through its childhood and adolescence. Currently, low-level laser (or light) therapy (LLLT), also known as “cold laser”, “soft laser”, “biostimulation” or “photobiomodulation” is practiced as part of physical therapy in many parts of the world. In fact, light therapy is one of the oldest therapeutic methods used by humans (historically as solar therapy by Egyptians, later as UV therapy for which Nils Finsen won the Nobel prize in 1904 [2]). The use of lasers and LEDs as light sources was the next step in the technological development of light therapy, which is now applied to many thousands of people worldwide each day. In LLLT the question is no longer whether light has biological effects but rather how energy from therapeutic lasers and LEDs works at the cellular and organism levels and what are the optimal light parameters for different uses of these light sources.

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One important point that has been demonstrated by multiple studies in cell culture [3], animal models [4] and in clinical studies is the concept of a biphasic dose response when the outcome is compared with the total delivered light energy density (fluence). The reason why the technique is termed **LOW**-level is that there exists an optimal dose of light for any particular application, and doses lower than this optimum value, or more significantly, **larger** than the optimum value will have a diminished therapeutic outcome, or for high doses of light a negative outcome may even result.

There are perhaps three main areas of medicine and veterinary practice where LLT has a major role to play (Figure 1). These are (i) wound healing, tissue repair and prevention of tissue death; (ii) relief of inflammation in chronic diseases and injuries with its associated pain and edema; (iii) relief of neurogenic pain and some neurological problems. The proposed pathways to explain the mechanisms of LLLT should ideally be applicable to all these conditions.

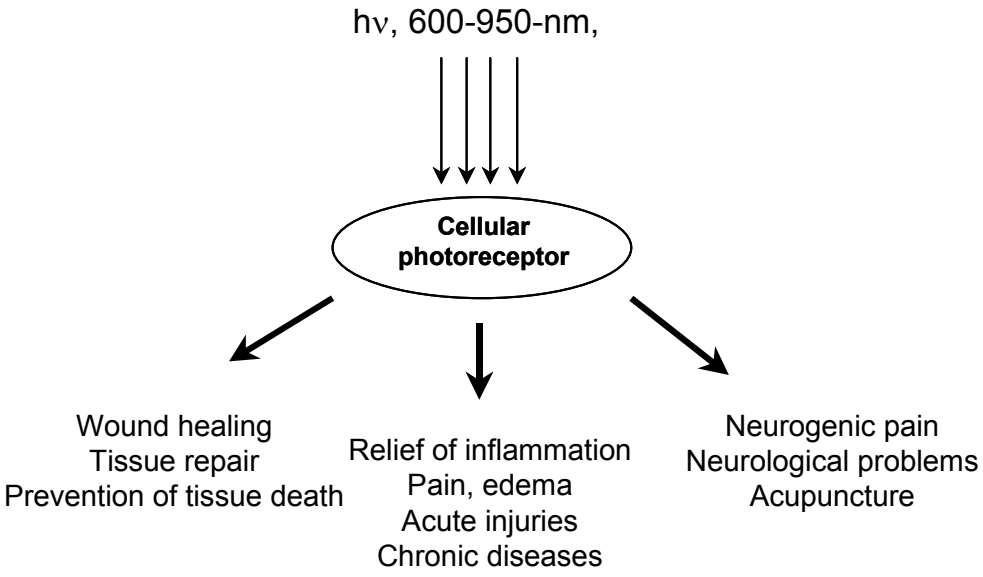

Figure 1. Schematic representation of the main areas of application of LLLT

2. BIOCHEMICAL MECHANISMS

2.1. Tissue photobiology

The first law of photobiology states that for low power visible light to have any effect on a living biological system, the photons must be absorbed by electronic absorption bands belonging to some molecular chromophore or photoacceptor [5]. One approach to finding the identity of this chromophore is to carry out action spectra. This is a graph representing biological photoresponse as a function of wavelength, wave number, frequency, or photon energy and should resemble the absorption spectrum of the photoacceptor molecule. The fact that a structured action spectrum can be constructed supports the hypothesis of the existence of cellular photoacceptors and signaling pathways stimulated by light.

The second important consideration involves the optical properties of tissue. Both the absorption and scattering of light in tissue are wavelength dependent (both much higher in the blue region of the spectrum than the red) and the principle tissue chromophore (hemoglobin) has high absorption bands at wavelengths shorter than 600-nm. For these reasons there is a so-called “optical window” The second important consideration involves the optical properties of tissue. Both the absorption and scattering of light in tissue are wavelength dependent (both much higher in the blue region of the spectrum than the red) and the principle tissue chromophores (hemoglobin and

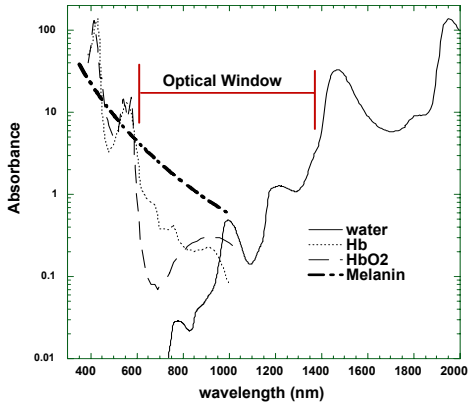

Figure 2. Optical window in tissue due to reduced absorption of red and near-infra-red wavelengths (600-1200 nm) by tissue chromophores

melanin) have high absorption bands at wavelengths shorter than 600-nm. Water begins to absorb significantly at wavelengths greater than 1150-nm. For these reasons there is a so-called "optical window" in tissue covering the red and near-infrared wavelengths, where the effective tissue penetration of light is maximized (Figure 2). Therefore although blue, green and yellow light may have significant effects on cells growing in optically transparent culture medium, the use of LLLT in animals and patients almost exclusively involves red and near-infrared light (600-950-nm).

2.2 Action spectra

It was suggested in 1989 that the mechanism of LLLT at the cellular level was based on the absorption of monochromatic visible and NIR radiation by components of the cellular respiratory chain [6]. The inner mitochondrial membrane contains 5 complexes of integral membrane proteins: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV), ATP synthase (Complex V) and two freely diffusible molecules ubiquinone and cytochrome c that shuttle electrons

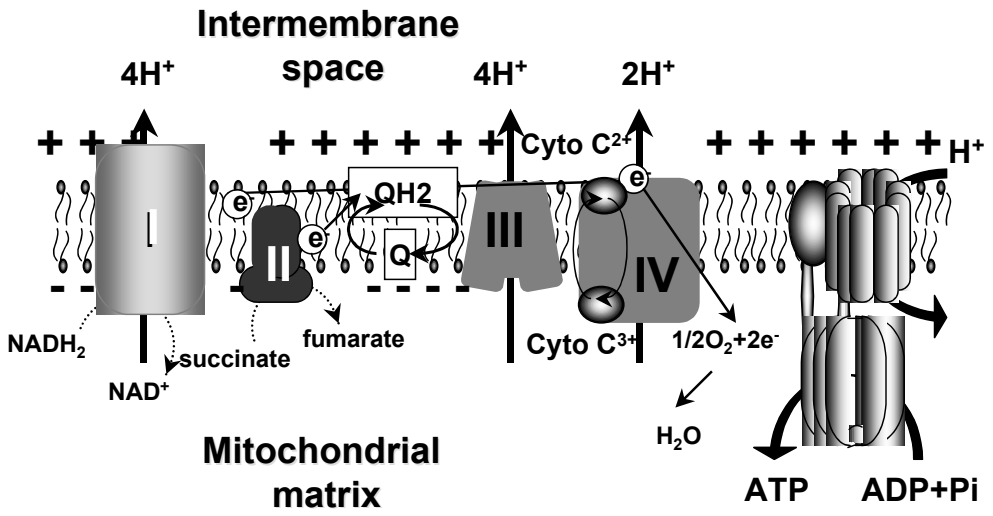

Figure 3. Structure of the mitochondrial respiratory chain

from one complex to the next (Figure 3). The respiratory chain accomplishes the stepwise transfer of electrons from NADH and $FADH_2$ (produced in the citric acid or Krebs cycle) to oxygen molecules to form (with the aid of

protons) water molecules harnessing the energy released by this transfer to the pumping of protons (H^+) from the matrix to the intermembrane space. The gradient of protons formed across the inner membrane by this process of active transport forms a miniature battery. The protons can flow back down this gradient, reentering the matrix, only through another complex of integral proteins in the inner membrane, the ATP synthase complex.

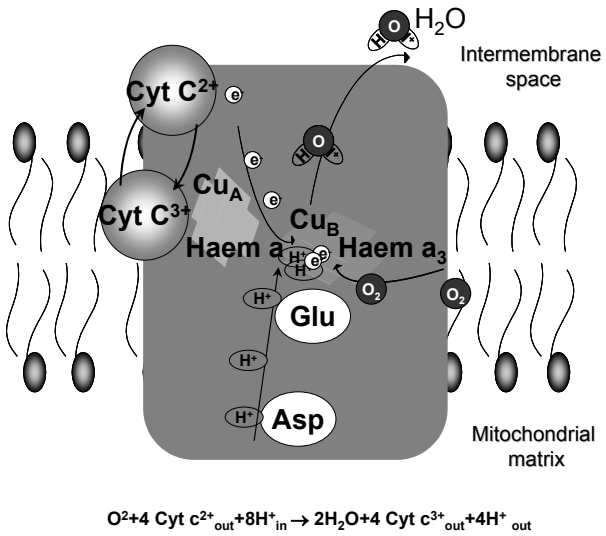

Figure 4. Structure and mode of action of cytochrome c oxidase

Absorption spectra obtained for cytochrome c oxidase in different oxidation states were recorded and found to be very similar to the action spectra for biological responses to light. Therefore it was proposed that cytochrome c oxidase is the primary photoacceptor for the red-NIR range in mammalian cells [7] (Figure 4). Cytochrome C oxidase contains two iron centers, haem *a* and haem a_3 (also referred to as cytochromes *a* and a_3), and two copper centers, Cu_A and Cu_B [8]. Fully oxidized cytochrome c oxidase has both iron atoms in the Fe(III) oxidation state and both copper atoms in the Cu(II) oxidation state, while fully reduced cytochrome c oxidase has the iron in Fe(II) and copper in Cu(I) oxidation states. There are many intermediate mixed-valence forms of the enzyme and other coordinate ligands such as CO, CN, and formate can be involved. All the many individual oxidation states of the enzyme have different absorption spectra [9], thus probably accounting for slight differences in action spectra of LLLT that have been reported. A recent paper from Karu's group [10] gave the following wavelength ranges for four peaks in the LLLT action spectrum: 1) 613.5 - 623.5 nm, 2) 667.5 - 683.7 nm, 3) 750.7 - 772.3 nm, 4) 812.5 - 846.0 nm.

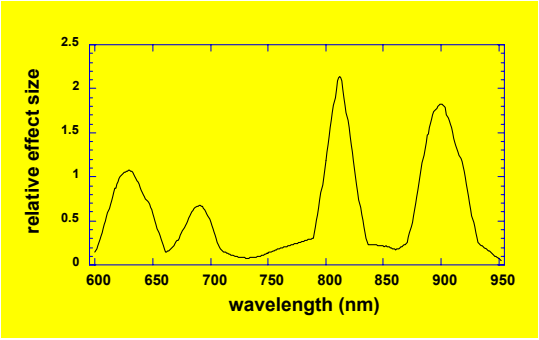

Figure 5. Generalized action spectrum for LLLT effects in cells, animals and patients. Data shown are an amalgamation of many literature reports from multiple laboratories.

A study from Pastore et al [11] examined the effect of He-Ne laser illumination on the purified cytochrome c oxidase enzyme and found increased oxidation of cytochrome c and increased electron transfer. Artyukhov and colleagues found [12] increased enzyme activity of catalase after He-Ne illumination.

Absorption of photons by molecules leads to electronically excited states and consequently can lead to acceleration of electron transfer reactions [13]. More electron transport necessarily leads to increased production of ATP [14]. Light induced increase in ATP synthesis and increased proton gradient leads to an increasing activity of the Na^+/H^+ and Ca^{2+}/Na^+ antiporters and of all the ATP driven carriers for ions, such as Na^+/K^+ ATPase and Ca^{2+} pumps. ATP is the substrate for adenyl cyclase, and therefore the ATP level controls the level of cAMP. Both Ca^{2+} and cAMP are very important second messengers. Ca^{2+} especially regulates almost every process in the human body (muscle contraction, blood coagulation, signal transfer in nerves, gene expression, etc.).

In addition to cytochrome c oxidase mediated increase in ATP production, other mechanisms may be operating in LLLT. The first of these we will consider is the "singlet-oxygen hypothesis." Certain molecules with visible absorption bands like porphyrins lacking transition metal coordination centers [15] and some flavoproteins [16] can be converted into a long-lived triplet state after photon absorption. This triplet state can interact with ground-state oxygen with energy transfer leading to production of a reactive species, singlet oxygen. This is the same molecule utilized in photodynamic therapy (PDT) to kill cancer cells, destroy blood vessels and kill microbes. Researchers in PDT have known for a long time that very low doses of PDT can cause cell proliferation and tissue stimulation instead of the killing observed at high doses [17].

The next mechanism proposed was the "redox properties alteration hypothesis" [18]. Alteration of mitochondrial metabolism and activation of the respiratory chain by illumination would also increase production of superoxide anions $O_2^{\bullet-}$. It has been shown that the total cellular production of $O_2^{\bullet-}$ depends primarily on the metabolic state of the mitochondria. Other redox chains in cells can also be activated by LLLT. NADPH-oxidase is an enzyme found on activated neutrophils and is capable of a non-mitochondrial respiratory burst and production of high amounts of ROS can be induced. [19]. These effects depend on the physiological status of the host organism as well as on radiation parameters.

The activity of cytochrome c oxidase is inhibited by nitric oxide (NO). This inhibition of mitochondrial respiration by NO can be explained by a direct competition between NO and O_2 for the reduced binuclear center CuB/a3 of cytochrome c oxidase and is reversible [20]. It was proposed that laser irradiation could reverse the inhibition of cytochrome c oxidase by NO and thus may increase the respiration rate ("NO hypothesis") [21]. Data published recently by Karu et al [21] indirectly support this hypothesis. Another piece of evidence for NO involvement in responses to LLLT is an increase in inducible nitric oxide synthase production after exposure to light (635 nm) [22]. While both observations support the hypothesis of NO dependent responses to LLLT, responses to different wavelengths of light in different models may be governed by distinct mechanisms.

2.3 Cell signaling

The combination of the products of the reduction potential and reducing capacity of the linked redox couples present in cells and tissues represent the redox environment (redox state) of the cell. Redox couples present in the cell include: nicotinamide adenine dinucleotide (oxidized/ reduced forms) NAD/NADH, nicotinamide adenine dinucleotide phosphate NADP/NADPH, glutathione/glutathione disulfide couple GSH/GSSG and thioredoxin/ thioredoxin disulfide couple Trx(SH)2/TrxSS [23]. Several important regulation pathways are mediated through the cellular redox state. Changes in redox state induce the activation of numerous intracellular signaling pathways, regulate nucleic acid synthesis, protein synthesis, enzyme activation and cell cycle progression [24]. These cytosolic responses in turn induce transcriptional changes. Several transcription factors are regulated by changes in cellular redox state. Among them redox factor –1 (Ref-1)- dependent activator protein-1 (AP-1) (Fos and Jun), nuclear factor κB (NF-κB), p53, activating transcription factor/cAMP-response element–binding protein (ATF/ CREB), hypoxia-inducible factor (HIF)-1α, and HIF-like factor. As a rule, the oxidized form of redox-dependent transcription factors have low DNA-binding activity. Ref-1 is an important factor for the specific reduction of these transcription factors. However it was also shown that low levels of oxidants appear to stimulate proliferation and differentiation of some type of cells [25-27]

It is proposed that LLLT produces a shift in overall cell redox potential in the direction of greater oxidation [28]. Different cells at a range of growth conditions have distinct redox states. Therefore, the effects of LLLT can

vary considerably. Cells being initially at a more reduced state (low intracellular pH) have high potential to respond to LLLT, while cells at the optimal redox state respond weakly or do not respond to treatment with light.

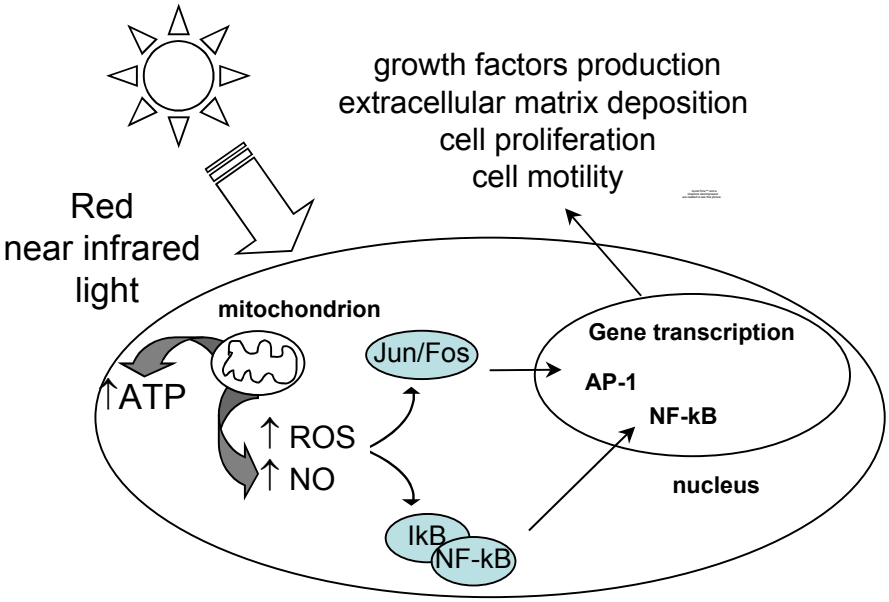

Figure 6. Cell signaling pathways induced by LLLT.

3. IN VITRO RESULTS

3.1 Cell types

There is evidence that multiple mammalian and microbial cell types can respond to LLLT. Much of Karu's work has used *Escherichia coli* (a Gram-negative aerobic bacterium) [29] and HeLa cells [30], a human cervical carcinoma cell line. However for the clinical applications of LLLT to be validated it is much more important to study the effects of LLLT on non-malignant cell types likely to be usefully stimulated in order to remedy some disease or injury. For wound healing type studies, these cells are likely to be endothelial cells [31], fibroblasts [32], keratinocytes [33] and possibly some classes of leukocytes such as macrophages [34] and neutrophils [35]. For pain relief and nerve regrowth studies these cells will be neurons [36-38] and glial cells [39]. For anti-inflammatory and anti-edema applications the cell types will be macrophages [34], mast-cells [40], neutrophils [41], lymphocytes [42] etc. There is literature evidence for in vitro LLLT effects for most of these cell types.

3.2. Isolated mitochondria

Since the respiratory chain and cytochrome c oxidase are located in mitochondria, several groups have tested the effect of LLLT on preparations of isolated mitochondria. The most popular system to study is the effects of HeNe laser illumination of mitochondria isolated from rat liver. Increased proton electrochemical potential and ATP synthesis was found [43]. Increased RNA and protein synthesis was demonstrated after 5 J/cm^2 [44]. Pastore et al [45] found increased activity of cytochrome c oxidase and an increase in polarographically measured oxygen uptake after 2 J/cm^2 of HeNe. A major stimulation in the proton pumping activity, about 55% increase of <--H+/e- ratio was found in illuminated mitochondria. Yu et al [13] used 660 nm laser at a power density of 10 mW/cm^2 and showed increased oxygen consumption (0.6 J/cm^2 and 1.2 J/cm^2), increased phosphate potential, and energy charge (1.8 J/cm^2 and 2.4 J/cm^2) and enhanced activities of NADH: ubiquinone oxidoreductase, ubiquinol: ferricytochrome C oxidoreductase and ferrocytochrome C: oxygen oxidoreductase (between 0.6 J/cm^2, and 4.8 J/cm^2).

3.3 LLLT cellular response

The cellular responses observed in vitro after LLLT can be broadly classed under increases in metabolism, migration, proliferation, and increases in synthesis and secretion of various proteins. Many studies report effects on more than one of these parameters. Yu et al reported [33] on cultured keratinocytes and fibroblasts that were irradiated with 0.5-1.5 J/cm^2 HeNe laser. They found a significant increase in basic fibroblast growth factor (bFGF) release from both keratinocytes and fibroblasts and a significant increase in nerve growth factor release from keratinocytes. Medium from HeNe laser irradiated keratinocytes stimulated [3H]thymidine uptake and proliferation of cultured melanocytes. Furthermore, melanocyte migration was enhanced either directly by HeNe laser or indirectly by the medium derived from HeNe laser treated keratinocytes.

The presence of cellular responses to LLLT at molecular level was also demonstrated [46]. Normal human fibroblasts were exposed for 3 days to 0.88J/cm^2 of 628 nm light from light emitting diode. Gene expression profiles upon irradiation were examined using a cDNA microarray containing 9982 human genes. 111 genes were found to be affected by light. All genes from antioxidant related category and genes related to energy metabolism and respiratory chain were upregulated. Most of the genes related to cell proliferation were upregulated too. Amongst genes related to apoptosis and stress response, some genes such as JAK binding protein were upregulated, others such as HSP701A, caspase 6 and stress-induced phosphoprotein were downregulated. It was suggested that LLLT stimulates cell growth directly by regulating the expression of specific genes, as well as indirectly by regulating the expression of the genes related to DNA synthesis and repair, and cell metabolism.

4. ANIMAL MODELS

There has been a large number of animal models that have been used to demonstrate LLLT effects on a variety of diseases, injuries, and both chronic and acute conditions, In this review we will therefore only discuss three particular applications for which there are good literature reports of efficacy.

4.1 Wound healing

The literature on LLLT applied to a stimulation of wound healing in a variety of animal models contains both positive and negative studies. The reasons for the conflicting reports, sometimes in very similar wound models, are probably diverse. It is probable that applications of LLLT in animal models will be more effective if carried out on models that have some intrinsic disease state. Although there have been several reports that processes such as wound healing are accelerated by LLLT in normal rodents [3, 34], an alternative approach is to inhibit healing by inducing some specific disease state. This has been done in the case of diabetes, a disease known to significantly depress wound healing in patients. LLLT significantly improves wound healing in both diabetic rats [35, 36] and diabetic mice [37, 38]. LLLT was also effective in X-radiation impaired wound healing in mice [39]. A study [47] in hairless mice found improvement in the tensile strength of the HeNe laser-irradiated wounds at 1 and 2 weeks. Furthermore, the total collagen content was significantly increased at 2 months when compared with control wounds. The beneficial effect of LLLT on wound healing can be explained by considering several basic biological mechanisms including the induction of expression cytokines and growth factors known to be responsible for the many phases of wound healing. Firstly there is a report [48] that HeNe laser increased both protein and mRNA levels of IL-1α and IL-8 in keratinocytes. These are cytokines responsible for the initial inflammatory phase of wound healing. Secondly there are reports [49] that LLLT can upregulate cytokines responsible for fibroblast proliferation and migration such as bFGF, HGF and SCF. Thirdly it has been reported [50] that LLLT can increase growth factors such as VEGF responsible for the neovascularization necessary for wound healing. Fourthly TGF-β is a growth factor responsible for inducing collagen synthesis from fibroblasts and has been reported to be upregulated by LLLT [51]. Fifthly there are reports [52, 53] that LLLT can induce fibroblasts to undergo the transformation into myofibloblasts, a cell type that expresses smooth muscle α-actin and desmin and has the phenotype of contractile cells that hasten wound contraction.

4.2 Neuronal toxicity

Studies from Whelan's group have explored the use of 670-nm LEDs in combating neuronal damage caused by neurotoxins. Methanol intoxication is caused by metabolic conversion to formic acid that produces injury to the retina and optic nerve, resulting in blindness. Using a rat model and the electroretinogram as a sensitive indicator of

retinal function, they demonstrated that three brief 670-nm LED treatments (4 J/cm(2)), delivered at 5, 25, and 50 h of methanol intoxication, attenuated the retinotoxic effects of methanol-derived formate. There was a significant recovery of rod- and cone-mediated function in LED-treated, methanol-intoxicated rats and histopathologic evidence of retinal protection [54]. A subsequent study [55] explored the effects of an irreversible inhibitor of cytochrome c oxidase, potassium cyanide in primary cultured neurons. LED treatment partially restored enzyme activity blocked by 10-100 microM KCN. It significantly reduced neuronal cell death induced by 300 μM KCN from 83.6 to 43.5%. LED significantly restored neuronal ATP content only at 10 microM KCN but not at higher concentrations of KCN tested. In contrast, LED was able to completely reverse the detrimental effect of tetrodotoxin, which only indirectly down-regulated enzyme levels. Among the wavelengths tested (670, 728, 770, 830, and 880 nm), the most effective ones (830 nm, 670 nm) paralleled the NIR absorption spectrum of oxidized cytochrome c oxidase.

4.3 Nerve regeneration

Animal models have been employed to study LLLT effects in nerve repair [56, 57]. Byrnes et al used 1,600 J/cm^2 of 810-nm diode laser to improve healing and functionality in a T9 dorsal hemisection of the spinal cord in rats [39]. Anders et al [58] studied LLLT for regenerating crushed rat facial nerves; by comparing 361, 457, 514, 633, 720, and 1064-nm and found best response with 162.4 J/cm^2 of 633-nm HeNe laser.

5. CLINICAL STUDIES

Low-power laser therapy is used by physical therapists to treat a wide variety of acute and chronic musculoskeletal aches and pains, by dentists to treat inflamed oral tissues and to heal diverse ulcerations, by dermatologists to treat edema, non-healing ulcers, burns, and dermatitis, by orthopedists to relieve pain and treat chronic inflammations and autoimmune diseases, and by other specialists, as well as general practitioners. Laser therapy is also widely used in veterinary medicine (especially in racehorse-training centers) and in sports-medicine and rehabilitation clinics (to reduce swelling and hematoma, relieve pain, improve mobility, and treat acute soft-tissue injuries). Lasers and LEDs are applied directly to the respective areas (e.g., wounds, sites of injuries) or to various points on the body (acupuncture points, muscle-trigger points). However one of the most important limitations to advancing the field into mainstream medical practice is the lack of appropriately controlled and blinded clinical trials. The trials should be prospective, placebo controlled and double blinded and contain sufficient subjects to allow statistically valid conclusions to be reached.

Clinical applications of low-power laser therapy are diverse. The field is characterized by a variety of methodologies and uses of various light sources (lasers, LEDs) with different parameters (wavelength, output power, continuous-wave or pulsed operation modes, pulse parameters). In recent years, longer wavelengths (~800 to 900 nm) and higher output powers (to 100 mW) have been preferred in therapeutic devices especially to allow deeper tissue penetration. In 2002 MicroLight Corp received 510K FDA clearance for the ML 830-nm diode laser for treatment of carpal tunnel syndrome. There were several controlled trials reporting significant improvement in pain and some improvement in objective outcome measures [59-61]. Since then several light sources have been approved as equivalent to an infrared heating lamp for treating a wide-range of musculoskeletal disorders with no supporting clinical studies.

6. UNRESOLVED QUESTIONS

6.1 Wavelength. This is probably the parameter where there is most agreement in the LLLT community. Wavelengths in the 600-700-nm range are chosen for treating superficial tissue, and wavelengths between 780 and 950 are chosen for deeper-seated tissues due to longer optical penetration distances through tissue. Wavelengths between 700 and 770-nm are not considered to have much activity.

6.2 Laser vs non-coherent light. One of the most topical and widely discussed issues in the LLLT clinical community is whether the coherence and monochromatic nature of laser radiation have additional benefits as compared with more broad band light from a conventional light source or LED with the same center wavelength and intensity. Two aspects of this problem must be distinguished: the coherence of light itself and the coherence of the interaction of light with matter (biomolecules, tissues).

6.3. Dose. Because of the possible existence of a biphasic dose response curve referred to above, choosing the correct dosage of light (in terms of energy density) for any specific medical condition is difficult. In addition there has been some confusion in the literature about the delivered fluence when the light spot is small. If 5J of light is

given to a spot of 5 mm^2 the fluence is 100 J/cm^2 which is nominally the same fluence as 100 J/cm^2 delivered to 10 cm^2, but the total energy delivered in the latter case is 200 time greater.

6.3 Pulsed or CW. There have been some reports that pulse structure is an important factor in LLLT; for instance Ueda et al [62, 63] found better effects using 1 or 2 Hz pulses than 8 Hz or CW 830-nm laser on rat bone cells, but the underlying mechanism for this effect is unclear..

6.4 Polarization status. There are some claims that polarized light has better effects in LLLT applications than otherwise identical non-polarized light (or even 90-degree rotated polarized light) [64]. However it is known that polarized light is rapidly scrambled in highly scattering media such as tissue (probably in the first few hundred μm), and it therefore seem highly unlikely that polarization could play a role except for superficial applications to the upper layers of the skin.

6.5. ***Systemic effects.*** Although LLLT is mostly applied to localized diseases and its effect is often considered to be restricted to irradiated area, there are reports of systemic effects of LLLT acting at a site distant from the illumination [65, 66].

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