Dose-Response (Prepress)
Formerly Nonlinearity in Biology, Toxicology, and Medicine
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ISSN: 1559-3258
DOI: 10.2203/dose-response.09-027.Hamblin

International Dose-Response Society
www.Dose-Response.org

BIPHASIC DOSE RESPONSE IN LOW LEVEL LIGHT THERAPY

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□ 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 cell death and tissue damage has been known for over forty years since the invention of lasers. Despite many reports of positive findings from experiments conducted in vitro, in animal models and in randomized controlled clinical trials, LLLT remains controversial in mainstream medicine. The biochemical mechanisms underlying the positive effects are incompletely understood, and 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. A biphasic dose response has been frequently observed where low levels of light have a much better effect on stimulating and repairing tissues than higher levels of light. The so-called Arndt-Schulz curve is frequently used to describe this biphasic dose response. This review will cover the molecular and cellular mechanisms in LLLT, and describe some of our recent results in vitro and in vivo that provide scientific explanations for this biphasic dose response.

1. INTRODUCTION

1.1. Brief history

Low level laser therapy (LLLT) is the application of light (usually a low power laser or LED in the range of 1mW – 500mW) to a pathology to promote tissue regeneration, reduce inflammation and relieve pain. The light is typically of narrow spectral width in the red or near infrared

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(NIR) spectrum (600nm – 1000nm), with a power density (irradiance) between 1mw-5W/cm^2. It is typically applied to the injury for a minute or so, a few times a week for several weeks. Unlike other medical laser procedures, LLLT is not an ablative or thermal mechanism, but rather a photochemical effect comparable to photosynthesis in plants whereby the light is absorbed and exerts a chemical change.

The phenomenon was first published by Endre Mester at Semmelweis University, Budapest, Hungary in 1967 a few years after the first working laser was invented (Mester *et al.* 1967). Mester conducted an experiment to test if laser radiation might cause cancer in mice. He shaved the hair off their backs, divided them into two groups and irradiated one group with a low powered ruby laser (694-nm). The treatment group did not get cancer and to his surprise, the hair grew back more quickly than the untreated group. He called this "Laser Biostimulation".

1.2. Evidence for effectiveness of LLLT

Since 1967 over 100 phase III, randomized, double-blind, placebo-controlled, clinical trials (RCTs) have been published and supported by over 1,000 laboratory studies investigating the primary mechanisms and the cascade of secondary effects that contribute to a range of local tissue and systemic effects.

RCTs with positive outcomes have been published on pathologies as diverse as osteoarthritis (Bertolucci and Grey 1995; Ozdemir *et al.* 2001; Stelian *et al.* 1992), tendonopathies (Bjordal *et al.* 2006b; Stergioulas *et al.* 2008; Vasseljen *et al.* 1992), wounds (Caetano *et al.* 2009; Gupta *et al.* 1998; Ozcelik *et al.* 2008; Schubert *et al.* 2007), back pain (Basford *et al.* 1999), neck pain (Chow *et al.* 2006; Gur *et al.* 2004), muscle fatigue (Leal Junior *et al.* 2008a; Leal Junior *et al.* 2008b), peripheral nerve injuries (Rochkind *et al.* 2007) and strokes (Lampl *et al.* 2007; Zivin *et al.* 2009); nevertheless results have not always been positive. This failure in certain circumstances can be attributed to several factors including dosimetry (inadequate or too much energy delivered, inadequate or too much irradiance, inappropriate pulse structure, irradiation of insufficient area of the pathology), inappropriate anatomical treatment location and concurrent patient medication (such as steroidal and non-steroidal anti-inflammatories which can inhibit healing) (Aimbire *et al.* 2006; Goncalves *et al.* 2007).

1.3. The medicine and the dose

As with other forms of medication, LLLT has its active ingredients or "medicine" (irradiation parameters) and a "dose" (the irradiation time). Table 1 lists the key parameters that define the medicine and Table 2 defines the dose. It is beyond the scope of this paper to exhaustively list and discuss every conceivable aspect of laser radiation or other light

TABLE 1. Parameters involved in determining the LLLT "medicine"

IRRADIATION PARAMETERS (*The Medicine*)		
Irradiation Parameter	Unit of measurement	Comment
Wavelength	nm	Light is electromagnetic energy which travels in discrete packets that also have a wave-like property. Wavelength is measure in nanometres (nm) and is visible in the 400-700 nm range.
Irradiance	W/cm^2	Often called Intensity, or Power Density and is calculated as Irradiance = Power (W)/Area (cm^2)
Pulse structure	Peak Power (W) Pulse freq (Hz) Pulse Width (s) Duty cycle (%)	If the beam is pulsed then the Power should be the Average Power and calculated as follows: Average Power (W) = Peak Power (W) × pulse width (s) × pulse frequency (Hz)
Coherence	Coherence length depends on spectral bandwidth	Coherent light produces laser speckle, which has been postulated to play a role in the photobiomodulation interaction with cells and subcellular organelles.
Polarisation	Linear polarized or circular polarized	Polarized light may have different effects than otherwise identical non-polarized light (or even 90-degree rotated polarized light). However, it is known that polarized light is rapidly scrambled in highly scattering media such as tissue (probably in the first few hundred μm).

sources however we believe we have captured the main elements with some comment on others.

Energy (J) or energy density (J/cm^2) is often used as an important descriptor of LLLT dose, but this neglects the fact that energy has two components, power and time,

$$\text{Energy (J)} = \text{Power (W)} \times \text{Time (s)}$$

and it has been demonstrated that there is not necessarily reciprocity between them; in other words, if the power doubled and the time is halved then the same energy is delivered but a different biological response is often observed.

It is our view LLLT is best described as two separate sets of parameters;

(a) The medicine (irradiation parameters)
(b) The dose (time)

This paper will mainly focus on irradiance and time, as it is beyond the scope of this paper to report in detail on the response to all aspects

TABLE 2. Parameters involved in determining the LLLT "dose"

IRRADIATION TIME OR ENERGY DELIVERED (*The Dose*)		
Irradiation Parameter	Unit of measurement	Comment
Energy (Joules)	J	Calculated as: Energy (J) = Power (W) x time (s) This mixes medicine and dose into a single expression and ignores Irradiance. Using Joules as an expression of dose is potentially unreliable as it assumes reciprocity (the inverse relationship between power and time).
Energy Density	J/cm^2	Common expression of LLLT "dose" is Energy Density This expression of dose again mixes medicine and dose into a single expression and is potentially unreliable as it assumes a reciprocity relationship between irradiance and time.
Irradiation Time	s	In our view the safest way to record and prescribe LLLT is to define the four parameters of the medicine (see table 1.) and then define the irradiation time as "dose".
Treatment interval	Hours, days or weeks	The effects of different treatment interval is underexplored at this time though there is sufficient evidence to suggest that this is an important parameter.

laser radiation listed in the "medicine" table; however there is evidence to show that different wavelengths, pulses, coherence, polarization have some effect on the magnitude of biomodulation (see sections 3 and 4).

2. MECHANISMS OF LOW LEVEL LIGHT THERAPY.

2.1. Cellular Chromophores and First Law of 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 photoacceptors, or chromophores (Sutherland 2002). A chromophore is a molecule (or part of a molecule) which imparts some decided color to the compound of which it is an ingredient. Chromophores almost always occur in one of two forms: conjugated pi electron systems and metal complexes. Examples of such chromophores can be seen in chlorophyll (used by plants for photosynthesis), hemoglobin, cytochrome c oxidase (Cox), myoglobin, flavins, flavoproteins and porphyrins (Karu 1999). Figure 1 illustrates the general concept of LLLT.

2.2. Action Spectrum and Tissue Optics

One important consideration should involve the optical properties of tissue. There is a so-called "optical window" in tissue, where the effective

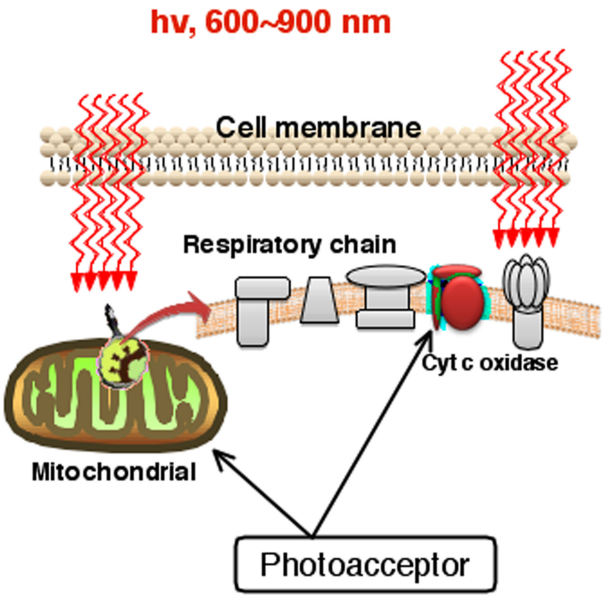

FIGURE 1. Schematic diagram showing the absorption of red and NIR light by specific cellular chromophores or photoacceptors localized in the mitochondrial respiratory chain

tissue penetration of light is maximized. This optical window runs approximately from 650 nm to 1200 nm. (Figure 2). The absorption and scattering of light in tissue are both much higher in the blue region of the spectrum than the red, because the principle tissue chromophores (hemoglobin and melanin) have high absorption bands at shorter wavelengths, tissue scattering of light is higher at shorter wavelengths, and furthermore water strongly absorbs infrared light at wavelengths greater than 1100-nm. Therefore the use of LLLT in animals and patients almost exclusively involves red and near-infrared light (600-1100-nm) (Karu and Afanas'eva 1995).

Phototherapy is characterized by its ability to induce photobiological processes in cells. Exact action spectra are needed for determination of photoacceptors as well as for further investigations into cellular mechanisms of phototherapy. The action spectrum shows which specific wavelength of light is most effectively used in a specific chemical reaction (Karu and Kolyakov 2005). The fact that defined action spectra can be constructed for various cellular responses confirms the first law of photobiology described above (light absorption by specific molecular chromophores).

2.3. Mitochondrial Respiration and ATP

Current research about the mechanism of LLLT effects inevitably involves mitochondria. Mitochondria play an important role in energy generation and metabolism. Mitochondria are sometimes described as

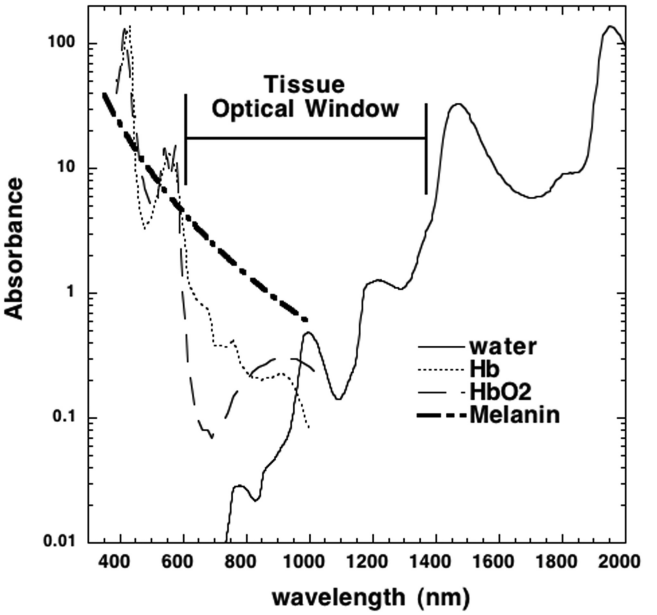

FIGURE 2. Absorption spectra of the main chromophores in living tissue on a log scale showing the optical window where visible and NIR light can penetrate deepest into tissue.

"cellular power plants", because they convert food molecules into energy in the form of ATP via the process of oxidative phosphorylation (see Figure 3 for an illustartion of the mitochondrial respiratory chain).

The mechanism of LLLT at the cellular level has been attributed to the absorption of monochromatic visible and NIR radiation by components of the cellular respiratory chain (Karu 1989). Several pieces of evidence suggest that mitochondria are responsible for the cellular response to red visible and NIR light. The effects of HeNe laser and other illumination on mitochondria isolated from rat liver, have included increased proton electrochemical potential, more ATP synthesis (Passarella *et al.* 1984), increased RNA and protein synthesis (Greco *et al.* 1989) and increases in oxygen consumption, membrane potential, and enhanced synthesis of NADH and ATP.

2.4. Cytochrome c oxidase and nitric oxide release

Absorption spectra obtained for cytochrome c oxidase (Cox) in different oxidation states were recorded and found to be very similar to the action spectra for biological responses to light (Karu and Kolyakov 2005). Therefore it was proposed that Cox is the primary photoacceptor for the red-NIR range in mammalian cells (Karu and Kolyakov 2005).

Nitric oxide produced in the mitochondria can inhibit respiration by binding to Cox and competitively displacing oxygen, especially in stressed

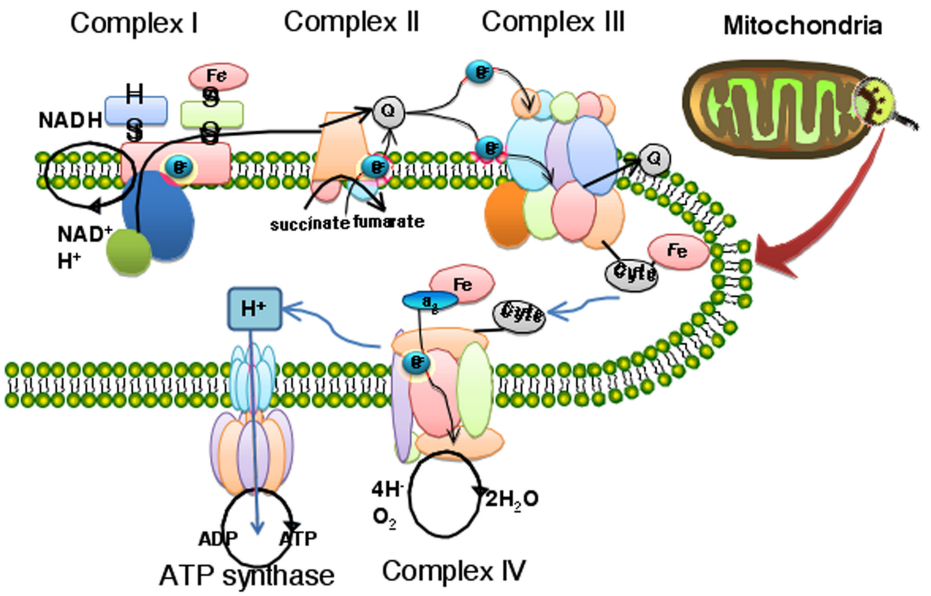

FIGURE 3. Mitochondrial respiratory chain consisting of contains five complexes of integral membrane proteins: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (ComplexV).

or hypoxic cells (Brown 2001). Increased nitric oxide (NO) concentrations can sometimes be measured in cell culture or in animals after LLLT due to its photo release from the mitochondria and Cox. It has been proposed that LLLT might work by photodissociating NO from Cox, thereby reversing the mitochondrial inhibition of respiration due to excessive NO binding (Lane 2006). Figure 4 illustrates the photodissociation of NO from its binding sites on the heme iron and copper centers where it cometively inhibits oxygen binding and reduces necessary enzymic activity, thus allowing an immediate influx of oxygen and resumption of respiration and generation of reactive oxygen species.

2.5. NO signaling

In addition to NO being photodissociated from Cox as described, it may also be photo-released from other intracellular stores such as nitrosylated hemoglobin and nitrosylated myoglobin (Shiva and Gladwin 2009). Light mediated vasodilation was first described in 1968 by R F Furchgott, in his nitric oxide research that lead to his receipt of a Nobel Prize thirty years later in 1998 (Mitka 1998). Later studies conducted by other researchers confirmed and extended Furchgott's early work and demonstrated the ability of light to influence the localized production or release of NO and stimulate vasodilation through the effect NO on cyclic guanine

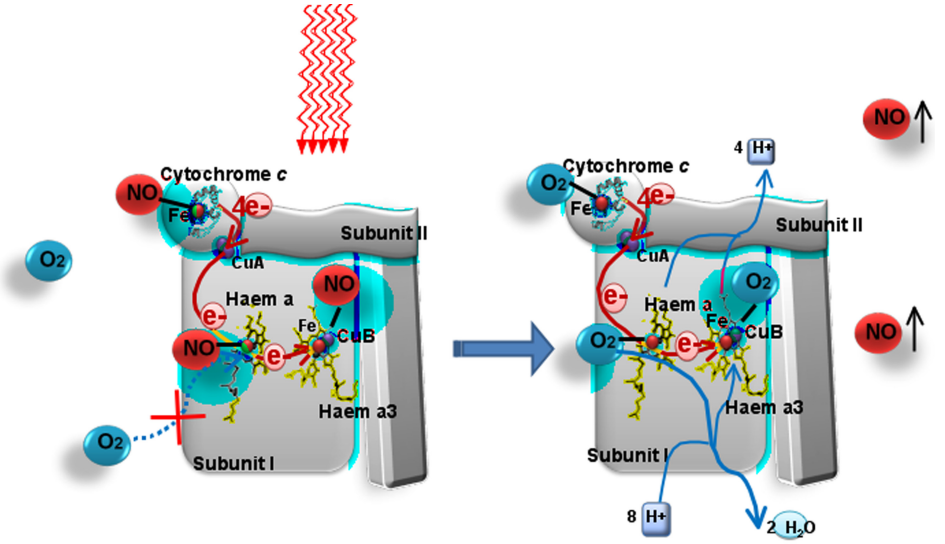

FIGURE 4. When NO is released from its binding to heme iron and copper centers in cytochrome c oxidase by the action of light, oxygen is allowed to rebind to these sites and respiration is restored to its former level leading to increased ATP synthesis.

monophosphate (cGMP). This finding suggested that properly designed illumination devices may be effective, noninvasive therapeutic agents for patients who would benefit from increased localized NO availability

2.6. Reactive oxygen species and gene transcription

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the signaling pathways from mitochondria to nuclei. Reactive oxygen species (ROS) are very small molecules that include oxygen ions such as superoxide, free radicals such as hydroxyl radical, and hydrogen peroxide, and organic peroxides. They are highly with biological molecules such as proteins, nucleic acids and unsaturated lipids. ROS form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling (Storz 2007), regulating nucleic acid synthesis, protein synthesis, enzyme activation and cell cycle progression (Brondon *et al.* 2005). LLLT was reported to produce a shift in overall cell redox potential in the direction of greater oxidation (Karu 1999) and increased ROS generation and cell redox activity have been demonstrated (Alexandratou *et al.* 2002; Chen *et al.* 2009b; Grossman *et al.* 1998; Lavi *et al.* 2003; Lubart *et al.* 2005; Pal *et al.* 2007; Zhang *et al.* 2008). These cytosolic responses may in turn induce transcriptional changes. Several transcription factors are regulated by changes in cellular redox state. But the most important one is nuclear factor B (NF-B). Figure 5 illustrates the effect of redox-sensitive transcription factor NF-κB

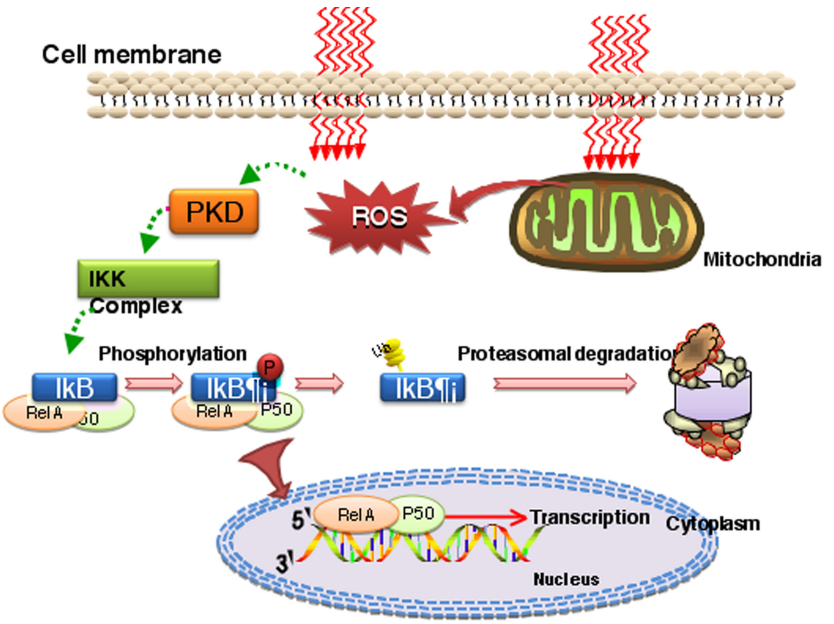

FIGURE 5. Reactive oxygen species (ROS) formed as a result of LLLT effects in mitochondria may activate the redox-sensitive transcription factor NF-κB (relA-p50) via protein kinase D (PKD).

activated after LLLT and is instrumental in causing transcription of protective and stimulatory gene products.

2.7. Downstream cellular response

Although the underlying mechanism of LLLT are still not completely understood, in vitro studies, animal experiments and clinical studies have all tended to indicate that LLLT delivered at low doses may produce a better result when compared to the same light delivered at high doses. LLLT can prevent cell apoptosis and improve cell proliferation, migration and adhesion at low levels of red/NIR light illumination (see Figure 6).

LLLT at low doses has been shown to enhance cell proliferation in vitro in several types of cells: fibroblasts (Lubart *et al.* 1992; Yu *et al.* 1994), keratinocytes (Grossman *et al.* 1998), endothelial cells (Moore *et al.* 2005), and lymphocytes (Agaiby *et al.* 2000; Stadler *et al.* 2000). The mechanism of proliferation was proposed to involve photostimulatory effects in mitochondria processes, which enhanced growth factor release, and ultimately led to cell proliferation (Bjordal *et al.* 2007). Kreisler et al showed (Kreisler *et al.* 2003) that the attachment and proliferation of human gingival fibroblasts were enhanced by LLLT in a dose-dependent manner. LLLT modulated matrix metalloproteinase activity and gene expression in porcine aortic smooth muscle cells (Gavish *et al.* 2006). Shefer at el. showed (Shefer *et al.* 2002) that LLLT could activate skeletal

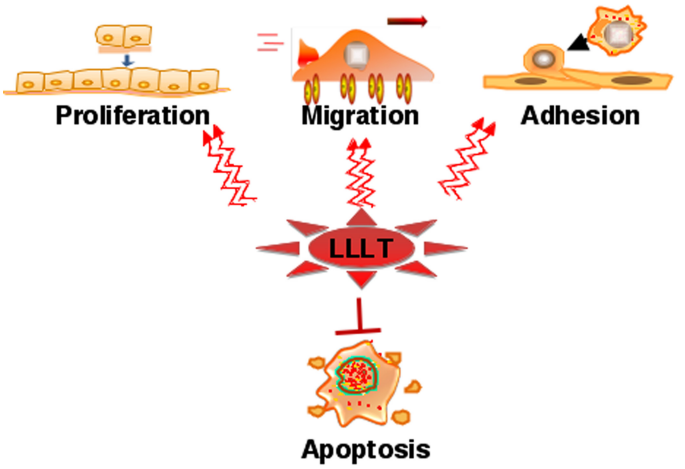

FIGURE 6. The downstream cellular effects of LLLT signaling include increases in cell proliferation, migration and adhesion molecules. Cell survival is increased and cell death reduced by expression of proteins that inhibit apoptosis.

muscle satellite cells, enhancing their proliferation, inhibiting differentiation and regulating protein synthesis.

2.8. Downstream tissue response

There have been a large number of both animal model and clinical studies that demonstrated highly beneficial LLLT effects on a variety of diseases, injuries, and has been widely used in both chronic and acute conditions (see Figure 7). LLLT may enhance neovascularisation, promote angiogenesis and increase collagen synthesis to promote healing of acute (Hopkins *et al.* 2004) and chronic wounds (Yu *et al.* 1997). LLLT provided acceleration of cutaneous wound healing in rats with a biphasic dose response favoring lower doses (Corazza *et al.* 2007). LLLT can also stimulate healing of deeper structures such as nerves (Gigo-Benato *et al.* 2004), tendons (Fillipin *et al.* 2005), cartilage (Morrone *et al.* 2000), bones (Weber *et al.* 2006) and even internal organs (Shao *et al.* 2005). LLLT can reduce pain (Bjordal *et al.* 2006a), inflammation (Bjordal *et al.* 2006b) and swelling (Carati *et al.* 2003) caused by injuries, degenerative diseases or autoimmune diseases. Oron reported beneficial effect of LLLT on repair processes after injury or ischemia in skeletal and heart muscles in multiple animal models in vivo (Ad and Oron 2001; Oron *et al.* 2001a; Oron *et al.* 2001b; Yaakobi *et al.* 2001). LLLT has been used to mitigate damage after strokes (in both animals (Lapchak *et al.* 2008) and humans (Lampl *et al.* 2007)), after traumatic brain injury (Oron *et al.* 2007) and after spinal cord injury (Wu *et al.* 2009).

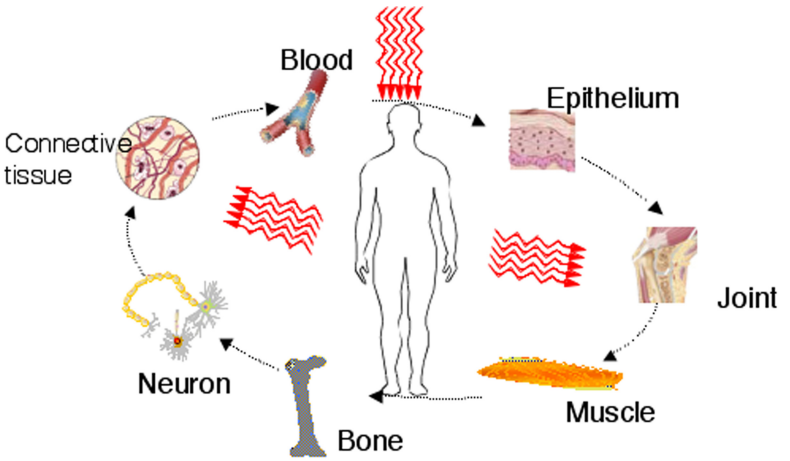

FIGURE 7. Beneficial tissue effects of LLLT can include almost all the tissues and organs of the body.

3. REVIEW OF BIPHASIC DOSE RESPONSES IN LLLT

3.1. Dose dependence and dose rate effects—the biphasic curve

A biphasic response has been demonstrated many times in LLLT research (Lanzafame *et al.* 2007; Oron *et al.* 2001a) and the "Arndt-Schulz Law" is frequently quoted as a suitable model to describe dose dependent effects of LLLT (Chow *et al.* 2006; Hawkins and Abrahamse 2006a; Hawkins and Abrahamse 2006b; Lubart *et al.* 2006; Sommer *et al.* 2001). The concept of the Arndt-Schulz Law dates from the years around the end of the nineteenth century, when H. Schulz published a series of papers that examined the activity of various kinds of poisons (iodine, bromine, mercuric chloride, arsenious acid, etc.) on yeast, showing that almost all these agents have a slightly stimulatory effect on the yeast metabolism when given in low doses (Schulz 1877; Schulz 1888). He then came into contact with the psychiatrist R. Arndt and together they developed a principle that later became known as the 'Arndt-Schulz law', stating that weak stimuli slightly accelerate vital activity, stronger stimuli raise it further, but a peak is reached and even stronger stimuli suppress it, until a negative response is finally achieved (Martius 1923). In 1960 Townsend and Luckey surveyed the field of classic medical pharmacology and published a list of 100 substances known to be capable of causing an inhibition at high concentrations and stimulation at low concentrations and termed the phenomenon "hormoligosis" (Townsend and Luckey 1960). The modern term "hormesis" was first used by Stebbing in 1982 (Stebbing 1982) and has been thoroughly reviewed by Calabrese (Calabrese 2001b; Calabrese 2002; Calabrese 2004a; Calabrese 2004b; Calabrese 2005).

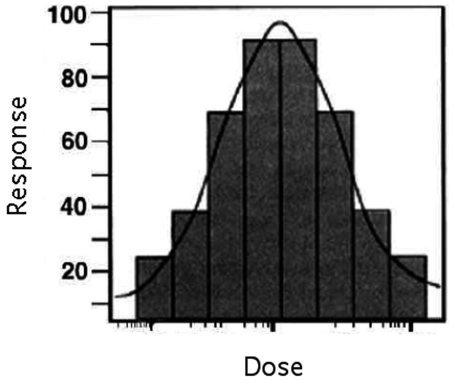

FIGURE 8. Idealized biphasic dose response curve (often termed Arndt-Schulz curve) typically reported in LLLT studies.

In the context of LLLT the increasing "stimulus" may be irradiation time or increased irradiance. This non-linear effect contradicts the Bunsen-Roscoe rule of reciprocity (which was originally formulated for visual detection of light by photoreceptors (Brindley 1952)), which predicts that if the products of exposure time in seconds and irradiance in mW/cm^2 are equal, i.e. the energy density is the same, then the changes in biological endpoint will be equal. This inverse linear relationship between irradiance and time has frequently failed in LLLT research (Karu and Kolyakov 2005; Lubart *et al.* 2006).

A "biphasic" curve can be used to illustrate the expected dose response to light at a subcellular, cellular, tissue or clinical level. Simply put, it suggests that if insufficient energy is applied there will be no response (because the minimum threshold has not been met), if more energy is applied the then a threshold is crossed and biostimulation is achieved but when too much energy is applied then the stimulation disappears and is replaced by bioinhibition instead. An idealized illustration (Figure 8) similar to that suggested by Sommer (Sommer *et al.* 2001) helps understand the concept.

3.2. Biphasic Response—irradiance

As early as 1978 Endre Mester observed a "threshold phenomenon" after laser irradiation of lymphocytes in vitro (Mester *et al.* 1978). Peter Bolton in 1991 irradiating macrophages with two different irradiances (W/cm^2) but the same energy density (J/cm^2) recorded different results (Bolton *et al.* 1991). Karu (Karu and Kolyakov 2005) found a dependence of stimulation of DNA synthesis rate on light intensity at a constant energy density 0.1 J/cm^2 with a clear maximum at 0.8 mW/cm^2. In another study (Karu *et al.* 1997) the same group found no less than seven maxima in the dose vs. biological effect curves using a pulsed 810-nm diode laser.

TABLE 3. Comparison of different irradiances and fluences of 810-nm laser on differentiation of normal human neural progenitor cells. Cells received light once a day for three days and neurite outgrowth was measured.

Anders *et al.* 2007 Average Summed Neurite Length Parameters						
	1 mW/cm^2	5 mW/cm^2	15 mW/cm^2	19 mW/cm^2	25 mW/cm^2	50 mW/cm^2
0.01 J/cm^2	NS	NS	—	NS	—	—
0.05 J/cm^2	NS	NS	NS	**S**	**S**	NS
0.2 J/cm^2	NS	NS	NS	**S**	**S**	**S**
1 J/cm^2	NS	NS	NS	NS	NS	**S**

NS: No statistical difference.
S: Groups significantly greater than Factors group.
(One way ANOVA *p<0.01, **p<0.001)

Four different biological models were used: luminol-amplified chemiluminescence measured in nucleated cells of murine spleen (splenocytes), bone marrow (karyocytes), and murine blood and adhesion of HeLa cells cultivated in vitro. The peaks coincided for all four models. Anders conducted the widest ranging in-vitro study (on normal human neural progenitor cells) with four different energy density groups, each group tested across a range of six different irradiance parameters (Anders *et al.* 2007) Table 3.

In 1979 Ginsbach found that laser stimulation of wound closure had "no reciprocity relation". His controlled experiments on rats with He-Ne laser at an energy density of 4 J/cm^2 found stimulation at an irradiance of 45 mW/cm^2 but not at 12.4 mW/cm^2 (Ginsbach 1979). Uri Oron (Oron *et al.* 2001a) showed different reductions of infarct size after induced heart attacks in rats. Keeping energy density constant and varying the irradiance he found that the beneficial effects were maximumal at 5 mW/cm^2 and significantly less effect both at lower irradiances (2.5 mW/cm^2) and also at higher irradiances (25 mW/cm^2). Ray Lanzafame (Lanzafame *et al.* 2007) conducted a study varying irradiance and interval on laser-induced healing of pressure ulcers in mice. Energy density (5 J/cm^2) was fixed but four different irradiance (0.7 – 40 mW/cm^2) parameters were tested with a significant improvement only occurring for 8 mW/cm^2

We know of only one human clinical trial which varied irradiance but this trial kept treatment time the same so energy density (J/cm^2) did not remain the same. This RCT by Hashimoto on the treatment of the stellate ganglion to reduce pain in patients with post herpetic neuralgia of the facial type. This study compared the effects of 830-nm lasers delivering 60 mW, 150 mW and placebo, each applied for 3 minutes to the anterior aspect of the lateral process of the 7th cervical vertebrae. Each patient

had three treatments (one treatment, three consecutive days), each treatment was with a different laser or placebo. The study was properly blinded and randomized. There was a significant difference in skin temperature of the forehead and in recorded pain scores. The greatest improvements were for the 150mW laser (Hashimoto *et al.* 1997).

There have been several systematic reviews and meta analyses of RCTs and these have revealed some irradiance dependant effects: Bjordal published a review of LLLT for chronic joint disorders and identified 14 RCTs of suitable methodological quality, 4 of which failed to report a significant effect because the irradiance was either too high or too low, and/or delivered insufficient energy, the remaining eight studies all produced positive effects (Bjordal *et al.* 2003). Tumilty reviewed 25 LLLT RCTs of tendinopathies,13 of which (55%) failed to produce a positive outcome, all of these negative/inconclusive studies that recorded irradiance (or could subsequently be established) had delivered an irradiance in excess of the guidelines set by the World Association for Laser Therapy (www.walt.nu) (Tumilty *et al.* 2009).

3.3. Biphasic Response—time or energy density

Again, Peter Bolton's study mentioned in 3.2 above had an energy density aspect showing a different response for each of the irradiances used. For the 400mW/cm^2 study he found increasing energy density from 2.4 J/cm^2 to 7.2 J/cm^2 increased fibroblast proliferation, in the 800 mW/cm^2 group increasing energy density from 2.4 J/cm^2 to 7.2 J/cm^2 decreased fibroblast proliferation (Bolton *et al.* 1991). Anders' study also mentioned in 3.2 above looked at four energy density groups, and for the irradiance parameters that produced significant results increasing energy density increased neurite length (Anders *et al.* 2007) Table 3. Yamaura and colleagues found a biphasic dose response in MTT activity in rheumatoid arthritis synoviocytes after 810-nm laser with a peak at 8 J/cm^2 and less effect at lower and higher fluences (Yamaura *et al.* 2009). Loevschall measured human oral mucosal fibroblast cell proliferation by incorporation of tritiated thymidine after varying fluences of 812-nm laser delivered at 4.5 mW/cm^2 and found a biphasic dose response with a distinct peak at 0.45 J/cm^2 (Loevschall and Arenholt-Bindslev 1994). Another study (al-Watban and Andres 2001) looked at chinese hamster ovary and human fibroblast proliferation after various fluences of He-Ne laser delivered at a constant irradiance of 1.25 mW/cm^2. Again they found a clear biphasic dose response with a peak at 0.18 J/cm^2. Zhang et al (Zhang *et al.* 2003) found a biphasic dose response in human fibroblast cell numbers after treatment with varying fluences of 628-nm light, with a maximum increase of 30% after 0.88 J/cm^2 and an actual reduction appearing at 9 J/cm^2. Brondon and colleagues (Brondon *et al.* 2005) found that two treatments per day

caused a bigger increase than 1 or 4 treatments per day measuring proliferation index in human HEP-2 and murine L-929 cell lines. They used a 670 nm light emitting diode device with an irradiance of 10 mW /cm^2 and each single treatment was 5 J/cm^2 and the course was stopped after 50 J/cm^2 had been given (at 10, 5 or 2.5 days).

Lopes-Martins showed a biphasic response to LLLT on the number of mononuclear cells that accumulate in pleural cavity after carrageenan injection. The results showed neutrophil influx mice treated with three different laser fluencies at 1, 2.5 & 5 J/cm^2) with 2.5 having the greatest effect (Lopes-Martins *et al.* 2005).

As stated in 3.2 above, Hashimoto reported on the laser treatment of the stellate ganglion to reduce pain in patients with post herpetic neuralgia of the facial type. The study compared the effects of 830-nm lasers delivering 60mW, 150mW and placebo, The greatest improvements were for the 150mW laser (Hashimoto *et al.* 1997). Again as stated in 3.2 above, there have been several systematic reviews and meta analyses of RCTs and these revealed some energy density dependant effects (Bjordal *et al.* 2003; Tumilty *et al.* 2009).

3.4. Beam measurement reporting errors

One notable aspect of the dose rate (W/cm^2) studies is the wide variation of "optimal" irradiances in vitro studies as they range from 1-800 mW/cm^2 in just the few papers referenced in this review. If the primary photo acceptor is cytochrome C oxidase as postulated here, then why would so many authors arrive at different conclusions for optimal parameters in vitro, should it not be the same for all of them?

Explanations may include, the slightly different wavelengths used or sensitivity due the redox state of mitochondia in the target cells (Tafur and Mills 2008), but we consider that the greater contributor may be laser beam measurement problems. It may be a surprise to non-physicists that diode laser beams are not inherently round, and even if circularizing lenses are used to correct this, then the beam intensity distribution is not homogeneous. Laser beams are brighter (higher irradiance) in the middle and weaker towards the edge. Cells in the centre of a culture well will be exposed to considerably higher irradiances than those on the periphery. Because the edge of a laser beam is hard to define and find this could mean that irradiance calculations are significantly different between research centers. Agreement on beam measurement and reporting of intensity distribution is needed to reduce these inconsistencies. This is important not only for in vitro studies but also in vivo and clinical trials as reporting of irradiance is just as important though we accept that tissue scattering diffuses the beam probably making non-homogenous sources less critical to clinical effectiveness.

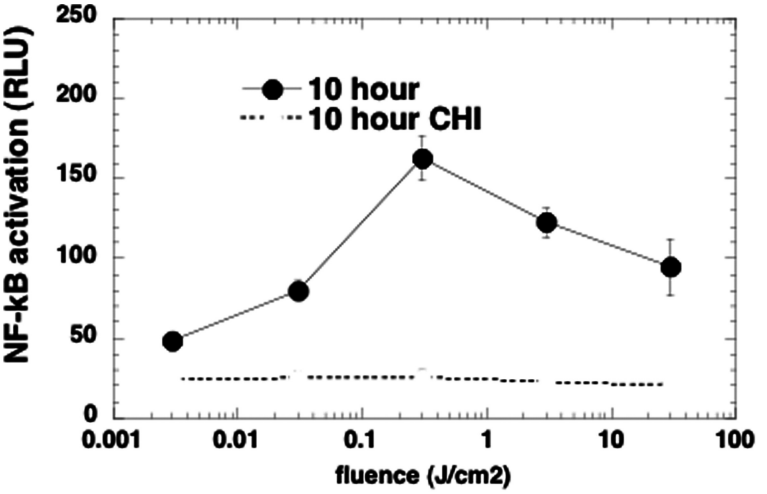

FIGURE 9. Biphasic dose response of NF-κB activation (measured by bioluminescence reporter assay) in mouse embryonic fibroblasts 10 hours after different fluences of 810-nm laser light. CHI is control where all protein synthesis has been inhibited.

4. BIPHASIC LLLT DOSE RESPONSE STUDIES IN OUR LABORATORY

4.1. In vitro activation of NF-κB

We developed the hypothesis (Chen *et al.* 2009a) that NIR light (810-nm laser) would activate the transcription factor NF-κB by generating reactive oxygen species from the mitochondria (see section 2.5). We tested this in mouse embryonic fibroblasts that had been genetically engineered to synthesize luciferase in response to NF-κB activation (Chen *et al.* 2009a). We used a wide range (four orders of magnitude) of delivered fluences by adjusting the laser power so that the illumination time was kept constant at 5 minutes. As shown in Figure 9 there was a biphasic dose dependent activation of NF-κB as measured by luciferase assay 10 hours after the illumination was completed. There was no significant increase at 0.003 J/cm^2 compared to the dark control, a small increase at 0.03 J/cm^2,, the maximum activation was observed at 0.3 J/cm^2, while at 3 J/cm^2 and even more so at 30 J/cm^2 there was a decrease in NF-κB activation, but the level was still higher than that found at 0.03 J/cm^2. The level of luciferase expression was also measured in the presence of cycloheximide (CHI) as a control. CHI is a protein synthesis inhibitor that removes even the background level of luciferase seen in dark control cells, as well as all the increases seen with the different fluences of 810-nm light.

We tested the hypothesis that the activation of NF-κB by LLLT was mediated by generation of ROS because NF-κB is known to be a redox-sensitive transcription factor (Schreck *et al.* 1992) and moreover ROS have previously been shown to be generated during LLLT (Alexandratou

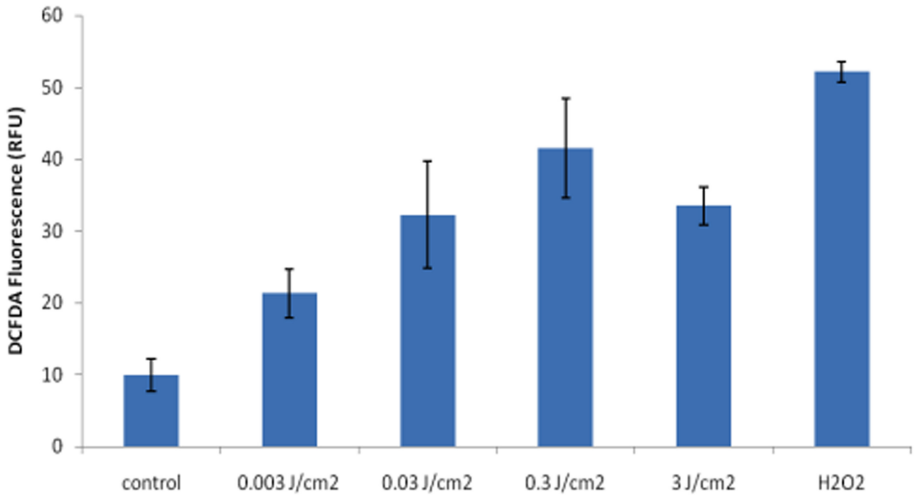

FIGURE 10. Biphasic dose response in generation of ROS as detected by fluorescence probe under same conditions as Fig 9 but measured at 5 minutes post-irradiation.

et al. 2002; Lubart *et al.* 2005; Pal *et al.* 2007). We used dichlorodihydrofluorescein diacetate (DCHF-DA) which is taken up into cells, hydrolyzed and oxidized to a fluorescent form by most species of ROS probably via lipid peroxides (Diaz *et al.* 2003). As can be seen in Figure 10 even the low fluence of 0.003 J/cm^2 produced detectable levels of ROS, greater at 0.03 J/cm^2 and maximum at 0.3 J/cm^2 with a slight decrease observed at 3 J/cm^2. The maximum level observed at 0.3 J/cm^2 was only slightly less than that observed inside the cells after addition of hydrogen peroxide to the extracellular medium.

4.2. Mouse wound healing

In an in vivo study (Demidova-Rice *et al.* 2007) we used a set of fluences of 635-nm (+/−15-nm) light delivered from a filtered lamp. The model was a full thickness dorsal excisional wound in BALB/c mice treated with a single exposure to light 30 minutes after wounding. These fluences were 1, 2, 10 and 50 J/cm^2 delivered at constant fluence rate of 100 mW/cm^2 and taking 10, 20, 100 and 500 seconds respectively. In this model the untreated wound tends to expand for 2-3 days after it was made, but even a brief exposure to light soon after wounding, reduces or stops the expansion of the wound and the integrated time course of the wound size can therefore be significantly reduced. Our hypothesis is that fibroblasts in the edge of the wounded dermis can be transformed into myofibroblasts, and the contractile nature of these cells with their smooth muscle actin fibers prevents the wound expanding. It should be noted that the fibroblast-myofibroblast transition can be mediated by NF-κB activation (Watson *et al.* 2008). As shown in Figure 11 there was a bipha-

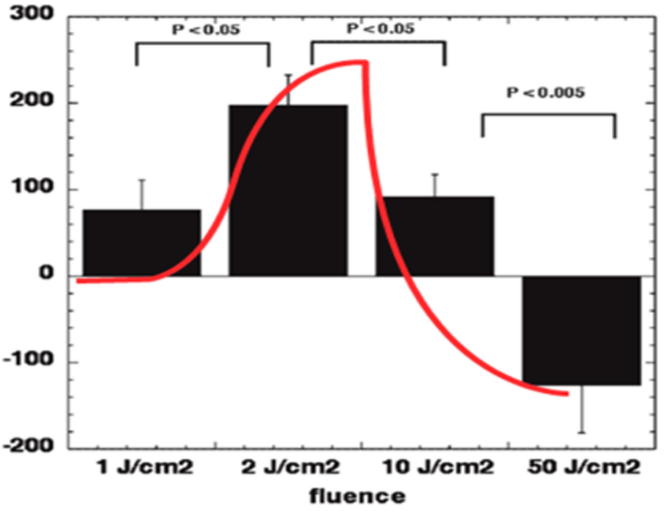

FIGURE 11. Biphasic dose response in measured difference in integrated area under the curve of time course of wound size compared to no treatment control, with a clear maximum seen at 2 J/cm^2, and the high dose of 50 J/cm^2 gave a worsening of the wound healing time curve.

sic dose response with positive effects (difference in integrated area under the curve of time course of wound size compared to no treatment control) seen in low doses with a clear maximum seen at 2 J/cm^2, and the high dose of 50 J/cm^2 actually gave a worsening of the wound healing time curve i.e. there was a greater expansion of the wound compared with non-treated controls.

4.3. Rat arthritis

In another in vivo study (Castano *et al.* 2007) we investigated whether LLLT using an 810-nm laser could have a therapeutic effect in a rat model of inflammatory arthritis caused by zymosan injected into their knee joints. In this model the severity of the arthritis is quantified by measuring the diameter of the swollen joint every day and plotting a time course for each joint. We compared illumination regimens consisting of a high and low fluence (3 and 30 J/cm^2), delivered at high and low irradiance (5 and 50 mW/cm^2) using 810-nm laser light daily for 5 days, with the positive control of conventional corticosteroid (dexamethasone) therapy.

As shown in Figure 12 three of the illumination regimens were effective in reducing the mean integrated knee swelling almost as much as the positive control of the powerful steroid, dexamathasone; these were 3 J/cm^2 delivered at 5 mW/cm^2 and 30 J/cm^2 delivered at 50 mW/cm^2 both of which took 10 minutes, and 30 J/cm^2 delivered at 5 mW/cm^2 which took 100 minutes. The only ineffective dose regimen was 3 J/cm^2

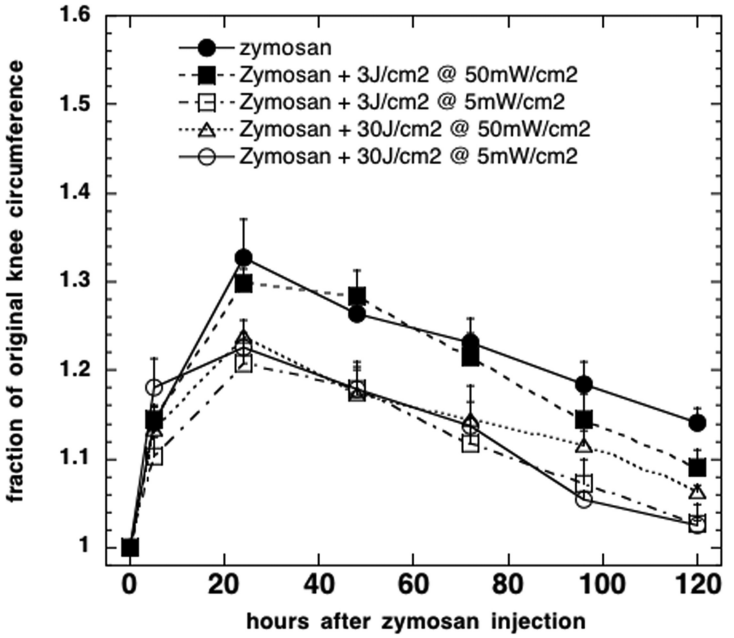

FIGURE 12. Dose response of illumination time found in a study of 810-nm laser to treat zymosan-induced arthritis in rats. Integrated curves of knee circumference versus time were compared. Three LLLT regimens were equally successful where the illumination time was either 100 minutes or 10 minutes, but the ineffective regimen only had a 1 minute illumination time.

delivered at 50 mW/cm^2 which took the comparatively short time of 1 minute to deliver. This observation led us to propose that the illumination time was an important parameter in some LLLT applications.

5. POSSIBLE EXPLANATIONS FOR BIPHASIC DOSE RESPONSE IN LLLT

The repeated observations that have been made of the biphasic dose response phenomenon in LLLT require some explanation. The natural assumption that is frequently made is, that if a small dose of red or near-infrared light produces a significant therapeutic effect, then a larger dose should produce an even more beneficial effect. This natural assumption is frequently not the case. We here propose three possible explanations for the existence of the biphasic dose response based upon mechanistic considerations outlined in section 2.

5.1. Excessive ROS

As discussed in 2.5 the light mediated generation of reactive oxygen species has been observed in many in vitro studies and has been proposed to account for the cellular changes observed after LLLT via activation of redox sensitive transcription factors (Chen *et al.* 2009a). The evidence of

ROS mediated activation of NF-κB in MEF cells presented in 4.1 provides additional support for this hypothesis (Chen *et al.* 2009a). It is well-accepted that ROS can have both beneficial and harmful effects (Huang and Zheng 2006). Hydrogen peroxide is often used to kill cells in vitro (Imlay 2008). Other ROS such as singlet oxygen (Klotz *et al.* 2003) and hydroxyl radicals (Pryor *et al.* 2006) are thought to be harmful even at low concentrations. The concept of biphasic dose response in fact is well established in the field of oxidative stress (Day and Suzuki 2005). If the generation of ROS can be shown to be dose dependent on the delivered energy fluence this may provide an explanation for the stimulation and inhibition observed with low and high light fluences.

5.2. Excessive NO

The other mechanistic hypothesis that is put forward to explain the cellular effects of LLLT relates to the photolysis of nitrosylated proteins that releases free NO (see section 2.6). Again the literature has many papers that discuss the so-called two-faced or "Janus" molecule NO (Anggard 1994; Lane and Gross 1999). NO can be either protective or harmful depending on the dose and particularly on the cell or tissue type where it is generated (Calabrese 2001a).

5.3. Activation of a cytotoxic pathway

The third hypothesis to explain the biphasic dose response of LLLT is the idea that the protective and stimulatory effects of light occur at low doses, but there is an additional pathway that leads to damaging effects of light that only occurs at high doses, and effectively overwhelms the beneficial effects of low doses of light. Work from South China Normal University provides some support for this hypothesis. Low doses of LLLT were found to phosphorylate hepatocyte growth factor receptor (c-Met), and initiate signaling via cyclic AMP and Jun kinase and Src (Gao and Xing 2009). By contrast, high dose LLLT was found to induce apoptosis via a mitochondrial caspase-3 pathway and cytochrome c release was attributed to opening of the mitochondrial permeability transition pore caused by high-level intracellular reactive oxygen species (ROS) generation (Wu *et al.* 2009). A secondary signaling pathway through Bax activation was observed (Wu *et al.* 2009).

6. SUMMARY AND CONCLUSION

LLLT delivered at low doses tends to work better than the same wavelength delivered at high levels, which illustrates the basic concept of biphasic dose response or hormesis (Calabrese 2001b). In general, fluences of red or NIR as low as 3 or 5 J/cm^2 will be beneficial in vivo, but a large dose

like 50 or 100 J/cm^2 will lose the beneficial effect and may even become detrimental. The molecular and cellular mechanisms LLLT suggest that photons are absorbed by the mitochondria; they stimulate more ATP production and low levels of ROS, which then activates transcription factors, such as NF-κB, to induce many gene transcript products responsible for the beneficial effects of LLLT. ROS are well known to stimulate cellular proliferation of low levels, but inhibit proliferation and kill cells at high levels. Nitric oxide is also involved in LLLT, and may be photo-released from its binding sites in the respiratory chain and elsewhere. It is possible that NO release in low amounts by low dose light may be beneficial, while high levels released by high dose LLLT may be damaging. The third possibility is that LLLT may activate transcription factors, upregulating protective proteins which are anti-apoptotic, and generally promote cell survival. In contrast, it is entirely possible that different transcription factors and cell-signaling pathways, that promote apoptosis, could be activated after higher light exposure. We believe that further advances in the mechanistic understanding of LLLT will continue to be made in the near future. These advances will lead to greater acceptance of LLLT in mainstream medicine and may lead to LLLT being used for serious diseases such as stroke, heart attack and degenerative brain diseases. Nevertheless the concept of biphasic dose response or LLLT hormesis (low levels of light are good for you, while high levels are bad for you) will remain.

ACKNOWLEDGMENTS

Research in the Hamblin laboratory is supported by the US NIH (grants R01CA/AI838801 and R01AI050875 to MRH).

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