SCIENTIFIC STUDIES

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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

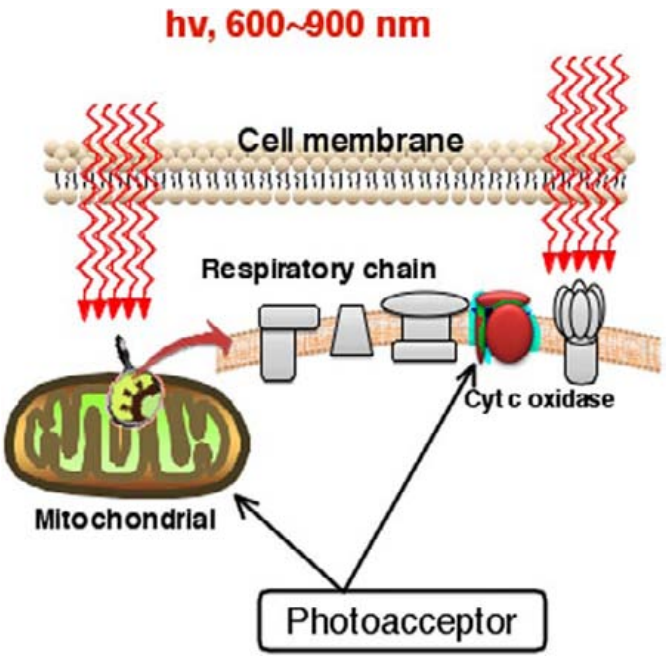

FIGURE 1. Schematic diagram showing the absorption of red and NIR light by specific cellular chromophores or photoacceptors localized in the mitochondrial respiratory chain

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

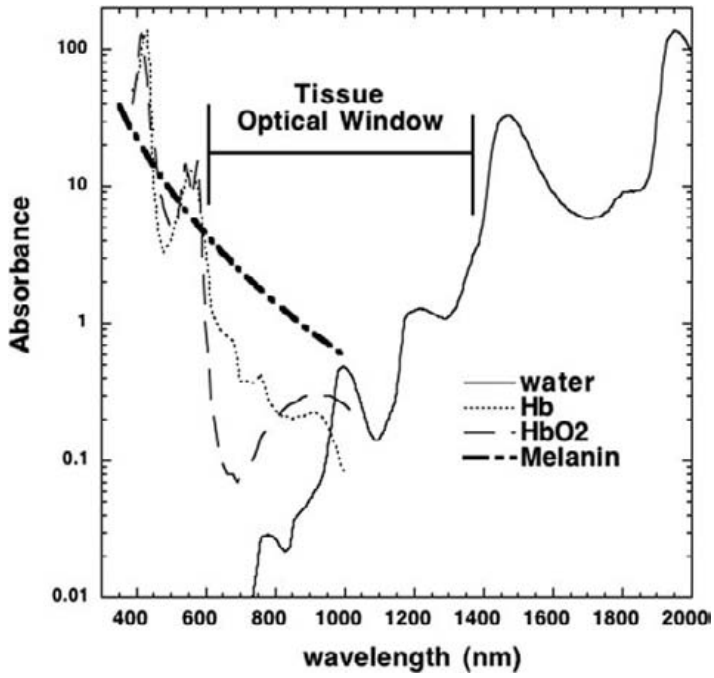

FIGURE 2. Absorption spectra of the main chromophores in living tissue on a log scale showing the optical window where visible and NIR light can penetrate deepest into tissue.

"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

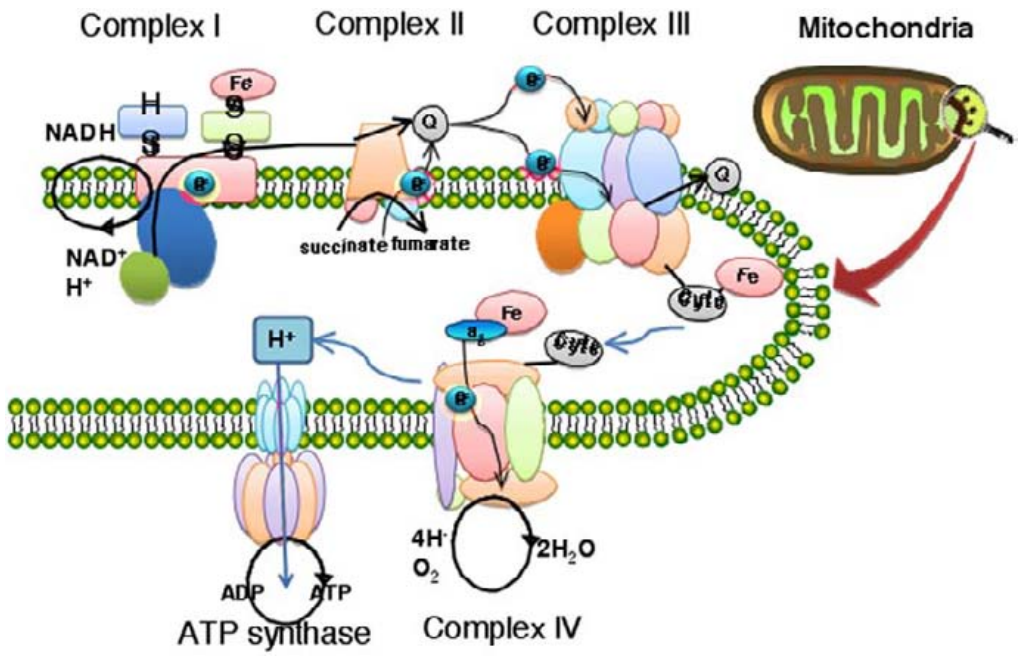

FIGURE 3. Mitochondrial respiratory chain consisting of contains five complexes of integral membrane proteins: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (ComplexV).

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

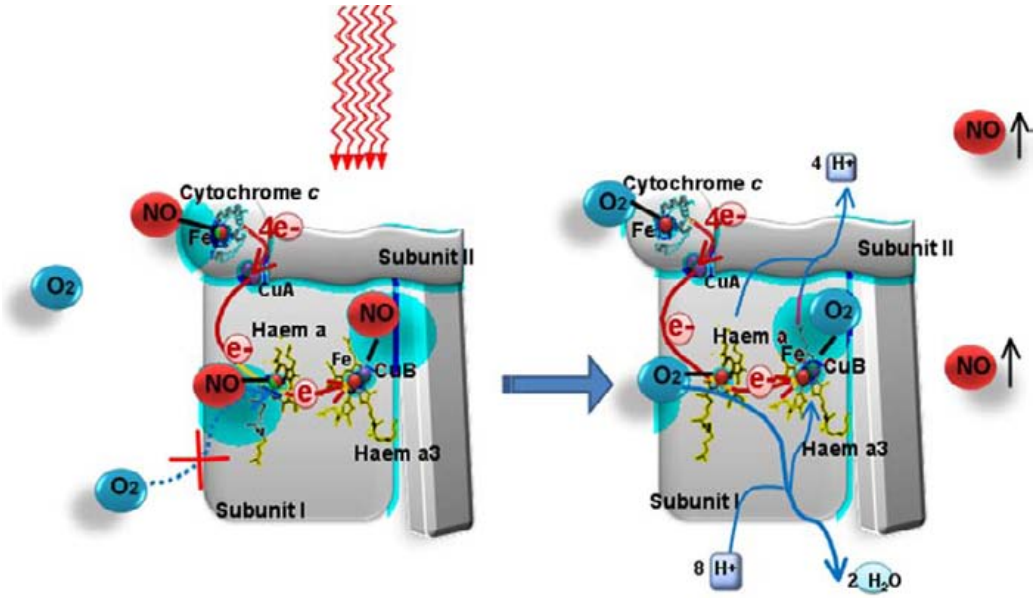

FIGURE 4. When NO is released from its binding to heme iron and copper centers in cytochrome c oxidase by the action of light, oxygen is allowed to rebind to these sites and respiration is restored to its former level leading to increased ATP synthesis.

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

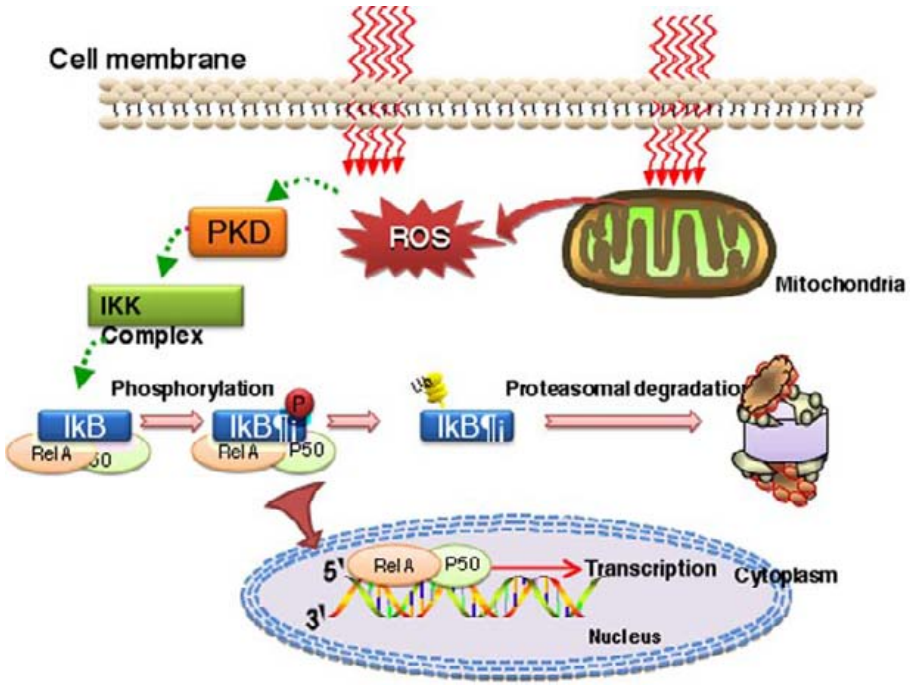

FIGURE 5. Reactive oxygen species (ROS) formed as a result of LLLT effects in mitochondria may activate the redox-sensitive transcription factor NF-κB (relA-p50) via protein kinase D (PKD).

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

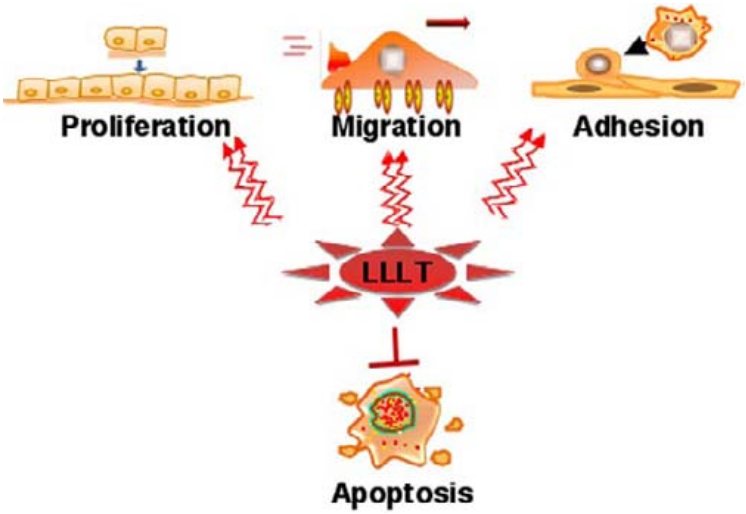

FIGURE 6. The downstream cellular effects of LLLT signaling include increases in cell proliferation, migration and adhesion molecules. Cell survival is increased and cell death reduced by expression of proteins that inhibit apoptosis.

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).

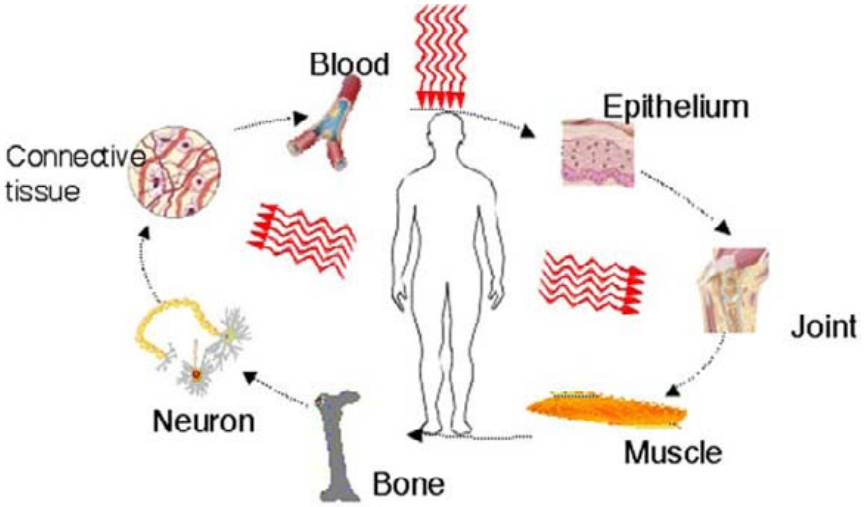

FIGURE 7. Beneficial tissue effects of LLLT can include almost all the tissues and organs of the body.

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).

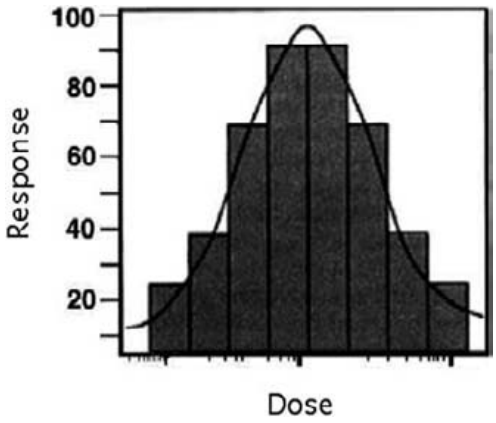

FIGURE 8. Idealized biphasic dose response curve (often termed Arndt-Schulz curve) typically reported in LLLT studies.

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.

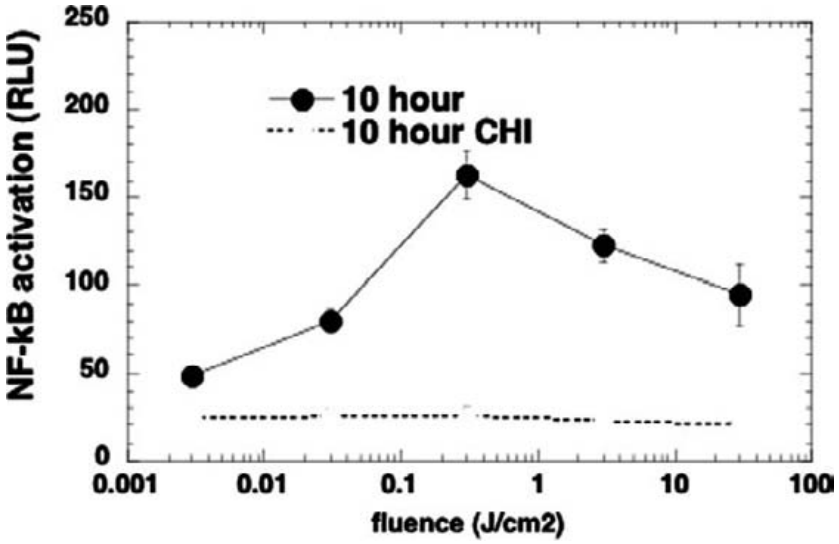

FIGURE 9. Biphasic dose response of NF-κB activation (measured by bioluminescence reporter assay) in mouse embryonic fibroblasts 10 hours after different fluences of 810-nm laser light. CHI is control where all protein synthesis has been inhibited.

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

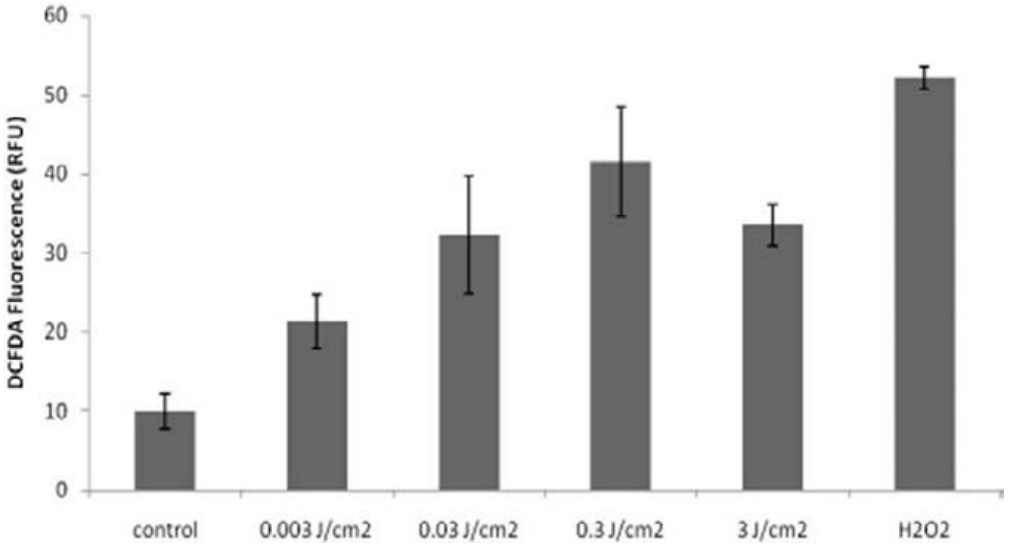

FIGURE 10. Biphasic dose response in generation of ROS as detected by fluorescence probe under same conditions as Fig 9 but measured at 5 minutes post-irradiation.

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-

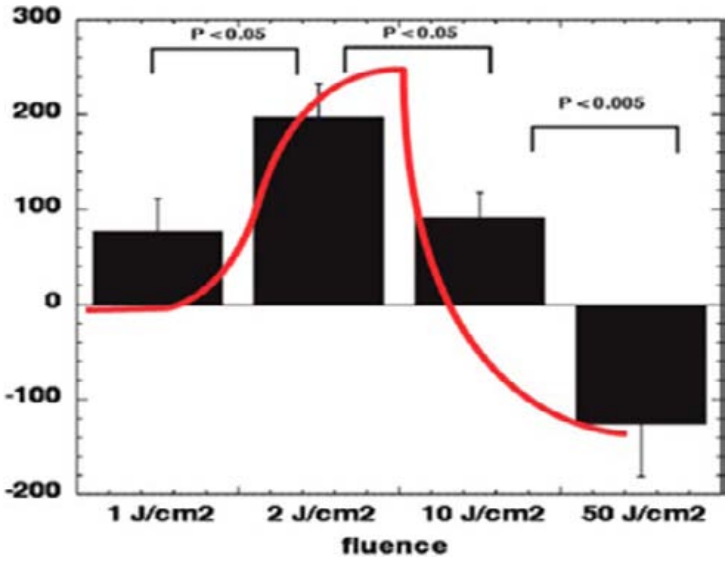

FIGURE 11. Biphasic dose response in measured difference in integrated area under the curve of time course of wound size compared to no treatment control, with a clear maximum seen at 2 J/cm^2, and the high dose of 50 J/cm^2 gave a worsening of the wound healing time curve.

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

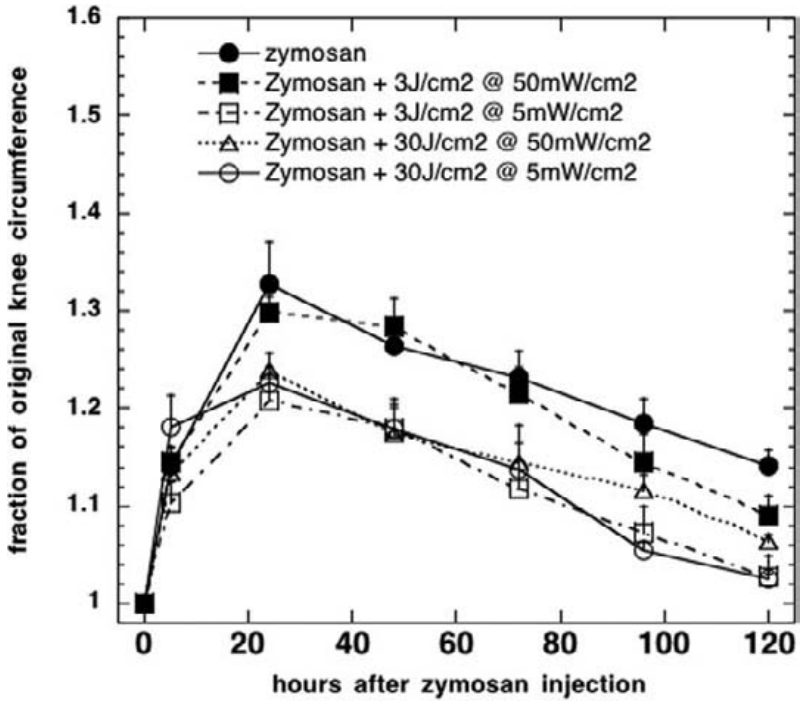

FIGURE 12. Dose response of illumination time found in a study of 810-nm laser to treat zymosan-induced arthritis in rats. Integrated curves of knee circumference versus time were compared. Three LLLT regimens were equally successful where the illumination time was either 100 minutes or 10 minutes, but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

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Low-level laser therapy with pulsed infrared laser accelerates third-degree burn healing process in rats

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Abstract—This study investigated the influence of pulsed low-level laser therapy (LLLT) on the healing of a third-degree burn in a rat model. Two third-degree burns (distal and proximal) were made in the skin of 74 rats. Rats were divided into four groups. In group 1, the distal burn received LLLT with laser switched off; in groups 2 and 3, distal burns were treated with a 3,000 Hz-pulsed infrared diode laser with 2.3 and 11.7 J/cm^2 energy densities, respectively. In group 4, the distal burns were treated topically with 0.2% nitrofurazone. The proximal burn of all groups was considered a control burn. We assessed the response to treatment both microbiologically and macroscopically. The chi-square test showed that the incidence of *Staphylococcus epidermidis*, *Lactobacillus*, and diphtheria decreased significantly in laser-treated groups compared with other groups. Independent sample *t*-test showed that LLLT with 11.7 J/cm^2 energy density significantly increased wound-closure rate at 3 and 4 weeks after burning compared with their relevant control burns ($p = 0.018$ and $p = 0.01$, respectively). Pulsed LLLT with 11.7 J/cm^2/890 nm of a third-degree burn in a rat model significantly increased wound-closure rate compared with control burns.

Key words: basic science, burn, infrared diode laser, in vivo, low-level laser therapy, microbiology, rat, third-degree burn, wound contraction, wound healing.

INTRODUCTION

Burns are among the most devastating of all injuries, with outcomes spanning the spectrum from physical impairments and disabilities to emotional and mental consequences [1–2]. In the United States, approximately 2.4 million burn injuries are reported each year. Nearly 650,000 persons with these injuries are treated by medical professionals through outpatient care and 750,000 through inpatient or hospital care. Of those persons hospitalized, 20,000 have major burns involving at least 25 percent of their total body surface. Between 8,000 and 12,000 of patients with burns die and approximately 1 million will sustain permanent disabilities resulting from burn wounds [3]. Third-degree or *full-thickness* burns involve the entire epidermis and dermis and may appear as white, thick brown, or tan and have a leathery texture [4].

Low-level laser therapy (LLLT) has been used clinically since the first successful cases reported by Professor Mester and colleagues [5–6]. Cameron et al. reported that the frequency of the laser light, as well as the type of tissue being irradiated, determines the depth to which light penetrates [7]. Laser light with a wavelength

Abbreviations: ANOVA = analysis of variance, CFU = colony-forming units, CW = continuous wave, GaAlAs = gallium aluminum arsenide, GaAs = gallium arsenide, LLLT = low-level laser therapy, LSD = least significant difference.

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between 600 and 1,300 nm optimizes the depth of penetration in human tissue at 1 to 4 mm and is therefore most frequently used in the clinical setting. Laser light with a longer wavelength, such as the (infrared) diode produced by the gallium arsenide (GaAs) or gallium aluminum arsenide (GaAlAs) laser, penetrates deeper [8], whereas laser light with a shorter wavelength, such as red light produced by the helium-neon laser, penetrates human skin very superficially [7]. Research findings have shown that 99 percent of low-level laser is absorbed in the superficial 3.6 mm of human skin [7].

Studies on the influence of continuous-wave (CW) diode lasers on burn healing were few and have shown inconsistent results [8–12]. While Cambier et al. [8], Schlager et al. [9–10], and Al-Watban and Delgado [11] reported irradiation of burns with different wavelengths, powers and energy densities produced no beneficial effects on the wound-healing process. Meireles et al. in a recent study indicate that a 660 nm laser effectively improved the healing of third-degree burns in diabetic rats [12]. Cambier et al. inflicted two burns on each rat: one was left untreated and the other was treated with a continuous GaAs diode laser with 0.210 J/cm^2 energy density [8]. Treatment frequency was 5 times a week over 6 weeks. No major stimulating effect was observed based on the size of index. Cambier et al. reported that type of burn or protocol parameters could be responsible for this lack of effect [8]. Schlager et al. investigated the effect of a CW low-power diode laser with a wavelength of 670 nm on the healing of burn wounds in rats [9]. The animals were burned on each flank. One of the burns was treated by laser irradiation, whereas the other burn received no treatment. Laser irradiation was performed daily with a 2 J/cm^2 energy density (dose). Neither macroscopic nor histological examination of the irradiated wound showed accelerated wound healing when compared with the control wound [9].

In another study, Schlager et al. investigated the effects of two different low-power diode laser lights on the healing process of rats [10]. The animals were burned on each flank and allocated to one of three groups. In group A, both wounds remained untreated. In groups B and C, one wound was irradiated with a CW low-power diode laser at a 690 nm wavelength and the other wound a 635 nm wavelength, respectively. Laser irradiation in both groups was performed daily with an energy density of 1.5 J/cm^2 at each treatment. Schlager et al. found that between and within each group, diameter, redness, and edema of the wound were similar throughout the entire observation period [10]. Schlager et al. mentioned that the reason such differences were obtained on the use of low-power laser light in the burn healing is unknown [9–10]. Al-Watban and Delgado initiated a study using a diode laser at varied doses on burn healing to determine optimum energy density and treatment schedule [11]. Burns on both flanks of rats were created and measured daily with a caliper. The right-side burns were irradiated. Slopes from the actual burn areas were obtained and compared with the control group, with the healing rats calculated and expressed in percent. Al-Watban and Delgado reported that with reference to the control group, they observed no significant difference in the healing process [11]. They also reported that in younger rats, they observed accelerated healing with the highest rates in the lower range of doses (1 and 5 J/cm^2), 12.4 and 11.6 percent, respectively. They concluded that their study affirms that the beneficial effect of laser on burn healing in rats is indeed affected by interplay of several factors [11].

Meireles et al. made a third-degree burn in the 55 diabetic rats [12]. They were divided into three groups that were or were not treated with LLLT (wavelength = 660 nm or wavelength = 780 nm, 35 mW; laser beam diameter = 2 mm, 20 J/cm^2). They found that the healing in animals receiving 660 nm laser energy was more apparent at early stages, with positive effects on inflammation, the amount and quality of granulation tissue, fibroblast proliferation, and collagen deposition and organization [12]. The studies on the influence of CW diode lasers on burn healing have apparently shown inconsistent results.

Baxter reported that although a large percentage of the diode low-level laser instruments used in clinical practice are CW output, most instruments now available in the United Kingdom have pulsed output [13]. The application of frequency is growing rapidly. In this regard, results of several cellular studies [14–16], an in vitro model of a fetal mouse limb growth [17], and three clinical trials [18–20] suggest that the frequency parameter is critical to at least some biological and medical effects of this parameter. Thawer and Houghton investigated the effects of a 904 nm GaAs laser on the growth and development of fetal limb tissue [17]. Organ culture dishes that contained ipsilateral forelimbs and hind limbs were exposed to laser irradiation. The limbs were assigned to receive energy densities of 0 (control), 0.23, 1.37, 2.75, 3.66, or 4.58 J/cm^2, with frequencies of 0 (control), 500,

3,000, 6,000, 8,000, and 10,000 Hz, respectively. Thawer and Houghton found that the dermal cell number and collagen fiber thickness increased after lower frequencies of laser (500 and 3,000 Hz) [17]. These laser frequencies also produced a greater amount of dermal collagen [17]. In another study, Karu et al. investigated the effects of 1,300 nm CW diode laser and 950 nm modulated superluminous diode laser, which had frequencies of 2, 26, 700, 1,000, and 5,000 Hz [21]. The effects of both diodes on the rate of *Escherichia coli* WP2 division were examined [21]. The radiation of CW mode of 1,300 nm laser increased the division of *Escherichia coli* in the dose range of 0.9 to 9.0 J/cm^2. The 950 nm-pulsed irradiation inhibited the division rate of bacteria at frequencies of 1,000 and 5,000 Hz. Karu et al. mentioned that their results indicate that one of the critical parameters of laser irradiation when acting on living cells is the pulse duration and/or frequency [21].

Review of the literature has revealed that no studies have been done regarding the effect of pulsed LLLT on burn healing. On the other hand, a number of studies have reported the effects of pulsed diode lasers on skin wound healing [22–24]. Al-Watban and Zhang evaluated the effects of pulsed CW and the role of wound healing in rats by using both pulsed and CW LLLTs [22]. An elliptic wound was made on the back of rats after anesthesia. The study was performed with the use of a pulsed laser at a wavelength of 635 nm. Pulse frequencies of 100, 200, 300, 400, and 500 Hz in CW were used in the study. Every rat in the treatment group was irradiated with a laser at a 0.89 mW/cm^2 power density for 18.7 minutes with a 1.0 J/cm^2 incident dose or energy density. They reported the percentage of relative wound healing was 4.32 in 100 Hz, 3.21 in 200 Hz, 3.83 in 300 Hz, 2.22 in 400 Hz, 1.73 in 500 Hz, and 4.81 in CW. Al-Watban and Zhang concluded that LLLT using pulsed CW laser at the appropriate dosimetry and frequency can accelerate wound healing in rats [22]. The 100 Hz frequency had a better effect than other pulse frequencies used in the study. The effects of CW laser treatment were higher than pulse frequency. The frequency of pulsed CW laser was not found to increase wound healing in rats compared with normal (not pulsed) CW laser [22].

Recently, Demir et al. investigated the effects of electrical stimulation and laser treatment on wound healing in rats [23]. They made a 6 cm linear incision at the dorsal skin of rats. Group 1 was given a constant direct current of 300 μA a day. Group 3 was treated with a GaAs laser device, delivering a 904 nm wavelength, 6 mW average power, 1 J/cm^2 dose, with a maximum frequency of 128 Hz. This dose was delivered continuously for 10 minutes each day for 10 days. Additional specifications of the laser device were an infrared GaAs laser tube, 6 mW mean and 27 mW maximum power, 15° emission angle, continuous and modulated output type, and 1 to 128 Hz frequency. Groups 2 and 4 were considered the control groups and received sham treatment. Demir et al. concluded that electrical current and laser treatment both benefited healing during the inflammation, proliferation, and maturation phases of a wound [23]. More recently, Matic et al. made a rectangular defect of all skin layers at the dorsal part of the rat neck under general anesthesia [24]. They used an 890 nm wavelength of a pulsed semiconductor laser, with a frequency of 1,500 Hz, impulse duration of 300 ns, maximum strength output of 36 mW, and medium strength of 15.4 mW. The exposure lasted for 5 minutes every day for 21 days. The control group was not exposed to any irradiation. Matic et al. found that the average surface area of the wounds in the laser-treated group decreased significantly more than that of the control group [24].

However, the benefits of pulsed diode lasers in the wound healing process are still controversial and many other investigators found no improvement in the wound-healing process [25–26]. Because of these contradictory results, still no consensus of the effects of LLLT in the wound-healing process exists. Recent studies of skin wound-healing and burn-healing processes have used various diode lasers with different wavelengths, laser power, and stimulation doses. Concerning the type of laser and sufficiency of wavelength, no clear recommendation can be made yet. On the other hand, low-level-pulsed diode laser has not been examined in burn-healing treatment yet. The recent investigations contain no burn healing for an 890 nm infrared diode laser with a 3,000 Hz frequency. Therefore, the present study aimed to examine the influence of LLLT using a 3,000 Hz-pulsed infrared diode on the healing of third-degree burns in rats. Infection is a major cause of morbidity and mortality in burns [27–28], so we also examined microbial flora of the burn.

MATERIALS AND METHODS

Animals and Study Design

We used 74 adult male Wistar rats, 4 months old and weighing 250 ± 30 g, in this study. (Values throughout the article are expressed as mean ± standard deviation.)

Rats were divided into groups 1 to 4. They were provided food and water ad libitum. Two third-degree burns were made at the dorsal proximal and distal regions of the thoracic region of each rat (Figure 1). Distal (experimental) burn of group 1 was treated with LLLT with the radiating head without the laser switched on and was considered the placebo group. Distal burns of groups 2 and 3 were treated with two different energy densities of infrared diode laser (experimental), so groups 2 and 3 and group 1 had no differences except LLLT. Distal burn of group 4 was treated three times a week with topical application of 0.2 percent nitrofurazone (Iran Nago Pharmaceutical Co; Tehran, Iran) during the study. Treatment was started in all groups immediately after burns were made. Proximal burn of all rats was considered as their relevant control burn. All burn wounds were examined macroscopically and microbiologically. Six rats of each group were randomly selected for day 7 (group A), six rats of each group were randomly selected for day 15 (group B), and remaining six rats of each group were selected for day 28 (group C). Two groups of microbiological examination had 7 rats. Groups A and B were used for microbiological examination, and group C was used for clinical examination. Table 1 gives the distribution of groups 1 to 4 by examination of study treatment.

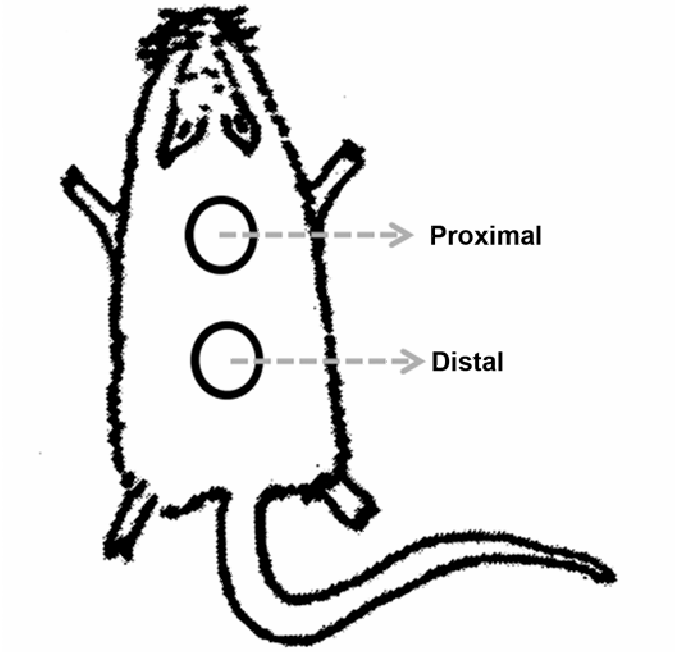

Figure 1.
Diagram of location of burns in rat model.

Burning of Animals

On day 0, all rats were anesthetized by 50 mg/kg ketamine hydrochloride injected intramuscularly along with 5 mg/kg diazepam. The dorsal hair of the rats' thoracic region was shaved and cleaned with povidone-iodine. Each rat was kept in a special box that had a 3 × 3 cm hole. At first, each rat's proximal and then distal part of thoracic region were exposed separately to the external tip of a 5 cm-long cylinder, 22 mm in diameter, and connected to a source (5 L kettle) of boiling water for 7 s (Figure 1). A pilot study was performed at the beginning of the current study and also during our previous study using histological examination that revealed that the epidermis and the whole thickness of the dermis were burned [29]. The burned area of the skin was 3.8 cm^2 [29]. The Medical Ethics Committee of Shahid Beheshti University, MC, approved all procedures.

Low-Level Laser Therapy

Distal burns of groups 2 and 3 were exposed to a pulsed infrared laser (MUSTANG 2000 with L 07 radiating head made by Technica Co; Moscow, Russia):

- Average power output: 70 W.
- Wavelength: 890 nm.
- Pulse frequency: 3,000 Hz.
- Spot size: 1 cm^2.
- Pulse duration: 180 μs.
- Duration of exposure for group 2: 62 s 3×/wk.
- Duration of exposure for group 3: 310 s 3×/wk.
- Energy density for group 2: 2.3 J/cm^2.
- Energy density for group 3: 11.7 J/cm^2.

LLLT was begun immediately after skin was burned. To administer laser irradiation, we divided the burned area and normal surrounding skin into eight equal squares (1 × 1 cm). Next, we held the tip of the laser source about 5 mm above the skin center of each square and directed it perpendicularly to the target tissue for the designated time just mentioned, i.e., 62 s for group 2 and 310 s for group 3 [30]. Note that LLLT was restricted to three times a week; duration of LLLT was calculated for 1 J/cm^2 energy density each day for group 2 and 5 J/cm^2 energy density each day for group 3 of each point (center of square) for 7 days, and then the time was divided by three. So energy density for groups 2 and 3 was 2.3 and 11.7 J/cm^2, respectively.

Microbiological Examination

On days 7 and 15, we took microbiological samples from the burned skin of groups A and B rats. Swabs were taken from burns under anesthesia. We cultured and tested the samples to identify *Staphylococcus epidermidis*, *Staphylococcus aureus, Staphylococcus saprophyticus, Bacillus subtilis*, *Lactobacillus*, diphtheria, and *Pseudomonas aeruginosa* using the routine methods of microbiology originally described by Fingold and Martin [31], Baron and Fingold [32], and Brooks et al. [33]. The number of rats in each microbiological group was six plus one additional rat on days 7 and 15. The data for each bacterium were compared between each group's distal burns and also between each group's proximal and distal burns with use of the χ^2 test. Also between study groups, we further compared bacteria assumed to be nonpathogenic (class 1: *Staphylococcus epidermidis, Staphylococcus saprophyticus, Bacillus subtilis, Lactobacillus*, and diphtheria) and organisms assumed to be pathogenic (class 2: *Staphylococcus aureus* and *Pseudomonas aeruginosa*). We statistically compared the data of classes 1 and 2 using the χ^2 test. Colony-forming units (CFU) of each sample were counted semiquantitatively. We compared the data of distal burn and proximal burn of rats and the data of distal burn of groups using independent sample Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Clinical Examination of Burn Size

The burn area of group C rats was photographed with a digital camera (5-megapixel Canon PowerShot G6; Ohta-ku, Tokyo, Japan), and the surface was measured with Adobe Photoshop CS3 (version 10; San Jose, California) extended image. Each rat was photographed five times on days 0, 7, 14, 21, and 28. To measure the burn area, we placed the photographed images on a grid, equally dividing each into four regions (Nos. 1, 2, 3, and 4). The holes of all regions completely occupied by the burn were counted. The holes of number 1 and holes of number 3 regions partially occupied by the burn were counted, too. The holes of numbers 2 and 4 regions partially occupied by the burn were not counted.

We calculated the percentage wound size using

$$S_n / S_0 \times 100(\%) , \quad (1)$$

where S_0 is the surface area of the wound on day 0 and S_n is the surface area of the wound on the indicated day [34].

We compared the surface area of the two burns in each rat of all groups using an independent sample Student *t*-test. The surface area of placebo, laser-treated burn, and nitrofurazone-treated burn study groups was analyzed with analysis of variance (ANOVA) in each week and between each group. Statistical significance was set at $p < 0.05$.

RESULTS

Microbiological Examination

Statistical analysis of the incidence of microbial flora is shown in Table 2. Significant differences were found between study groups: The incidence of *Staphylococcus epidermidis* and also *Lactobacillus* decreased significantly in group 3 compared with group 1 on day 7 (both $p = 0.046$). The incidence of diphtheria increased significantly in group 2 compared with group 4 on day 15 ($p = 0.018$).

Table 1.
Distribution of rats in study periods and groups.

Day	Group 1: Placebo	Group 2: 2.3 J/cm^2 LLLT	Group 3: 11.7 J/cm^2 LLLT	Group 4: Nitrofurazone
7	Microbiological examination	Microbiological examination	Microbiological examination	Microbiological examination
15	Microbiological examination	Microbiological examination	Microbiological examination	Microbiological examination
28	Clinical examination	Clinical examination	Clinical examination	Clinical examination

LLLT = low-level laser therapy.

Table 2.
Number of rats from which bacteria were cultured by study group.

Day	Bacteria	Group 1: Placebo (n = 6)	Group 2: 2.3 J/cm^2 LLLT (n = 6)	Group 3: 11.7 J/cm^2 LLLT (n = 6)	Group 4: Nitrofurazone (n = 7)
7	*S. epidermidis*	6	5	5	7
	Lactobacillus	2	2	2	2
	Bacillus subtilis	0	0	0	0
	S. saprophyticus	0	0	0	0
	Diphtheria	2	3	3	0
	S. aureus	1	0	1	1
	P. aeruginosa	0	1	0	0
15	*S. epidermidis*	6	5	3	7
	Lactobacillus	3	2	0	3
	Bacillus subtilis	0	0	0	0
	S. saprophyticus	0	0	0	0
	Diphtheria	0	0	0	0
	S. aureus	0	2	0	1
	P. aeruginosa	0	0	0	0

LLLT = low-level laser therapy, *P.* = *Pseudomonas*, *S.* = *Staphylococcus*.

The χ^2 test of *Staphylococcus epidermidis* and also *Lactobacillus* differed significantly between groups 1 and 3 (both $p = 0.046$). Also, a significant difference of diphtheria was found between groups 2 and 4 ($p = 0.018$).

Colony-Forming Units Count

Day 15

Statistical analysis of the incidence of CFU count of flora is shown in Table 3. Student *t*-test showed that CFU count of *Staphylococcus epidermidis* of nitrofurazone-treated burns was significantly lower than that of control burns ($p = 0.025$). Student *t*-test also showed CFU count of *Staphylococcus epidermidis* differed significantly between group 4 and its control burn ($p = 0.025$).

Day 7

Student *t*-test showed that CFU count of *Lactobacillus* in group 3 was significantly lower than that of group 1 ($p = 0.041$). *Staphylococcus epidermidis* in group 4 was significantly lower than that of group 1 ($p = 0.017$).

Independent sample Student *t*-test of CFU count of *Lactobacillus* differed significantly between groups 3 and 1 ($p = 0.025$).

Clinical Examination

Between Groups

Statistical analysis of the wound-closure examination for weeks 1 to 4 is shown in Figures 2 to 5 and Table 4. In week 1, no significant differences were found between groups. In week 2, independent sample Student *t*-test indicated that the wound-closure rate of experimental (laser-treated) burns was significantly higher than that of the relevant control burn in group 2 ($p = 0.028$). In weeks 3 and 4 after burning, the experimental wound-closure rate compared with its relevant control burn rate increased significantly in group 3 ($p = 0.018$ and $p = 0.01$, respectively). In week 4 alone, the rate also increased significantly in group 4 ($p = 0.005$) compared with that of its relevant control burn.

Comparing experimental burns in group 4 with placebo burns of group 1, we found that the ANOVA test increased significantly in wound-closure rate of 3 weeks after burning (least significant difference [LSD] test, $p = 0.013$). In addition, in groups 3 and 4, the statistical analysis showed a significant increase in wound-closure rate of experimental burns 4 weeks after burning compared with that of group 1 (ANOVA test: $p = 0.005$; LSD tests: $p = 0.028$ and $p = 0.007$, respectively). Significant increase of wound-closure rate was also found in experimental burn of groups 3 and 4 compared with that of group 2 (ANOVA test: $p = 0.005$; LSD tests: $p = 0.028$ and $p = 0.007$, respectively).

Within Groups

ANOVA test differed significantly within each group between sequential intervals in most cases (ANOVA test:

Table 3.
Mean ± standard deviation of colony-forming units of study groups at days 7 and 15.

Day	Bacteria	Group 1 (n = 6)		Group 2 (n = 6)		Group 3 (n = 6)		Group 4 (n = 7)	
		Placebo	Control	2.3 J/cm^2 LLLT	Control	11.7 J/cm^2 LLLT	Control	Nitrofurazone	Control
7	*S. epidermidis*	383.3 ± 312.5	333.3 ± 150.5	233.3 ± 296.0	583.3 ± 780.0	66.6 ± 81.6	366.0 ± 492.0	250.0 ± 4.0	121.4 ± 107.0
	Lactobacillus	16.7 ± 40.8	100.0 ± 89.4	25.0 ± 41.0	33.3 ± 51.6	60.0 ± 0.0	25.0 ± 41.8	28.6 ± 39.3	35.7 ± 47.6
	Bacillus subtilis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 20.4	40.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	S. saprophyticus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Diphtheria	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14.3 ± 37.8	14.2 ± 37.8
	S. aureus	0.0 ± 0.0	0.0 ± 0.0	175.0 ± 304.0	343.0 ± 812.0	16.7 ± 40.8	16.7 ± 40.8	14.3 ± 37.8	14.3 ± 3.8
	P. aeruginosa	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15	*S. epidermidis*	1,038.3 ± 491.6	1,083.0 ± 491.6	660.0 ± 466.6	1,040.0 ± 638.0	400.0 ± 344.4	783.3 ± 676.0	642.0 ± 350.0	1,142.0 ± 378.6
	Lactobacillus	75.0 ± 75.8	25.0 ± 41.8	30.0 ± 44.7	20.0 ± 44.7	50.0 ± 83.6	366.6 ± 804.0	428.6 ± 48.8	57.1 ± 97.6
	Bacillus subtilis	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ± 22.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	S. saprophyticus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Diphtheria	133.3 ± 196.6	100.0 ± 200.0	110.0 ± 134.3	310.0 ± 439.3	333.3 ± 51.6	66.7 ± 103.0	0.0 ± 0.0	14.3 ± 37.8
	S. aureus	166.7 ± 408.0	166.7 ± 408.0	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 20.4	8.3 ± 20.4	14.3 ± 37.8	28.6 ± 75.6
	P. aeruginosa	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 223.6	20.0 ± 44.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

LLLT = low-level laser therapy, *P.* = *Pseudomonas*, *S.* = *Staphylococcus*.

p = 0.001). However, no significant differences were found in—

- Group 1: 2 and 3 weeks after burning.
- Group 2: 1 and 2 weeks after burning.
- Group 3: 0 and 1 day and 2 weeks after burning.
- Group 4: 0 days and 1 week after burning.

DISCUSSION

Despite the failure of some studies [8–11] to show beneficial effect of CW low-level diode lasers on burn healing in healthy animals, the present study for the first time demonstrated that pulsed LLLT can significantly accelerate the wound-closure rate of a third-degree burn model in healthy rats.

The biostimulatory effect of pulsed LLLT in the current study is demonstrated by the significant increase of the wound-closure rate of laser-treated burns compared with the placebo group 1, 3, and 4 weeks after burning, while nitrofurazone-treated burns significantly increased the wound-closure rate compared with placebo burns only 4 weeks after burning. Apparently, LLLT was more effective than nitrofurazone ointment in healing a third-degree burn model. LLLT, when used appropriately, can stimulate the healing of injured tissue such as those of dermis [35]. Investigations into the mechanisms involved have shown that many of the cell types whose interactions repair the dermis can be therapeutically stimulated by treatment with LLLT both in vitro and in vivo. Mast cells and macrophages can be stimulated to release growth factors and other substances, whereas the proliferation of fibroblasts, endothelial cells, and keratinocytes maintained during adverse conditions can also be stimulated. The development of granulation tissue is mainly controlled by growth factors released from macrophages [35].

In the present investigation, we found that the effects of 2.3 J/cm^2 LLLT of third-degree burns are more evident only at the early stage of the burn-healing process; however, we cannot find a significant effect of 2.3 J/cm^2 LLLT at the late stage of burn healing compared with its control burns. One proposed mechanism by which LLLT stimulates the wound-healing process is light energy absorbed by mitochondria, which increases cell energy and stimulates the release of chemical mediators [36–38]. Apparently, such a mechanism did not occur in the 2.3 J/cm^2 laser-treated burns of the present study, as well as of the Cambier et al., Schlager et al., and Al-Watban and Delgado studies [8–11]. This finding may be due to insufficient light energy reaching the cells. Allendorf et al. have suggested that laser light penetrations of tissue and eschar debridement are involved in wound healing [39]. Wounds that are not debrided, such as wounds in the current study, may not allow the maximum amount of light

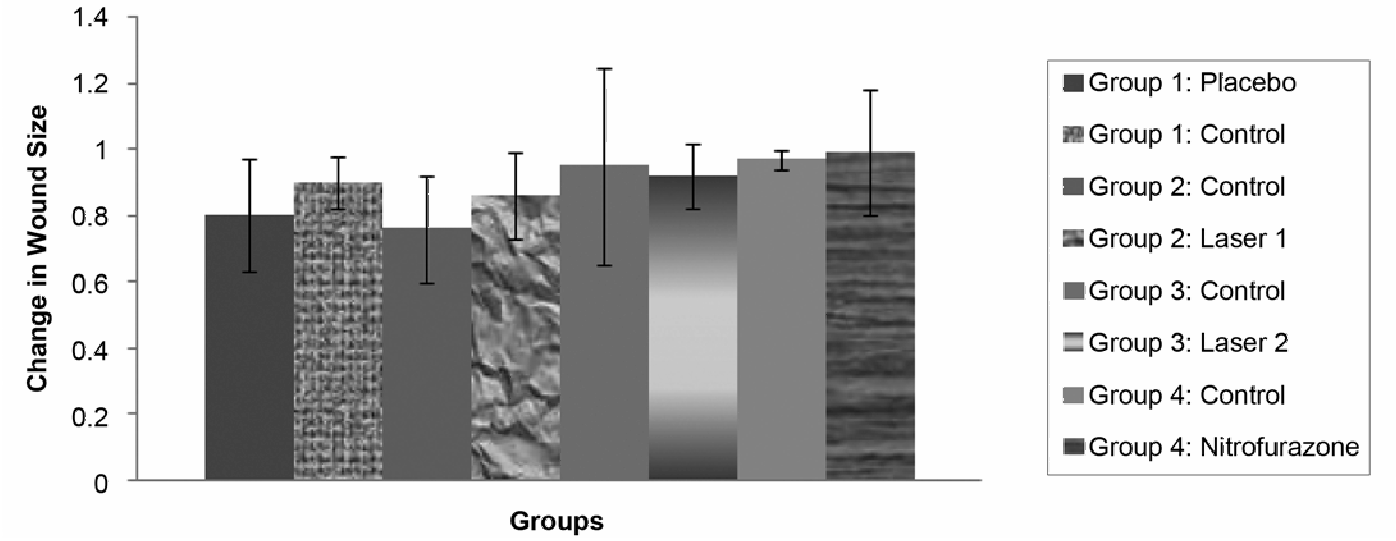

Figure 2.
Wound-closure rate represented as percentage of wound size after burn induction at week 1 between groups. No significant differences were found between groups.

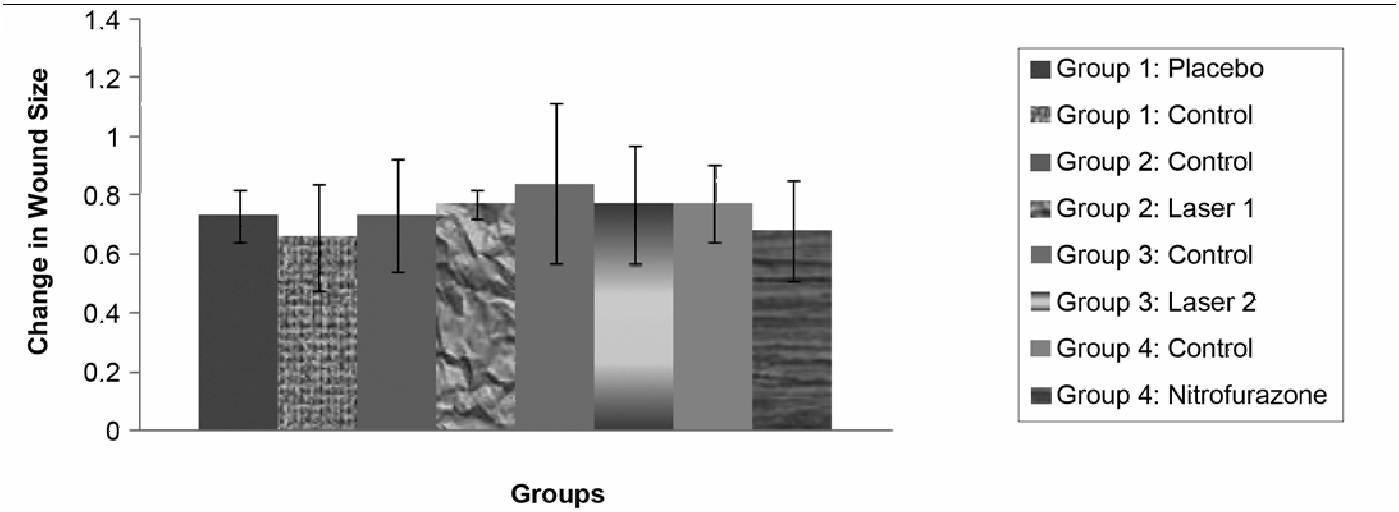

Figure 3.
Wound-closure rate represented as percentage of wound size after burn induction at week 2 between groups: independent sample Student *t*-test showed significant differences between control and experimental (laser-treated) burns of group 2 ($p = 0.028$).

to reach the tissue. Our results suggest that pulsed LLLT at a 11.7 J/cm^2 dose significantly increases the wound-closure rate. Our results also confirm Matic et al.'s findings that pulsed LLLT significantly accelerates the wound-closure rate of a surgically induced cutaneous wound [24]. Other studies failed to show positive effect of pulsed LLLT on the impaired wound-healing process [25–26], whereas the results of the present study and of Matic et al.'s study confirmed positive effect of pulsed LLLT on burn and acute skin wound Using a GaAlAs 890 nm multidiode (n = 60) array unit (270 Hz; maximum rated output 300 mW), Lowe et al. examined wound healing in mice that had been exposed to X-ray irradiation [25]. They found that although wounds treated

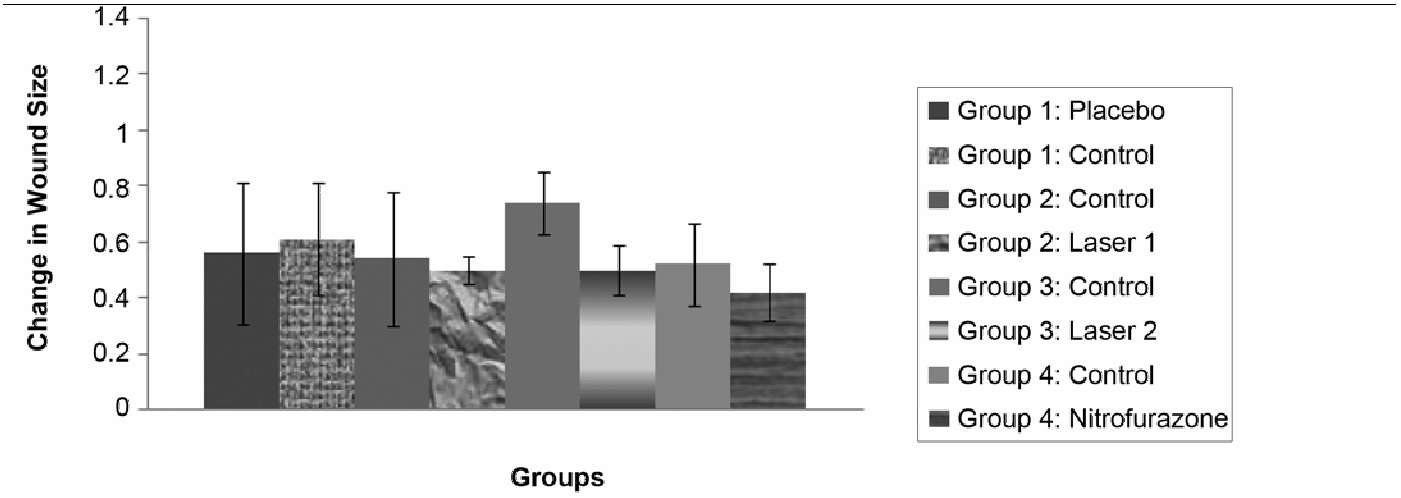

Figure 4.
Wound-closure rate represented as percentage of wound size after burn induction at week 3 between groups. Significant differences were found between control and experimental (laser-treated) burns of group 3 at week 3 after burning ($p = 0.018$, $p = 0.01$, respectively).

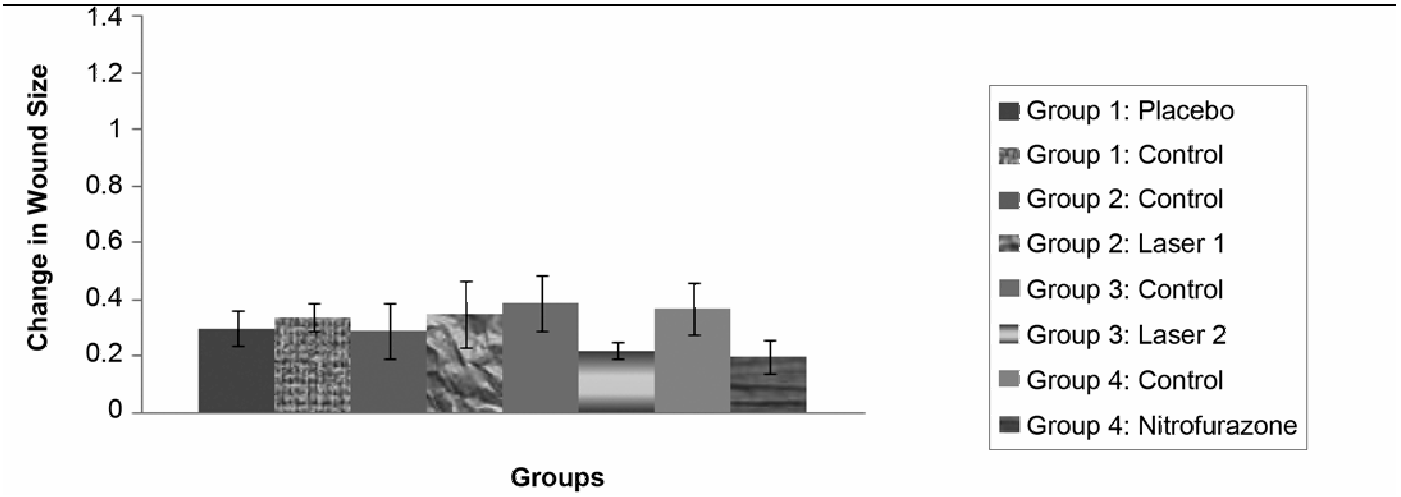

Figure 5.
Wound-closure rate represented as percentage of wound size after burn induction at week 4 between groups. Significant differences were found between control and experimental (nitrofurazone-treated) burns of group 4 after burning ($p = 0.003$). Analysis of variance (ANOVA) test showed significant differences between experimental burns of groups 3 and 4 and that of group 1 after burning (ANOVA test: $p = 0.005$, least significant difference (LSD) test: $p = 0.028$ and $p = 0.007$, respectively). Significant differences were also found between groups 3 and 4 and group 2 (ANOVA test: $p = 0.005$, LSD test: $p = 0.028$ and $p = 0.007$, respectively).

with X-ray irradiation showed delayed wound-healing treatment with 890 nm, light therapy did not significantly affect wound closure at doses of 0.18 and 0.54 J/cm^2 and only further delayed wound healing at a dose of 1.54 J/cm^2 [26]. Using a similar animal model of radiation-impaired wound healing in mice, Walker and colleagues found no hastening in wound healing with 660 nm GaAlAs laser (5 kHz; 15 mW; 0.5, 1.5, and 4.0 J/cm^2 for three groups) [26].

The statistically significant difference found in wound-closure rate of burns between laser-treated (distal) and control (proximal) burns in group 3 of the current study clearly rejects the probable systemic effect of LLLT. Rochkind et al. reported that irradiation of low-power

Table 4.
Mean ± standard deviation of wound-closure rate represented as percentage of wound size after burn induction at weeks 1, 2, 3, and 4 within each group. ANOVA test ($p = 0.001$) also showed significant differences within each group between sequential intervals in most cases.*

Weeks After Burning	Group 1 ($n = 6$)		Group 2 ($n = 7$)		Group 3 ($n = 6$)		Group 4 ($n = 6$)	
	Placebo	Control	2.3 J/cm^2 LLLT	Control	11.7 J/cm^2 LLLT	Control	Nitrofurazone	Control
1	0.90 ± 0.08	0.8 ± 0.17	0.86 ± 0.13	0.76 ± 0.16	0.92 ± 0.10	0.95 ± 0.30	0.99 ± 0.19	0.97 ± 0.03
2	0.66 ± 0.18	0.73 ± 0.09	0.77 ± 0.05	0.73 ± 0.19	0.77 ± 0.21	0.84 ± 0.27	0.68 ± 0.17	0.77 ± 0.13
3	0.61 ± 0.20	0.56 ± 0.25	0.50 ± 0.05	0.54 ± 0.24	0.50 ± 0.09	0.74 ± 0.11	0.42 ± 0.10	0.52 ± 0.15
4	0.34 ± 0.05	0.30 ± 0.06	0.35 ± 0.12	0.29 ± 0.10	0.22 ± 0.03	0.39 ± 0.10	0.2 ± 0.06	0.37 ± 0.09

*No significant differences were found in group 1, between 2 and 3 weeks after burning; group 2, between 1 and 2 weeks after burning; group 3, between day 0 and 1 week and 2 weeks after burning; and group 4, day 0 and 1 week after burning.
ANOVA = analysis of variance, LLLT = low-level laser therapy.

laser on a crushed injured sciatic nerve in a right leg of a bilaterally inflicted crush injury significantly increased the compound action potential in the left nonirradiated leg as well [40].

Microbiological examination showed that the control burns had few pathogen microorganisms; however, pulsed LLLT significantly decreased incidences of *Staphylococcus epidermidis* and *Lactobacillus* compared with group 1 (control burns), incidence of diphtheria compared with nitrofurazone-treated burns, and CFU of *Lactobacillus* compared with placebo burns. The current results provide little evidence of inhibitory effect of pulsed LLLT on microbial flora of a third-degree burn model.

Examining the burns using a histological method may help detect differences between study groups at the cellular level; therefore, further histological studies are suggested.

CONCLUSIONS

We conclude that irradiation of a third-degree burn model with an 11.7 J/cm^2/890 nm-pulsed low-level laser in rats significantly increased wound-closure rate compared with control burns. In addition, the inhibitory effect of the LLLT on microbial flora of the burn was minimal.

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Drafting of manuscript: M. Bayat.
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Bjordal:

Systemic Review – Chronic Joint Disorders

A systematic review of low level laser therapy with location-specific doses for pain from chronic joint disorders

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We investigated if low level laser therapy (LLLT) of the joint capsule can reduce pain in chronic joint disorders. A literature search identified 88 randomised controlled trials, of which 20 trials included patients with chronic joint disorders. Six trials were excluded for not irradiating the joint capsule. Three trials used doses lower than a dose range nominated a priori for reducing inflammation in the joint capsule. These trials found no significant difference between active and placebo treatments. The remaining 11 trials including 565 patients were of acceptable methodological quality with an average PEDro score of 6.9 (range 5-9). In these trials, LLLT within the suggested dose range was administered to the knee, temporomandibular or zygapophyseal joints. The results showed a mean weighted difference in change of pain on VAS of 29.8 mm (95% CI, 18.9 to 40.7) in favour of the active LLLT groups. Global health status improved for more patients in the active LLLT groups (relative risk of 0.52; 95% CI 0.36 to 0.76). Low level laser therapy with the suggested dose range significantly reduces pain and improves health status in chronic joint disorders, but the heterogeneity in patient samples, treatment procedures and trial design calls for cautious interpretation of the results. **[Bjordal JM, Couppé C, Chow RT, Tunér J and Ljunggren AE (2003): A systematic review of low level laser therapy with location-specific doses for pain from joint disorders. *Australian Journal of Physiotherapy* 49: 107-116]**

Key words: Inflammation; Joint Diseases; Lasers; Meta-Analysis

Introduction

Chronic joint disorders represent some of the most prevalent pain conditions treated in primary care (Carmona et al 2001, Mantyselka et al 2001). They constitute several entities, with the common factor that pain is located at the articular structures.

Osteoarthritis is probably the most common entity and the prevalence of osteoarthritis is rising parallel to the increasing age of the population (Felson et al 2000). Temporomandibular joint disorders, patellofemoral pain syndrome and mechanical spine disorders are other examples of chronic joint disorders. These conditions can be associated with impaired muscular stabilisation (Cowan et al 2001, Radebold et al 2001), reduced range of motion (McNamara et al 1996, Steultjens et al 2000) and inflammation of the joint capsule (Speldewinde et al 2001, Suenaga et al 2001, Vaatainen et al 1998).

A link has been established between synovial inflammatory activity and worsening of cartilage degeneration in osteoarthritis (Chikanza and Fernandes 2000). In this context, it is interesting to investigate if an anti-inflammatory action can be induced clinically by electrophysical agents.

Controlled laboratory trials have found that LLLT can reduce inflammation through reduction of PGE_2-levels and inhibition of cyclooxygenase-2 (COX-2) in cell cultures (Campaña et al 1993, Honmura et al 1993, Sakurai et al 2000, Shimizu et al 1995). The transformation of encouraging laboratory results into clinical effectiveness has been a difficult task (Basford 1995), and clinical effectiveness of LLLT has been questioned in systematic reviews on a broad range of conditions (de Bie et al 1998, Del Mar et al 2001, Gam et al 1993). A recent Cochrane systematic review on LLLT found a minor positive effect on rheumatoid arthritis, but the material on osteoarthritis was conflicting (Brosseau et al 2000). In the following review, our hypothesis is that laser irradiation of the joint capsule can reduce pain in chronic joint disorders if the dose is adjusted to inhibit inflammatory activity in the joint capsule.

Materials and methods A detailed review protocol was specified prior to conducting the review. This included a sequential four-step reviewing procedure involving predetermination of an optimal dose range, conduct of a sensitive literature search, application of a pre-specified inclusion/exclusion procedure, and testing of differences between trials with and without optimal dose.

The optimal dose range was derived from successful laboratory trials prior to the literature search. In the first step of the reviewing procedure, an optimal dose range was

Table 1. Suggested range of power densities and dose for the most common joints for infrared GaAlAs and Nd:YAG (continuous) lasers with wavelength 820, 830 and 1060 nm; infrared GaAs (pulse) lasers with wavelength 904 nm; and red HeNe (continuous) lasers with wavelength 632 nm.

Location	IR 820, 830, 1060 nm Power density (mW/cm^2)	IR 820, 830, 1060 nm Dose (Joules)	IR 904 nm Power density (mW/cm^2)	IR 904 nm Dose (Joules)	HeNe 632 nm Power density (mW/cm^2)	HeNe 632 nm Dose (Joules)
Finger/toe/ temporomandibular 1 point/1 cm^2 Depth 2 mm	15 - 105	0.5 - 15	6 - 42	0.2 - 1.4	30 - 210	6 - 30
Knee 3 points/3 cm^2 Depth 4 mm	30 - 210	6 - 180	12 - 60	1.2 - 84	90 - 500	9 - 2700
Cervical spine 3 points/3 cm^2 Depth 12 mm	50 - 350	11 - 360	24 - 60	0.8 - 56	150 - 500	5 - 150
Lumbar spine 3 points/3 cm^2 Depth 30 mm	180 - 500	48 - 480	30 - 210	15 - 105	Not applicable as optimal power density is above safety regulations for laser	Not applicable

determined at the target location and then adjusted according to energy loss estimates for each anatomical location and the size of affected peripheral, facial and spinal joints.

Determination of possible anti-inflammatory LLLT dose at target location In *in vitro* trials, LLLT has been reported to suppress inflammation by a reduction of PGE_2 in ligament cell cultures (Sakurai et al 2000, Sattayut et al 1999, Shimizu et al 1995). Low level laser therapy has also been found to reduce PGE_2 levels in the joint capsule of animals in *in vivo* trials (Campaña et al 1993 and 1999, Honmura et al 1993, Sakurai et al 2000). This effect was reported within a range between 0.4 and 19 J and a power density of 5-21.2 mW/cm^2. The lower range limits for PGE_2 reduction were identified because data showed no effect below this threshold. Upper range limits could not be identified, as there were no data available to show when or if this effect would level off. However, it has been shown that power densities above 20 mW/cm^2 temporarily inhibit fibroblast metabolism (van Breugel and Bar 1992), and numerous fibroblast cells are found in the joint capsule. We assumed doses of 0.4-19 J and power density of 5-21 mW/cm^2 would be capable of reducing inflammation at the target joint capsule without compromising fibroblast metabolism.

Location-specific dose adjustment for energy loss and anatomical size Data on beam diameter and laser output were collected from the manufacturers' manuals. Power density and dose were calculated according to the following formulas:

Power density for GaAs 904 nm pulse lasers (mW/cm^2) = (peak power pulse x pulse duration x pulses frequency) /spot size on skin.

Power density for lasers with continuous output (mW/cm^2) = mean power/spot size on skin.

Dose (J) = mean power x treatment time per session.

Measurement of light penetration and absorption in biological tissue is dependent on several variables. Two anatomical factors are essential to LLLT dose calculations: distance from skin to synovia and size (area) of the affected synovia. For knee (anteromedial and anterolateral part), finger, toe and temporomandibular joint, the distance from skin ranges from 1.5 to 5 mm (authors' unpublished data; 10 persons scanned by 7.5 MHz ultrasound imaging). The distance from skin surface to the zygapophyseal joints was 8 to 20 mm for the cervical spine and 25-35 mm for the lumbar spine. Another variable that affects penetration is the wavelength of the laser. Infrared laser light has been demonstrated to have a typical penetration depth (ie the distance which reduces the incident energy to 37%) of nearly 3 mm, while red laser light has a penetration depth of 1 mm (Kolari and Airaksinen 1988). Although energy loss is exponential near the laser source, optical measurements have demonstrated that energy loss is nearly linear at greater distances (Faris et al 1991). In an experimental porcine tongue model, a 200 mW GaAlAs laser had intensity reduced to 16 mW after the first 15 mm, which is within our suggested optimal dose range (Bradley et al 1998, Gursoy and Bradley 1994). From this depth, intensities fell at a slower almost linear rate to 1.4 mW at 35 mm. In vivo trials with 904 nm pulse lasers have

Table 2. List of excluded studies.

Author	Joint (s)	Result	Reason for exclusion
Gallachi 1981	Cervical and lumbar	No significant differences	Acupuncture and trigger point exposure only
Lewith 1981	Knee	LLLT significantly better than placebo	Trigger point exposure only
Walker 1983	Not stated	LLLT significantly better than placebo	Peripheral nerve exposure only, randomisation doubtful
Waylonis 1988	Low back	No significant differences	Trigger point exposure only
Snyder-Mackler 1989	Lumbar and cervical	LLLT significantly better than placebo	Trigger point exposure only
Rogvi-Hansen 1991	Knee	No significant differences	Did not irradiate joint, but peripheral nerves and top of patella only

demonstrated that these lasers achieve similar effects on collagen production with far lower doses on the animals' skin than lasers with continuous output (Enwemeka 1991, van der Veen and Lievens 2000). This effect can be attributed to the photobleaching phenomenon, where the first strong pulse bleaches the opaque barrier of tissue, letting the second pulse pass through the tissue barrier with less loss of energy (Kusnetzow et al 2001).

We postulate that energy loss due to the skin barrier for continuous HeNe (632nm) laser is 90%, for continuous GaAlAs (820nm) and NdYag IR lasers, 80% and for GaAs (904 nm) infrared pulse laser, 50%. Further energy loss is, according to the porcine penetration model, postulated to be linear at 5% per mm of tissue for infrared lasers. For red HeNe laser we postulate that further energy loss is 10% per mm of tissue.

The synovial area is rather small in finger, toe and temporomandibular joints, and we postulate that at least one single point is necessary to deliver an optimal dose of LLLT in these locations. We also postulate that a minimum of three points of the synovial membranes of the knee and the zygapaphyseal joints of the spine must be irradiated to provide a sufficient dose for these locations.

Estimations of dose and power densities required for the different anatomical locations are shown in Table 1.

Literature search A pre-specified literature search was performed from 1980 through to November 2001 on MEDLINE, Embase, CINAHL, PEDro and the Cochrane Controlled Trials Register (Central) for randomised controlled clinical trials.

Key words were: Low level laser therapy, low intensity laser therapy, low energy laser therapy, HeNe laser, IR laser, GaAlAs, GaAs, diode laser, osteoarthritis, chronic joint disorder, temporomandibular joint, hip, knee, thumb, spine. Hand searching was also performed on national physiotherapy and medical journals from Norway, Denmark, Sweden, The Netherlands, Germany, Switzerland, England, USA, Canada and Australia. Additional information on randomised controlled trials was gathered from researchers in the field. The literature search was concluded by the end of November 2001.

Methods

Inclusion criteria The trials were subjected to six inclusion criteria: joint disorder of more than six months duration or osteoarthritis verified by x-ray, random allocation of patients to groups, control group received identical placebo treatment, blind patients and outcome assessors, laser exposure of skin overlying inflammatory joint capsule, and outcome measure of pain and change in health status.

Assessment of methodological quality A criteria list of 10 methodological criteria developed for the PEDro database of physiotherapy trials at The University of Sydney, Australia, was used (Moseley et al 2002). Assessments of methodology were made by an assessor who was blinded to the trial results. No specific cut-off limit for method scores was pre-planned as a criterion for exclusion.

Outcome measures We selected pain on a visual analogue scale (VAS) as the first of two main outcome measures. In trials where several aspects of pain were measured, measures of pain during physical activity were preferred. Variance was calculated from post-treatment data and given as 95% confidence intervals (95% CI) in millimetres on VAS. Results were presented as weighted mean differences (WMD), ie a pooled estimate of the difference in mean change of the treatment and the placebo groups weighted by the inverse of the variance using a random effects model. Variance was calculated from the standard deviation (SD) of post-treatment data and given as 95% CIs. If variance data were reported as interquartiles, then the average SD from the other included trials was used for the statistical pooling.

The second main outcome measure was categorical data of change in global health status. Improved global health status was defined as any one of the following categories: "improved", "good", "better", "much improved", "pain-

Table 3. List of included trials with treatment specifications.

First author and year of publication	Location	Laser type, manufacturer	Laser continuous output (maximum pulse) and treatment time	Power density (mW/cm^2)	Dose (Joule)	No. of sessions/ sessions per week	Co-interventions
Basford 1987	*Thumb*	*632 nm (P) Dynatronics*	*0.4 mW 1 min*	*90*	*0.0135**	*9/3*	*Drugs registered*
Jensen 1987	*Knee*	*904nm (P) Space CEB*	*0.3 mW*(2W) (200 Hz) 6 min*	*0.3*	*0.05**	*5/5*	*Analgesics registered*
Klein 1990	*Lumbar spine*	*904 nm (P) Physio Technology*	*0.4mW* (2W) 4 min*	*0.4*	*0.1 **	*8/2*	*Exercises NSAIDs*
Stelian 1991	Knee	630 nm (P) 820 nm (P) Amcor	75 mW 25 mW 15 min	34 11	10.3 11.1	20/10	Analgesics
Nivbrant 1992	Knee	904 nm (P) ASA	4 mW(10 W) 5000 Hz, 3 min (C)	57	2.1	6/3	Analgesics registered, NSAIDs not allowed
Bulow 1994	Knee	830 nm (P) Unilaser	25 mW 15 min (C)	110	22.5	9/3	Drugs registered
Gray 1994	TMJ	904 nm (P) Space CEB	4 mW(27 W)* 3 min	57	0.7*	12/3	Not registered
Toya 1994	Lumbar Cervical Extremity	830 nm (P) OhLase3D1	60 mW 9 min	3000	48-60	1/1	Not allowed
Bertolucci 1995	TMJ	904 nm ASA	4 mW (10W) (700 Hz) 9 min	57	2.1	9/3	Not registered
Gøtte 1995	Knee	904 nm Felas	12 mW (25W) 13 min	4	12*	12/3	NSAIDs not allowed
Conti 1997	TMJ	830 nm (P) Omnilase	100mW 40 sec	38887	4	4/1	Not registered
Soriano 1998	Lumbar spine	904 nm (P) Brand missing	40 mW (20W)10 kHz	40	16*	10/5	NSAIDs and physiotherapy not allowed
Basford 1999	Lumbar spine	NdYag Laser Biotherapy	1626 mW, 6 min (C)	542	48.8	12/3	NSAIDs allowed
Özdemir 2001	Cervical spine	830 nm (P) Enraf Nonius	50 mW 3 min	390	10.8	10/7	Not registered

Trials with dose or power density outside suggested range in Italics. NSAID, non-steroidal anti-inflammatory drug; P, pointer; *, dose revised by reviewers; TMJ, temporomandibular joint.

free", "excellent". If sufficient data from the trial reports were provided, then the proportions of "improved" and "not improved" patients were pooled and expressed as a relative risk. A random effects model was used for statistical pooling.

Results

Included studies The literature search identified 88 randomised controlled trials of LLLT, of which 20 included chronic joint disorders. Six trials were excluded for not irradiating the skin directly overlying the joint capsule (Gallacher et al 1981, Lewith and Machin 1981, Rogvi Hansen et al 1991, Snyder-Mackler et al 1989, Walker 1983, Waylonis et al 1988) (Table 2).

A total of 14 trials with 695 patients (Basford et al 1987 and 1999, Bertolucci and Grey 1995, Bulow et al 1994, Conti 1997, Gray et al 1994, Götte et al 1995, Jensen et al 1987, Klein and Eek 1990, Nivbrant and Friberg 1992,

Table 4. Method scores (PEDro scale).

First author	Random-isation performed	Concealed allocation to groups	Baseline similarity	Patient blinded	Therapist blinded	Observer blinded	With-drawals/ dropouts < 15%	Intention-to-treat-analysis	Between-groups difference tested statistically	Mean and variability data	Total score
Basford 1987	*1*	*0*	*1*	*1*	*1*	*1*	*1*	*0*	*1*	*1*	*8**
Jensen 1987	*1*	*0*	*0*	*1 (0)*	*0*	*1 (0)*	*1*	*0*	*1*	*0*	*5(3*)*
Klein 1990	*1*	*0*	*1*	*1*	*1*	*1*	*0*	*0*	*1*	*1*	*7**
Stelian 1991	1	0	1	1	1	1	1	0	1	1	8
Nivbrant 1992	1	0	1	1	1	1	1	0	0	1	7
Bulow 1994	1	0	0	1	1	0	1	0	1	1	6
Gray 1994	1	0	0	0	1	1	0	0	1	1	5*
Toya 1994	1	1	1	1	1	1	1	0	1	1	9*
Bertolucci 1995	1	0	0	1	1	0	1	0	1	1	6
Gøtte 1995	1	0	1	1	0	1	1	0	1	1	7
Conti 1997	1	0	1	1	0	1	1	1	0	0	6
Soriano 1998	1	0	1	1	1	1	0	0	1	1	7*
Basford 1999	1	0	1	1	1	1	1	0	1	1	8*
Özdemir 2001	1	0	1	1	1	0	1	0	1	1	7

Trials shown in Italics gave treatment outside suggested dose range. * indicates that the same method scores have been given by PEDro reviewers. (*) indicates method score by PEDro reviewers where disagreement with our assessment existed.

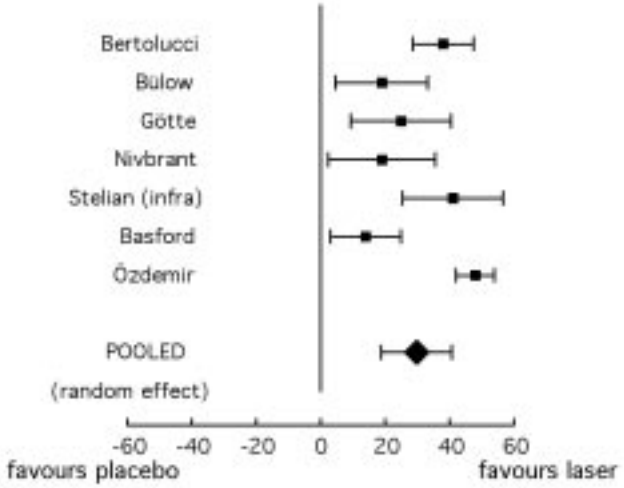

Figure 1. Effect of low level laser therapy on pain (mm on a 100 mm VAS).

Özdemir et al 2001, Soriano and Rios 1998, Stelian et al 1992, Toya et al 1994) satisfied our inclusion criteria. A list of included trials and their treatment characteristics is summarised in Table 3.

Dose assessment The results of the dose assessment revealed that three trials (Basford et al 1987, Jensen et al 1987 and Klein and Eek 1990) did not use doses inside our dose suggested range. These trials are indicated in Italics in Table 3. The remaining 11 trials, which included 565 patients, adhered to the suggested dose range.

Method scoring Method scores for trials that used the suggested dose range satisfied on average 6.9 out of 10 possible criteria on the PEDro scale, while the remaining three trials satisfied six out of all 10 criteria on the PEDro scale. Seven trials had previously been assessed by PEDro reviewers. For one trial, our assessment differed from the PEDro database scores (Jensen et al 1987). Missing concealed allocation to groups and intention to treat analysis were the most frequent shortcomings in the included trials. The results of the method scoring is summarised in Table 4.

Pain reduction on VAS Nine trials provided data of pain on VAS (Table 5). Two trials used a dose outside our suggested dose range and both reported a non-significant difference in pain reduction (Conti 1997, Klein and Eek 1990). Of the remaining eight trials with LLLT dose inside our suggested dose range, one trial reported variance data as interquartiles (Bulow et al 1994). These variance data were substituted by the average SD of the other six trials in the statistical pooling. By using a random effects model, WMD in change of pain on a 100 mm VAS was calculated to 29.8 mm (95% CI 18.9 to 40.7) in favour of active laser (Figure 1).

Table 5. List of included trials with data on treatment outcome.

First author	No. of patients	Condition	Mean pain (mm) before treatment	Mean pain (mm) after treatment	Mean change in pain (mm)	Proportions of patients improved	Author's test of significance
Basford 1987	*81*	*Active* *Placebo*	*53* *48*	*(missing data)* *(missing data)*		*22/47* *16/34*	*N.S.*
Jensen 1987	*29*		*No separate pain score (medication included)*				*N.S.*
Klein 1990	*20*	*Active* *Placebo*	*40* *44*	*23* *28*	*17* *16*		*N.S.*
Stelian 1991	50	Active(Red) Active(Infra) Placebo	65 72 62	33 32 63	32 40 -1		$p < 0.0001$ (Before/ after)
Nivbrant 1992	30	Active Placebo	67 58	44 54	23 4		$p < 0.01$ (Before/ after)
Bulow 1994	29	Active Placebo	82 71	61 69	21 2	7/14 9/15	N.S.
Gray 1994	55	Active Placebo				20/29 14/26	$p < 0.001$
Toya 1994	115	Active Placebo				43/59 16/56	$p < 0.0001$
Bertolucci 1995	32	Active Placebo				40 2	$p < 0.01$
Gøtte 1995	40	Active * Placebo*	69 70	42 68	27 2	13/20 2/20	"Significant" (no *p*-value)
Conti 1997	20	Active Placebo	58 49	27 38	31 11		N.S.
Soriano 1998	71	Active Placebo	79 81	(missing data) (missing data)		27/38 12/33	$p < 0.007$
Basford 1999	63	Active Placebo	35 37	17 33	18 4		$p < 0.001$
Özdemir 2001	60	Active Placebo	77 73	24 68	53 5		$p < 0.001$

Randomised placebo-controlled trials where LLLT has been used for CJD. Outcome data are extracted from trial reports. Trials shown in Italics gave treatment outside suggested dose range. *Visual estimates of data from graphs in trial report. Blank boxes indicate missing data in trial report. N.S. = not statistically significant.

Health status Six trials measured change in health status, and provided data that made it possib le to calculate the number of patients that impro ved their health status in active LLLT groups and placebo laser g roups (Table 5). In one trial that used a lo wer dose than our suggested dose range, no signif icant difference was registered between groups (44% versus 47% with improved status; Basford et al 1987). The remaining six trials used doses within our suggested dose range. In one of them (Basford et al 1999), the data showed a significant effect in favour of active laser but the presentation of the data did not allo w for identification of the number of patients w ho experienced improvement. Five trials repor ted improved health status for a total of 110 patients in the active LLLT groups versus 53 in the placebo g roups. Health status remained unchanged for 50 patients in the acti ve LLLT-groups and 97 patients in the placebo g roups. The pooled estimate of the change of health status w as significantly in f avour of active LLLT with a relati ve risk of 0.52 (95% CI 0.36 to 0.76) when calculated b y a random ef fects model. The results for health status are summarised in F igure 2.

Duration of pain r elief Six trials with assumed optimal treatment employed follow-up measurement of at least three weeks. Four of these trials repor ted pain relief under blinded conditions (Basford et al 1999, Götte et al 1995, Gray et al 1994, Ni vbrant and F riberg 1992). Two trials with intensive, daily treatment regimens (Soriano and Rios 1998, Stelian et al 1992) repor ted pain reduction from LLLT for four to six months, but e valuation in the follow-up period was unblinded.

Side effects and adverse reactions In terms of side effects, six of the LLL T trials with optimal dose (Basford et al

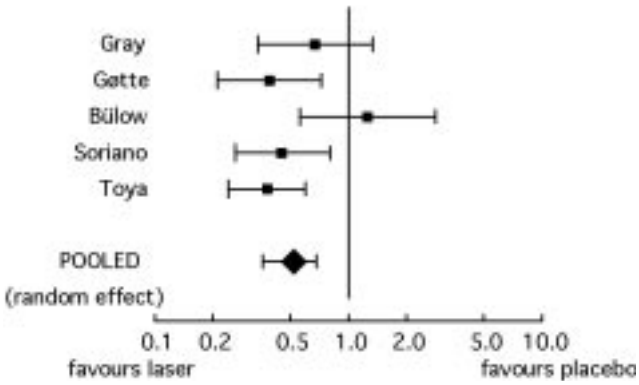

Figure 2. Effect of low level laser therapy on health status (Relative risk of not improving)

1999, Bulow et al 1994, Götte et al 1995, Nivbrant and Friberg 1992, Soriano and Rios 1998, Stelian et al 1992) explicitly stated in their report that no adverse effects were observed. One trial reported an incident of transient adverse effects for one patient in each group (Basford et al 1987).

Discussion

The results of this review were surprisingly unequivocal in favour of active LLLT when dosage was titrated above the suggested lower dose limit for reduction of inflammation. In our opinion, many trial authors and reviewers have investigated clinical effects without having a hypothesis of which biological action they expect from LLLT. They have often disregarded the fact that LLLT dose is affected by physical and anatomical penetration characteristics. Although we have tried to cater for these factors, it must be remembered that our estimate range of laser penetration (Table 1) is hypothetical. We currently lack hard data on what biological effects laser causes at certain depths and tissues in the human body.

Perhaps the weakest point of this review is the heterogeneity in treatment procedures and within the patient sample. The latter is reflected by mean baseline pain scores that ranged from 35 mm to 82 mm on VAS (Table 5). In two trials it was explicitly stated that patients were excluded if they experienced an acute episode of exacerbation (Basford et al 1999, Klein and Eek 1990). For the other trials, baseline pain was above 48 mm on the VAS.

Another issue that can partly explain heterogeneity in results is that only some trials prohibited co-intervention by anti-inflammatory drugs. The overall effect in trials that explicitly allowed anti-inflammatory drugs was poorer than those which did not allow for this co-intervention. This adds support for our hypothesis that pain reduction from LLLT is achieved through an anti-inflammatory action.

The differences in numbers and frequencies of the treatment sessions may also increase heterogeneity in results. However, the majority of trials involved treatment for two to four weeks, and only one trial (Toya et al 1994) treated once and measured the immediate effect of LLLT. We were in doubt whether this trial should be removed from the calculations of improved health status.

The structures which contribute to neck pain or low back pain are disputed, but both muscular and articular structures seem to be involved. The majority of patients with chronic spinal pain in our review had an x-ray confirmed diagnosis of osteoarthritis (Basford et al 1999, Ozdemir et al 2001, Soriano and Rios 1998). The presence of inflammation, however variable in activity, is a cardinal sign in osteoarthritis (Pelletier and Martel-Pelletier 2002). For this reason, we decided to include these trials as chronic joint disorders trials.

We think that the inclusion of pain from the temporomandibular joint is fairly uncontroversial. It is a common condition and, like other chronic joint disorders, is characterised by pain, synovial inflammation and decreased range of motion (Rauhala et al 2000).

Assessing scientific evidence from clinical trials is always a complex matter. We do agree that the methodological quality of trials is important, and have assessed the trials according to a widely accepted standard (the PEDro scale). Fortunately, the included trials were all of acceptable methodological quality, which made it unnecessary to exclude any of them from our conclusion. Six of the trials have been assessed by PEDro reviewers and confirm our method scores. For one trial we found that partial blinding was performed, which contradicts the PEDro review. In addition, two other trials (Bertolucci and Grey 1995, Stelian et al 1992) have previously been assessed by other reviewers who found that they fulfilled more than half of the quality criteria on the Jadad and Maastricht lists, respectively. There is, however, genuine disagreement between our method score and the score of a Swedish trial (Nivbrant et al 1992) in another review (de Bie et al 1998). This may be attributed to linguistic difficulties, or the fact that two reports have been published from this trial.

There is some evidence that LLLT may inhibit fibroblast activity (Loevschall and Arenholt-Bindslev 1994) when dose exceeds 4 J. As the joint capsule is populated by fibroblasts, future research is needed to clarify the matter of optimal balance between biological effects such as COX-2 inhibition and fibroblast activity.

Laser dosage is a complex topic, and missing parameters can give a misleading picture if they are not fully reported. We have retrieved the missing laser parameters by getting specifications from the manufacturers of all the lasers used in the included trials and we have recalculated all power densities, dose per treatment sessions and weekly doses. However, it is a weakness that testing and calibration of laser output was only performed in two of the clinical trials (Basford et al 1999, Bulow et al 1994).

In five of six LLLT trials with follow-up, pain reduction remained significant for three weeks, and unblinded follow-up suggested significant pain reduction for up to six months (Stelian et al 1992).

The literature on LLLT is full of conflicting reports, and we believe that much of this is caused by the lack of dosage consensus. One large, well-designed trial found no effect from LLLT on ankle sprains (de Bie et al 1998). In our opinion, the poor results may have been caused by insufficient irradiation, because only a single 1 cm^2 point of the swollen joint capsule was treated by LLLT. In a recent review on LLLT effectiveness (Brosseau et al 2000), results for osteoarthritis were conflicting. This review lacked procedural assessment of the laser exposure technique, and dose analysis was not used to adjust for differences in energy loss for each anatomical location. In addition, our literature search is more recent and extensive and includes two more trials on osteoarthritis of the knee (Götte et al 1995, Nivbrant and Friberg 1992), in addition to trials with spinal and temporomandibular joint disorders.

Conclusion

Although the heterogeneity of the trial results calls for caution in interpretation, LLLT seemed to be effective in reducing pain from chronic joint disorders. The hypothesis that LLLT acts through a dose-specific anti-inflammatory effect in the irradiated joint capsule is a potential explanation of the positive results. This hypothesis needs to be verified or refuted in studies where outcome measures of inflammatory activity are used. More and larger trials are needed to precisely determine optimal treatment procedures for LLLT and possible interaction with other therapies for chronic joint disorders.

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Byrnes:

Regeneration after Spinal Cord Injury

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Light Promotes Regeneration and Functional Recovery and Alters the Immune Response After Spinal Cord Injury†

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Background and Objectives: Photobiomodulation (PBM) has been proposed as a potential therapy for spinal cord injury (SCI). We aimed to demonstrate that 810 nm light can penetrate deep into the body and promote neuronal regeneration and functional recovery.

Study Design/Materials and Methods: Adult rats underwent a T9 dorsal hemisection, followed by treatment with an 810 nm, 150 mW diode laser (dosage = 1,589 J/cm^2). Axonal regeneration and functional recovery were assessed using single and double label tract tracing and various locomotor tasks. The immune response within the spinal cord was also assessed.

Results: PBM, with 6% power penetration to the spinal cord depth, significantly increased axonal number and distance of regrowth ($P < 0.001$). PBM also returned aspects of function to baseline levels and significantly suppressed immune cell activation and cytokine/chemokine expression.

Conclusion: Our results demonstrate that light, delivered transcutaneously, improves recovery after injury and suggests that light will be a useful treatment for human SCI. Lasers Surg. Med. 36:171–185, 2005.

Key words: astrocytes; corticospinal tract; footprint analysis; low power laser irradiation; macrophage; microglia; photobiomodulation; rat; retrograde and anterograde tract tracing

INTRODUCTION

Damaged central nervous system axons fail to regenerate following spinal cord injury (SCI) in adult mammals. Despite vigorous research, including use of anti-inflammatory drugs [1], X-irradiation [2,3], elimination of inhibitory factors in the spinal cord [4–9], provision of neurotrophic factors [10–14], and cell transplantation [15–22], there currently is no cure for the sensory or motor deficits seen following injury. After SCI, a secondary injury occurs that is mediated in part by the immune response [23] and magnifies the impairment [23–25].

Photobiomodulation (PBM), also known as light therapy, low power laser irradiation, or low level laser irradiation, is an effective treatment for cutaneous wounds and promoting peripheral nerve regeneration [26–29]. This modulation in recovery is attributed to a light absorption mechanism [30] rather than through the production of heat [29,31,32]. Research has shown that dosages of 0.001–10 J/cm^2 stimulate cellular activity (such as DNA, RNA, and protein production, proliferation, and motility) while dosages greater than 10 J/cm^2 inhibit activity [33].

Following SCI, high dosage PBM in combination with transplantation resulted in an increase in axonal sprouting, decreased scar formation, and improved weight bearing and step taking in dogs and rats in comparison to transplantation alone [34–36]. These studies indicate that PBM may have a number of therapeutic effects following SCI, potentially by decreasing the inflammatory response at the spinal cord lesion site.

Invasion/activation of immune cells has been under investigation as a potential mediator of secondary injury [23]. A variety of cell types invade or are activated within the first hours to days after SCI, including neutrophils, macrophages, microglia, astrocytes, and T and B lymphocytes [25,37–46]. These cells are primarily activated or drawn into the lesion area by pro-inflammatory cytokines and chemokines, expressed within the first few hours after injury [42,47–49]. Recent evidence suggests that alteration of cell invasion/activation after SCI improves functional recovery. Research demonstrated that depletion of macrophages improved locomotion, spared white matter, preserved myelinated axons, supported axonal sprouting and reduced cavitation [50]. Anti-inflammatory drugs also increased tissue sparing [51] and promoted functional recovery [21,52].

To date, no study has assessed the axonal regrowth of specific tracts or the recovery of specific locomotor functions

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in the spinal cord after acute injury and PBM. Additionally, no study has investigated the mechanisms of light therapy's effect within the injured nervous system. Here, we show that light applied transcutaneously at the site of SCI is able to penetrate to the level of the spinal cord and significantly improves axonal regeneration and restores specific locomotor functions while altering the immune response after injury.

MATERIALS AND METHODS

Subjects

Eighty-five adult female Sprague–Dawley rats (200–300 g, Taconic Farms, Germantown, NY) were used in this study under an approved Uniformed Services University IACUC protocol. Food and water were provided ad libitum and the rats were exposed to 12 hour reversed cycles of light and dark periods. For all experimental procedures, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on isothermal heating pads warmed to 37°C.

Spectrophotometric and Power Measurement

An incoherent broadband white light was directed at the surface of the skin in the low thoracic vertebral level of adult, Sprague–Dawley rats ($n = 5$). A smart, tissue-activated optical fiber probe [53] was inserted sequentially into the skin (layer 1; ~1 mm thick), sub-cutaneous connective tissue layer (2; ~1 mm thick), deep connective tissue layer (3; ~1 mm thick), muscle (4; ~15 mm thick), and the spinal cord within the vertebral column (5; ~10 mm thick). At each of these layers, a transmission spectrum in the range of 500–1,200 nm was collected while white light was applied to the skin surface.

Corticospinal Tract (CST) Lesion

Rats were randomly assigned to control ($n = 40$) or PBM (PBM; $n = 40$) groups. Dorsal hemisection was performed by an investigator blinded to group assignment. A laminectomy followed by a dorsal hemisection was performed at vertebral level T9 by passing a 6-0 suture (Nurulon; Ethicon, Inc., Piscataway, NJ) beneath the dorsal funiculus and carefully incising the entire dorsal portion of the spinal cord with iridectomy scissors. This lesion results in transection of the CST, which lies in the base of the dorsal funiculus. Complete transection was assured by lifting the suture through the lesion. Inspection of the lesion and visualization of the central gray commissure verified that the CST had been transected.

Retrograde Labeling

At the time of CST lesion, gelfoam soaked in hydroxystilbamidine methanesulfonate (HM, also known as fluorogold; 3% in 0.9% saline; Molecular Probes, Eugene, OR) was inserted into the lesion site of 20 rats ($n = 10$/experimental group). Ten weeks after the surgery, a laminectomy was performed approximately 24 mm caudal to the original lesion site (vertebral level L3). The dura was incised and 1 μl of a 2% fast blue solution (in PBS, Sigma, St. Louis, MO) was injected bilaterally at 0.5 mm lateral to the midline into the gray matter (0.5 μl into each side) of the spinal cord at a depth of 1.3 mm [54,55]. This injection would result in the spread of the dye to label axonal terminations in Rexed's laminae 7–9.

Anterograde Labeling

Five weeks after CST lesion, 5% tetramethylrhodamine biotinylated dextran (mini-ruby, Molecular Probes) was injected into the motor cortex of one group of 10 rats using stereotaxic coordinates ($n = 5$/experimental group). The skin overlying the skull was shaved and swabbed with alcohol pads. A midline incision was made in the skull and a total of six holes were drilled through the skull at the following stereotaxic coordinates to ensure that the axonal tracer was injected into the primary motor cortex: from bregma, −0.11 AP and ±1.60 ML; −1.33 AP and ±1.50 ML; −2.85 AP and ±1.40 ML. The needle of a Hamilton syringe was placed in each hole at a depth of 1.0–1.2 mm. Two microliters of the mini-ruby solution were injected into the primary motor cortex through each hole, for a total injection of 12 μl into the primary motor cortex. The skull was covered with bone wax, and the skin was sutured.

Light Treatment

Beginning within 15 minutes after spinal cord dorsal hemisection, rats randomly assigned to the PBM group were transcutaneously irradiated at the lesion site. Irradiation was applied daily for 14 consecutive days with a continuous wave 810 nm diode laser (Thor International, UK; 200 mW output, modified and homogenized with a delivery optical fiber resulting in an output power of 150 mW, 2,997 seconds treatment time/day). The dosage applied to the surface of the skin was 1,589 J/cm^2 per day (dose = [energy × time]/treatment area; 0.53 W/cm^2, 450 J). This is the dosage found to improve functional recovery after injury in previous studies [35]. During treatment, the 0.3 cm^2 spot was centered on the skin directly above the location of the spinal cord hemisection, with the expectation that the spot size would spread as it progressed through the tissue, while maintaining enough power to reach the spinal cord, as is presented in our accompanying paper. Power output of the light source was measured with a power meter to ensure that power delivery was consistent (Ultima Labmaster, Coherent, Inc., Auburn, CA). Prior to treatment, all animals were lightly anesthetized with sodium pentobarbital (20 mg/kg, i.p.) and placed on isothermal heating pads. All treatments were done in the dark. Rats in the control group were handled identically, except they did not receive light treatment. Using these treatment parameters, no adverse effects were noted at the skin surface at any time during or after treatment (data not shown).

Previous studies in our laboratory have determined that this level of irradiation does not induce significant heating at the level of the spinal cord, with an average temperature increase of 0.350 ± 0.01°C over the entire treatment time (data not shown). At the skin surface, the average temperature increase is 1.832 ± 0.06°C (data not shown). Other

investigators have determined that heating in this range does not have the same effect as light treatment, suggesting that the effects observed are due to light interaction rather than heating [31,32].

Labeling Assessment

Rats were intracardially perfused with 4% paraformaldehyde 8 days after injection of mini-ruby or fast blue. Work in our laboratory had determined that 8 days was a sufficient time for mini-ruby labeling of the thoracic spinal cord from the motor cortex (data not shown). Previous studies [56–58] have shown that 8 days is also sufficient for fast blue labeling of the cortex. Coronal sections of the brain through the motor cortex, 20 μm thick, and longitudinal sections of spinal cords, including 3 mm rostral to and 16 mm caudal to the lesion, were collected. These 20 μm thick sections were collected from the dorsal aspect of the spinal cord through the level of the gray commissure.

Mini-ruby labeled spinal cord sections, including the lesion site and 16 mm caudal, were collected at a ratio of 1/6. Mini-ruby labeled axons were counted at 0.5 mm intervals from the lesion site through 16 mm caudal to the lesion using an RITC filter (excitation 528–553 nm) and 20× magnification, as described previously [21]. Total axons counted were then averaged/section and, as the total number of sections required to encompass the entire CST was found to be 24, the average was multiplied by 24 for a final average axon count/animal. Axon counts were compared at 1 mm intervals for statistical analysis, and average distance of regeneration was established for each animal in each group. Axonal counts are presented as mean ± SEM. Axonal count data were analyzed using one-way ANOVA, with Bonferroni post-test.

For neuronal counting, cortical sections were collected and mounted at a ratio of 1/8. The fractionator method of unbiased stereology [59] was used to count HM and/or fast blue labeled neurons in the motor cortex at a magnification of 20× (2.6 mm from midline to lateral edge of brain per hemisphere). Every eighth section from Bregma to Bregma—2.5 mm was assessed using a random start site. Two filters, with excitation ranges of 330–380 nm and 450–490 nm, were used to identify single (HM or fast blue) and double labeled neurons. Double labeling was described as those neurons with a blue cytoplasm with green punctate labeling in the cytoplasm, as reported previously [60]. The percentage of neurons that regenerated an axon was calculated according to the following calculation:

$$\frac{\text{Double labeled neurons}}{\text{Fast Blue} + \text{HM} + \text{Double labeled neurons}} \times 100$$

Neuronal counts are presented as mean percentage of total neuronal number counted ± SEM. This calculation was based on an unbiased stereological technique that uses a dissector method and extrapolates the total number of objects from a representative sample of the whole. The total number of objects = the sum of the objects counted×1/(the number of sections sampled/total number of sections)×1/(the total area sampled/total area on all sampled sections)×1/(the height of the dissector/total section thickness). Neuronal count data were analyzed using Mann–Whitney *U*-analysis.

Only tissue in which cortical and spinal cord injection sites were without leakage of the tracer large distances away from the injection site and with adequate uptake into the intended neurons were included in the final analysis.

Functional Testing

One week prior, and 1 and 9 weeks after dorsal hemisection, the same rats undergoing retrograde labeling ($n = 10$/experimental group) were trained for 3 days and then tested for 2 days (five trials per day) to walk across a ladder beam (Columbus Instruments, Columbus, OH) that recorded the crossing time and footfalls. Footfalls were assessed as the number of time paws failed to grasp a ladder rung and fell below the plane of the ladder. Crossing time was assessed as the amount of time in seconds required to cross the ladder and reach a dark box at the end. This test was videotaped for confirmation. Rats also underwent footprint analysis and base of support (distance between central pads of the hind paws), stride length (distance between the central pads of two consecutive prints) and angle of rotation (angle formed by the intersection of the line through the print of the third digit and the line through the central pad parallel to the walking direction) were analyzed in a method modified from that of Metz et al. [61]. Briefly, hind paws of rats were inked and rats were allowed to walk across a narrow runway covered in white paper to a safety cage. All testing was done in triplicate on two consecutive days, and testing at nine weeks was completed prior to administration of the second retrograde tracer to avoid complications from a second surgery. Data are presented as mean percentage of pre-surgical measurement to control for variations among animals. Functional data were analyzed using Repeated Measures ANOVA with Newman–Keuls post-test to assess changes over time or one-way ANOVA with Tukey post-test to assess differences between groups at individual time points.

Immunohistochemistry

Spinal cord tissue from rats was collected at 48 hours, 14 and 16 days post-injury (DPI). At each time point, five rats per experimental group were deeply anesthetized with 10% chloral hydrate (1 ml/100 g, i.p.) and euthanized via intracardiac perfusion with 4% paraformaldehyde. The thoracic spinal cord at the lesion site, which was typically approximately 2 mm long and was identifiable by visible scar tissue, and 3 mm rostral and 5 mm caudal to the lesion site was dissected, post-fixed for 24 hours in 4% paraformaldehyde, and cryoprotected for 24 hours in 30% sucrose. Twenty micrometer longitudinal sections were collected from the dorsal aspect of the spinal cord through the level of the gray commissure. Sections were serially mounted onto 10 slides, with three sections per slide. One slide from each rat was processed for each cell type under investigation. Immunolabeling was repeated for each animal to ensure labeling efficacy. Negative controls, in which primary antibody was not added during immunohistochemistry, were run for each cell type. The tissue was rehydrated and

blocked with an appropriate blocking solution. Tissue was incubated overnight with primary antibodies for macrophages/activated microglia (ED1, 1:175, Serotec, Inc., Raleigh, NC), neutrophils (RP3, 1:30, BD Pharmingen, San Diego, CA), T lymphocytes (UCHL1, 1:25, Dako Corp, Carpinteria, CA), B lymphocytes (L26, 1:75, Dako Corp), Schwann Cells (S100, 1:100, Santa Cruz, Santa Cruz, CA), or astrocytes (GFAP, 1:100, Dako Corp) followed by incubation with an appropriate fluorescently labeled secondary antibody (Jackson Immunochemicals, West Grove, PA) at room temperature for 30 minutes.

The lesion epicenter and adjacent 1 mm of tissue of at least six sections per animal per antibody were digitally photographed using a Leica/Spot system (Version 2.2 for Windows, Diagnostic Instruments, Inc., Sterling Heights, MI). The proportional area of tissue occupied by immunohistochemically stained cellular profiles within a defined target area (the lesion site and surrounding tissue) was measured using the Scion Image Analysis system (http://rsb.info.nih.gov/nih-image/) using a method modified from that described by Popovich et al. [46]. Briefly, tissue regions were scanned and the proportion of the area that included positive immunolabeling was measured. All tissue sections were coded prior to measurement to prevent bias and all image backgrounds were normalized prior to quantitation. Area of spinal cord occupied by cell type is expressed as mean ± SEM. Kruskal–Wallis statistical analysis with Dunn's post-test was used to compare means. Student's *t*-test was also used for detection of differences at individual time points.

RT-PCR

At 6 hours or 4 DPI, five rats/time point/group were deeply anesthetized and euthanized by decapitation. The 5 mm of the spinal cord encompassing the lesion site and the area immediately rostral and caudal was dissected and placed in 500 μl of RNAlater solution (Amnion, Austin, TX). Total cellular RNA was extracted and reverse transcribed using First-Strand Synthesis beads (Marsha Pharmacia, Piscataway, NJ) as per the protocol of the manufacturers (Nitrogen, Carlsbad, CA and Amersham Pharmacia). Briefly, tissue was homogenized in TRIzol (Invitrogen) using a FastPrep machine (Qbiogene, Carlsbad, CA). RNA was then extracted using the chloroform/isopropanol method and purified with a 75% ethanol wash prior to being resuspended. RNA was transferred to tubes containing First-Strand Synthesis beads (Amersham Pharmacia) and Random Hexamers (Invitrogen) and incubated at 1 hour at 37°C. Resultant cDNA was amplified using the CytoXpress Multiplex Inflammatory Set 1 (Biosource, Camarillo, CA) or monocyte chemoattractant protein–1 (MCP-1; 5′ CTTCTGGGCCTGTTGTTCAC 3′; 5′ GGGACGCCTGCTGCTGGTGATTC 3′), macrophage inflammatory protein 1α (MIP1α; 5′ TTTTGAGACCAGCAGCCTTT 3′; 5′ CTCAAGCCCCTGCTCTACAC 3′), or inducible nitric oxide synthase (iNOS; 5′ CCCTTCCGAAGTTTCTGGCAGCAGC 3′; 5′ GGGTGTCAGAGTCTTGTGCCTTTGG 3′). PCR products were quantified as previously described [62,63]. Briefly, pixel density for each band was measured the Scion Image program and normalized against the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data is presented as the ratio of gene of interest to GAPDH ± SEM. Resultant relative gene expression is presented as mean ratio ± SEM. One-way ANOVA was used to compare groups, with Tukey's post-test for comparison of individual groups.

Statistical Analysis

All statistical tests were performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc., San Diego, CA) and SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois).

RESULTS

Light Penetrates to the Spinal Cord

Ex and in vivo spectrophotometric and power transmission analyses were performed to assess the extent to which transcutaneous 810 nm laser light, with an output power of 150 mW, penetrates to the depth of the spinal cord (Fig. 1a,b). Analysis of the transmission spectra revealed the range of transmission, or penetration, was highest through all tissue layers overlying the spinal cord (Fig. 1c) and through blood (Fig. 1d) between the 770 and 850 nm wavelengths. The transmission of light through tissue is heavily influenced by the absorption of light by blood (Fig. 1d), which is reflected by the similarity between the two peaks of transmission as well as the relatively flat transmission spectra by skin (Fig. 1c, layer 1). Analysis of power penetration revealed that 6% of the power of a 150 mW 810 nm laser was transmitted through all of the layers of tissue between the dorsal skin surface and the ventral side of the spinal cord. These data show that 810 nm light is within the optimal range for light penetration to the spinal cord level if applied transcutaneously, and that 9 mW of energy will reach the spinal cord if the initial output is 150 mW.

Light Improves Axonal Regrowth

To determine if application of 810 nm light to the injured spinal cord increased axonal growth, an anterograde tracer, mini-ruby, was injected bilaterally into the motor cortex 5 weeks after a CST lesion. Analysis revealed that mini-ruby labeled axons were found in the white matter, in the region of the spinal cord normally occupied by the CST (i.e., in the dorsal funiculus, between the dorsal horns; Fig. 2a,d,e). These axons were observed to pass the lesion site ventral, dorsal or around the remaining cavity (Fig. 2a), or to traverse the lesion through a tissue bridge (Fig. 2b), as has been reported previously [64–67]. There were few mini-ruby labeled axons caudal to the lesion in the control group (Fig. 2c,g), with 16.32 ± 8.53 found at 1 mm, 8.61 ± 5.76 at 4 mm, and 0 axons found from 7 to 16 mm caudal to the lesion (Fig. 2g). These labeled axons were calculated to extend an average distance of 2.9 ± 0.8 mm caudal to the lesion (Fig. 2f), which is comparable to previously reported spontaneous post-lesional sprouting [68].

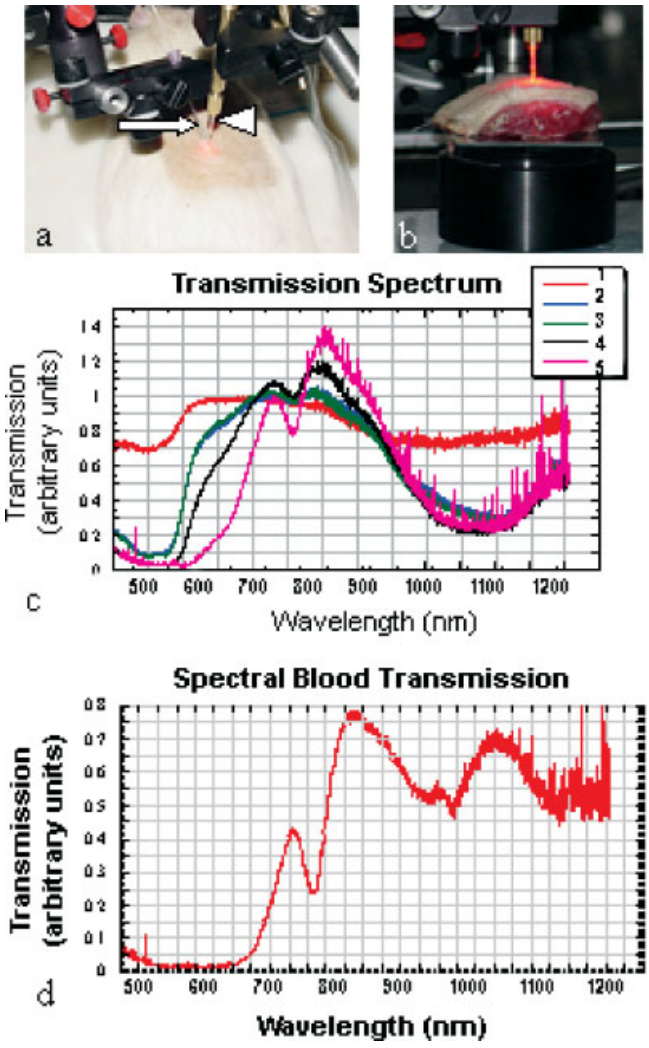


Fig. 1. Light penetration analysis. **a**: Photograph of spectrophotometric analysis experimental set-up. The smart fiber (arrow) is inserted below the skin of the rat, the light source (arrowhead) is positioned above the skin for transcutaneous application of light. **b**: Ex vivo power analysis, a cross section of the rat's dorsal thoracic region was placed between the light source and a power meter. Graphical representation of transmission (in arbitrary units) through each layer of tissue (**c**) or through blood (**d**), depending on wavelength (nm). Layer 1, skin; 2, loose connective tissue; 3, dense connective tissue; 4, muscle; 5, vertebral column and spinal cord.

Analysis of axonal number from 1 to 16 mm caudal to the lesion revealed that there were significantly more mini-ruby labeled axons in the PBM group than the control group ($P < 0.05$; Fig. 2g), with an average axonal count ranging from 71.76 ± 17.7 to 120.7 ± 18.5 axons counted per mm. No significant difference was found between the groups from 10 to 16 mm caudal to the lesion, although mini-ruby labeled axons were only found at these distances in the PBM group. The mini-ruby labeled axons in the PBM group extended an average of 8.7 ± 0.8 mm caudal to the lesion, a significantly increased length over the control group ($P < 0.05$; Fig. 2f).

Anterograde analysis demonstrates the presence of axons caudal to a transection; however, to determine if PBM promotes regeneration of transected axons, a double label, retrograde tracing analysis was performed. Based on the anterograde tracing data, axons in the PBM group were calculated to grow at a rate of 0.25–0.4 mm per day. Thus, axons would require approximately 10 weeks to reach the mid-lumbar region and innervate interneurons or motor neurons responsible for lower limb function [69]. At the time of CST lesion, transected neurons were labeled by inserting HM into the lesion. Ten weeks after CST lesion, axons terminating at vertebral level L3, approximately 24 mm caudal to the initial lesion, were labeled by injecting fast blue into the ventral horn. Numbers of single (HM or fast blue) and double (HM and fast blue; neurons with axons that were transected and regrew to L3) labeled neurons in the motor cortex were assessed using unbiased stereology. Due to insufficient labeling in two animals and the deaths of two animals prior to tract tracing analysis, all data presented for double-labeling assessment is for an n of 7 in the control group and an n of 9 in the PBM group.

Analysis of single labeled neurons (HM or fast blue) revealed no significant difference ($P > 0.05$) between control and PBM groups, demonstrating no difference in labeling efficacy between groups (Fig. 3a,b,c). The average number of HM labeled neurons is $8{,}860 \pm 3{,}408$ in the control group and $13{,}270 \pm 3{,}236$ in the PBM group, which is comparable to the number of CST axons reported in the lower thoracic region of the spinal cord [70,71]. The average number of fast blue labeled neurons is 129 ± 109 in the control group and 131 ± 120 in the PBM group, which is comparable to the number of neurons found in the motor cortex after injection of a retrograde tracer into the ventral portion of the CST at vertebral level L4 [70]. Since fibers of the dorsal and ventral CST originate from the same area of the motor cortex [70] and the lesioning procedure used in this study transects the dorsal CST but not the ventral CST, it is likely that these fast blue labeled neurons are from the unlesioned ventral CST.

Double labeled neurons, with both HM and fast blue labeling, were found only in the PBM group (Fig. 3d,e,f). In the PBM group, a maximal number of 543 double labeled neurons were counted, with an average of 70.5 ± 59.6 for the entire group. The percentage of double labeled neurons represented a statistically significant increase in comparison to the control group ($P < 0.05$; Fig. 3d). This increase in double labeling indicates that only CST axons in the PBM group regrew and terminated in the gray matter of vertebral level L3 after transection.

Light Improves Locomotor Function

To determine if PBM resulted in functional improvement, performance of rats in two functional tests, the ladder/grid walking test and footprint analysis, was

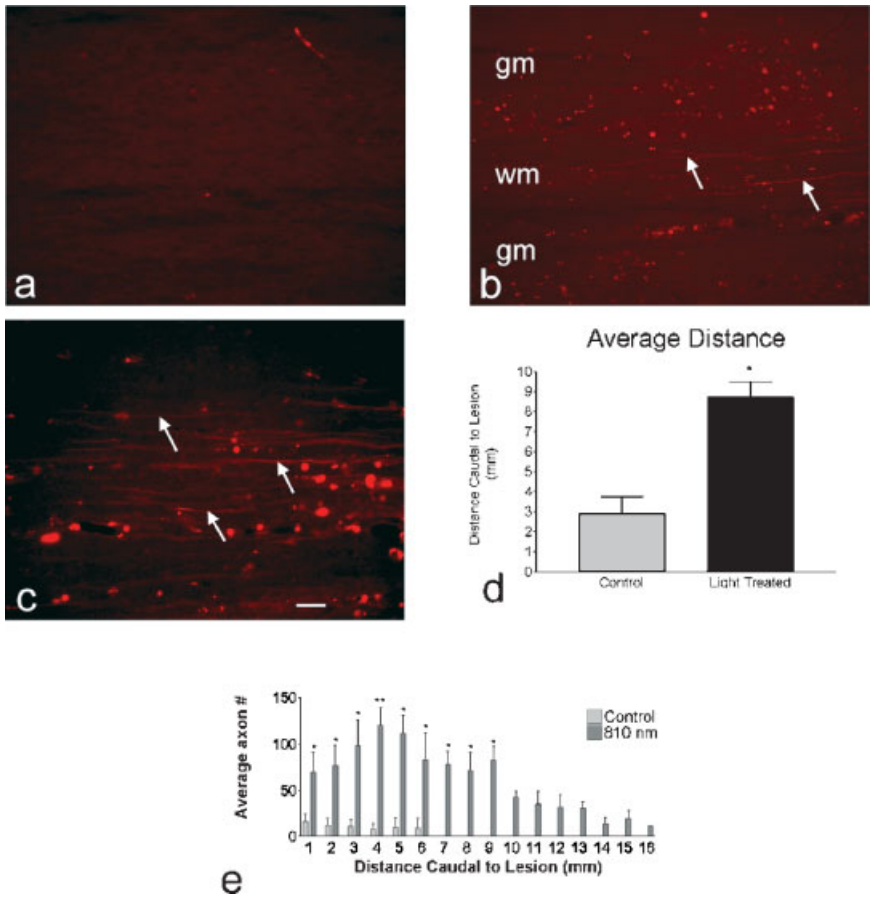


Fig. 2. Mini-ruby labeled axons and related quantitation at 5 weeks post-injury. Photomicrograph of two lesion sites (**a**, **b**), with axons passing around (a) or through (b) the lesion site (arrows). Photomicrograph of white matter 4 mm caudal to the lesion site in control rat (**c**) and photobiomodulation (PBM) rat (**d**, **e**). Note that mini-ruby labeled axons in the dorsal funiculus white matter (wm) between the dorsal horn gray matter (gm), indicated with arrows, are found at this distance only in the PBM group. Bar = 23 μm (c, e); 11.8 μm (a, b, d). Comparisons of average axon number/animal (**f**) and average distance caudal to the lesion (**g**) are shown. $^{*}P < 0.05$, $^{**}P < 0.001$. $N = 5$/group. Bars represent mean ± SEM.

assessed prior to and after CST lesion. Five measurements were taken, including footfalls, time to cross the ladder, base of support, stride length, and angle of rotation.

One week after CST lesion, rats had significant impairments in angle of rotation ($P < 0.05$; Fig. 4a) in the control group and footfalls ($P < 0.05$; Fig. 4b) in the control and PBM groups in comparison to pre-surgical measurements. An increase in ladder cross time was also observed in both groups at this time point (Fig. 4c). However, there was no significant difference between pre- and post-surgical angle of rotation in the PBM group at 1 week post-injury ($P > 0.05$; Fig. 4a).

At 9 weeks post-injury, angle of rotation remained at the baseline level ($P > 0.05$; Fig. 4a,d) and ladder beam cross time had returned to pre-surgical values ($P > 0.05$; Fig. 4c) in PBM animals, demonstrating a recovery of these functions. Control animals had measurements that remained at elevated levels ($P < 0.05$; Fig. 4a,c,d). Comparison of these measurements in PBM and control groups revealed a significant improvement in the PBM group ($P < 0.05$; Fig. 4a,c). There was a significant increase in footfalls in both control and PBM animals post-surgery ($P < 0.05$; Fig. 4b), but no significant difference between these two groups. No significant change was found in stride length or base of support in either group at any time point after CST lesion ($P > 0.05$). These functions have been found to be under the control of tracts other than CST, and are not normally affected by CST lesion alone [11], confirming the specificity of this lesion model for the CST.

Light Alters the Immune Response

To explore the potential mechanism of PBM's effects after SCI, the immune response within the spinal cord was assessed. Immunolabeling was quantified in order to determine the invasion/activation of different cell types in the spinal cord at 48 hours, 14 and 16 days after SCI.

Due to the clustering of cells surrounding the lesion following SCI, assessment of numbers of individual cells was not possible. Therefore, measurement of tissue area occupied by immuno-positive label within a defined target

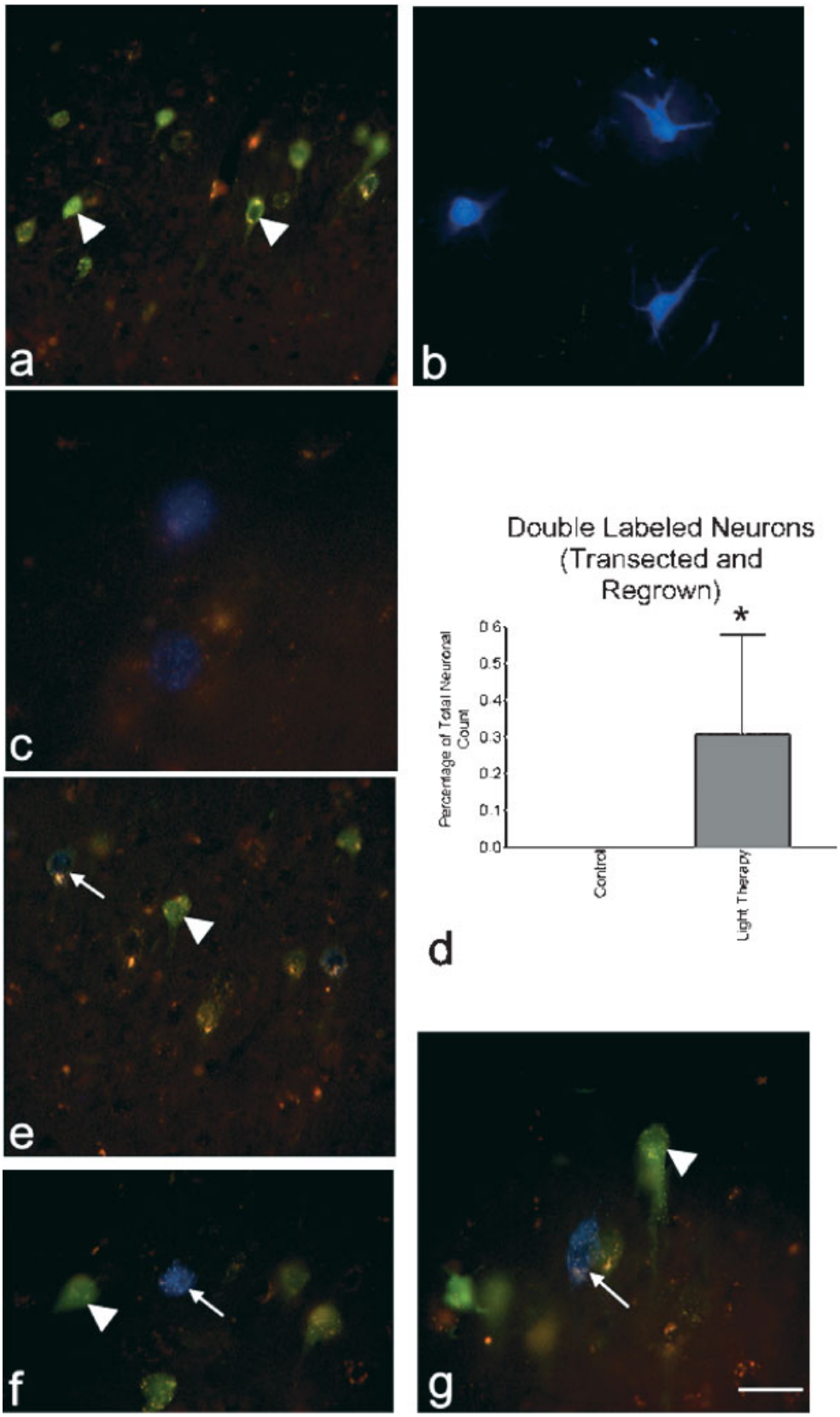
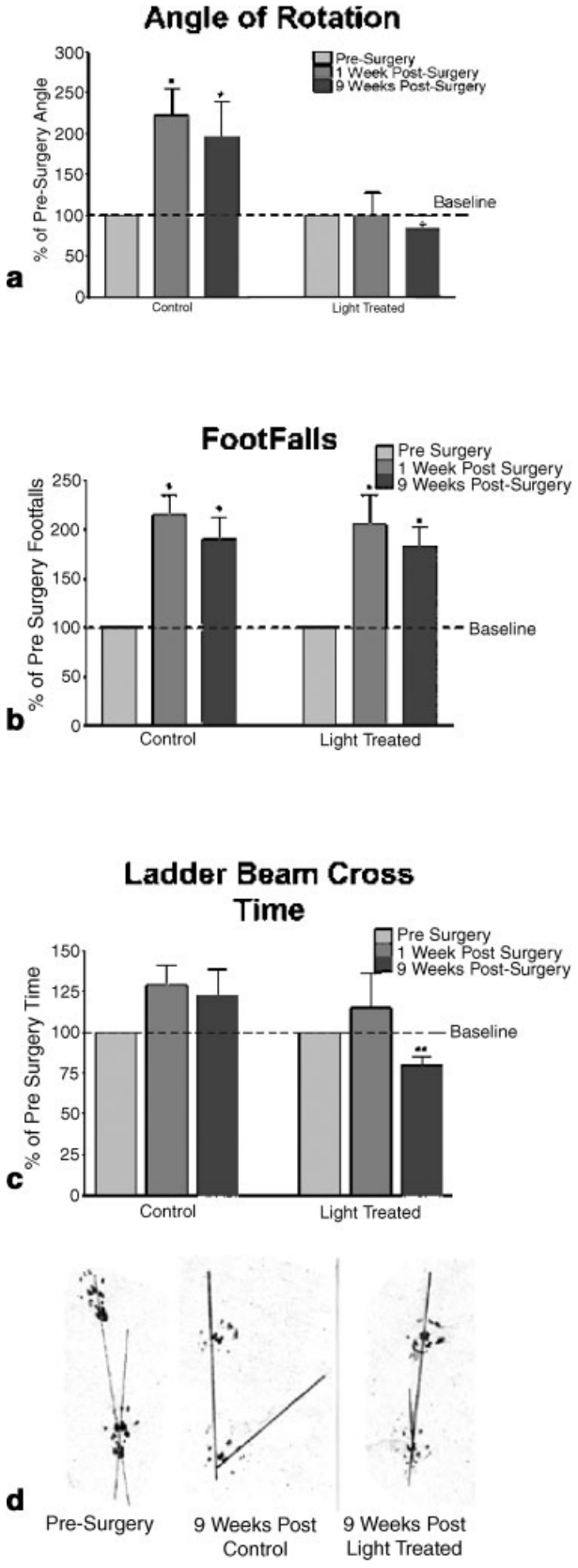


Fig. 3. Single and double labeled neurons at 10 weeks post-injury. HM labeled neurons in motor cortex (**a**, arrowheads), fast blue labeled neurons at L3 injection site (**b**), and fast blue labeled neurons in motor cortex (**c**). **d**: Graphical representation of double labeled neurons in PBM and control groups. $^*P < 0.05$; Mann–Whitney U. Bar represents mean percentage of counted neurons $\pm$SEM. **e**–**g**: Double labeled neurons (arrows), found only in motor cortex of PBM rats. Bar = 134 μm (a); 67 μm (b, f, g); 45 μm (c); 89 μm (e).



space (i.e., within the lesion and adjacent tissue area) was used to assess cell invasion/activation. As an increase in immunolabeling does not necessarily reflect an increase in cell number, this measurement is a method of quantifying the magnitude of a cellular response, both in terms of cell invasion and activation. The current study does not attempt to distinguish between these two cellular response parameters.

Macrophages and activated microglia are not distinguishable from each other in the mammalian CNS since activated microglia express the same cellular surface molecules and have the same round morphology as blood borne macrophages [41,46]. Immunolabeling for ED1, an antibody against a macrophage/microglia lysosomal glycoprotein, revealed many of these large, amoeboid cells in the injured spinal cord located in and around blood vessels, in the dorsal roots, along the edges of the lesion site, within the lesion site, and infiltrating into the surrounding tissue at 14 DPI and later. At many of the time points, there were observably fewer labeled macrophages/activated microglia in the PBM group than in the control group (Fig. 5a,b). In both control and PBM groups, ED1 expression was highest at 48 hours post-injury and 14 DPI. Both peaks were reduced in the PBM group, with significant reductions in ED1 expression at 48 hours and 14 DPI in the PBM group ($P < 0.001$; Fig. 5e). No significant difference between groups was found at 16 DPI ($P > 0.05$).

Astrocytes were detected using an antibody against GFAP, an intermediate filament primarily expressed in astrocytes. At 48 hours post-injury, heavy GFAP positive labeling was found to demarcate the lesion in all rats of the control group, with GFAP positive processes throughout the 10 mm section in three of the five rats (Fig. 5c). PBM tissue, however, had only a light band of GFAP positive label near the lesion edge and along the meninges/blood vessels in all five rats (Fig. 5d). In both groups, immunolabeling for GFAP decreased over the remaining time periods ($P < 0.05$), although there was a slight increase ($P < 0.05$) in the PBM group in comparison to the control group at 16 DPI.

T lymphocytes were detected in spinal cord tissue using UCHL1, an antibody against the surface glycoprotein CD45. Cells that were immuno-positive for UCHL1, were small, round, and found in very low numbers. T lympho-

Fig. 4. Functional analysis. **a**: Angle of rotation, (**b**) footfalls, and (**c**) ladder beam crossing time measurements are presented for pre-injury, and 1 and 9 weeks post-injury time points. Graph bars are mean percentage of pre-surgical measurements $\pm$ SEM. $^{*}P < 0.05$, repeated measures ANOVA with Newman–Keuls post-test between time points. $^{**}P < 0.05$, one way ANOVA with Tukey post-test between control and PBM group at 9 week time point. N = 10/group. **d**: Representative footprints from pre-injury and 9 weeks post-injury. Notice the increased angle of rotation at 9 weeks in the control group. In the PBM group, the angle returns to pre-surgical values.

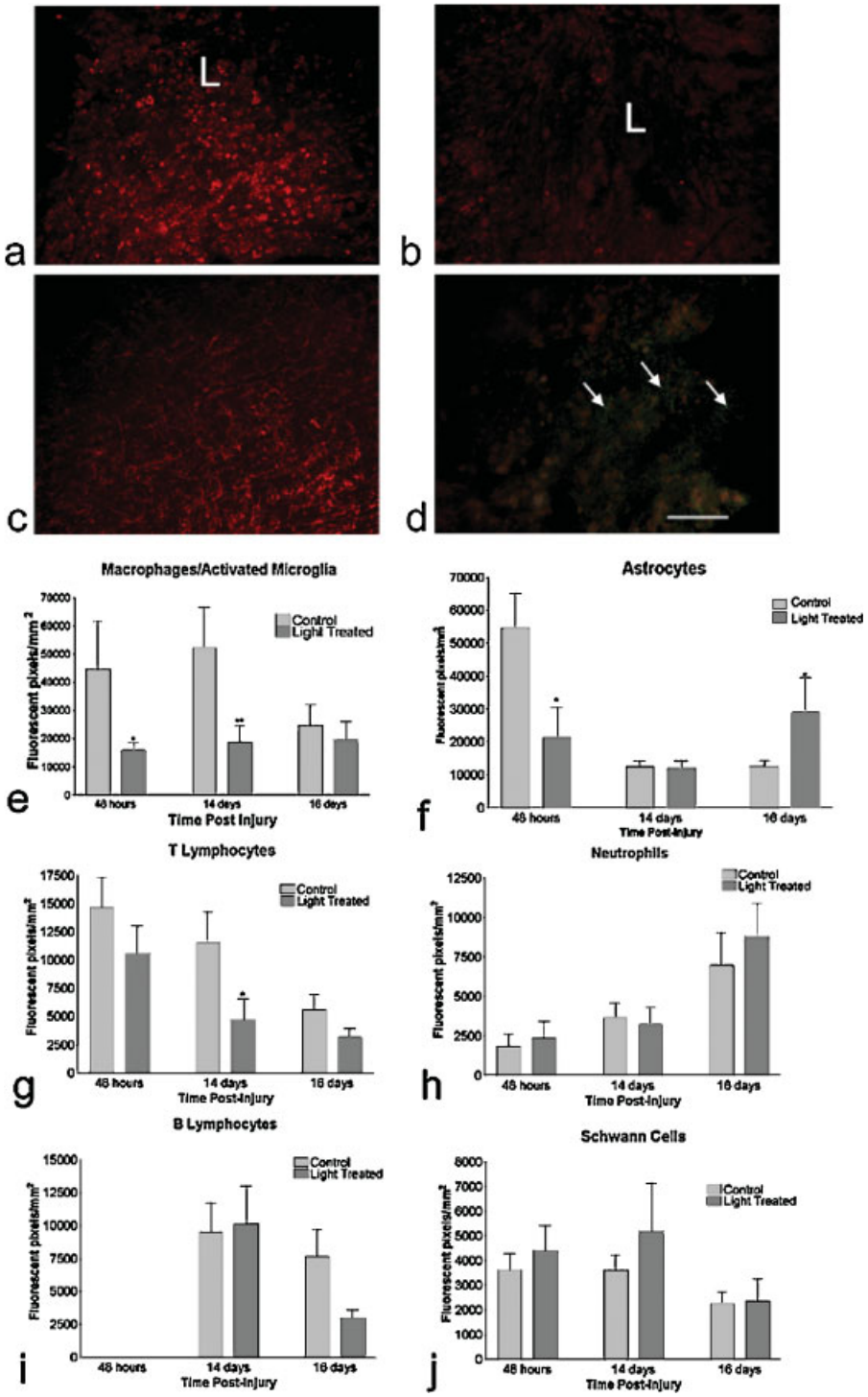


Fig. 5. Light suppresses cell invasion/activation. Immunolabeling for macrophages/activated microglia at 14 DPI in control (**a**) or PBM tissue (**b**), demonstrating cells in and around the lesion site. L indicates lesion site. **c**: Heavy GFAP labeling (Cy3, red) 5 mm caudal to the lesion site in control tissue at 48 hours post-injury. **d**: GFAP labeling (arrows, FITC, green) adjacent to the lesion site (*) in PBM tissue 48 hours post-injury. Quantitation for macrophage/activated microglia (**e**), astrocytes (**f**), T lymphocytes (**g**), neutrophils (**h**), B lymphocytes (**i**), and Schwann cells (**j**). $^{*}P < 0.05$, $^{**}P < 0.001$. $N = 5$/group. Graph bars represent mean ± SEM. Bar = 96 μm.

cytes were restricted to the lesion edge and in the acellular matrix within the lesion cavity. Statistical analysis of UCHL1 expression revealed that there was a peak in both the control and PBM groups at 48 hours post-injury, with a decline in expression through 16 DPI ($P < 0.05$ between 48 hour data and 16 DPI data, regardless of group, and between 14 day control data and 16 day data; Fig. 5g). UCHL1 expression in the PBM group was significantly decreased at 14 DPI ($P < 0.001$).

Three cell types investigated, neutrophils, B lymphocytes, and Schwann cells, were not significantly affected by PBM. Immunohistochemical labeling for neutrophils revealed small, round, cellular profiles that were detected bordering the lesion site or adjacent to the meninges at all time points investigated in both control and PBM groups. A non-significant increase in neutrophil immunolabeling was found at 16 DPI, which may be due to a reported suppression of neutrophil invasion and activity by sodium pentobarbital, the anesthetic used for all treatments from day 1 through 14 post-injury [72,73]. B lymphocytes, small, round cells near the edges of the spinal cord lesion or within the cavity, demonstrated 1–2 mm migration caudal to the lesion in the white matter tract at 16 DPI in the control group only. There was no migration observed in the PBM group. Also present in very low numbers were Schwann cells, identified by antibody labeling of S100, a neural specific Ca^{2+} binding protein. These small, circular cells were found at all time points investigated, primarily along the edges of the lesion, without any migration rostral or caudal to the lesion. No quantitative difference was found in the immunolabeling of these cell types between PBM and control tissue at any time point ($P > 0.05$; Fig. 5h,i,j).

To further clarify the effect of PBM on the injured spinal cord, RT-PCR was performed to quantify changes in expression of genes involved in the immune response. Analysis of gene expression at 6 hours and 4 DPI was performed and compared to expression of GAPDH, which demonstrated no significant difference between the control and PBM groups ($P = 0.6740$; Fig. 6a).

The expression of iNOS, transforming growth factor β (TGFβ), four pro-inflammatory cytokines (interleukin 1β (IL1β), tumor necrosis factor α (TNFα), interleukin 6 (IL6), and granulocyte-macrophage colony stimulating factor (GM-CSF)), and two chemokines (MIP1α and MCP-1) was assessed at 6 hours and 4 DPI. PBM resulted in a significant suppression ($P < 0.001$; Fig. 6a,b) of IL6 expression at 6 hours post-injury, with a 171-fold decrease in expression of IL6. PBM also resulted in a significant decrease in MCP-1 at this time point ($P < 0.01$; Fig. 6c), in which the control group had 66% greater expression of MCP-1. A fivefold suppression of iNOS transcription at 6 hours post-injury ($P < 0.01$; Fig. 6d) was found in the PBM group in comparison to the control group. By 4 DPI, transcription of IL6, MCP-1 and iNOS had significantly decreased in the control group ($P < 0.001$; Fig. 6b,c,d), while the expression in the PBM group remained depressed. There was no significant difference between control and PBM groups in expression of TNFα, IL1β, GM-CSF, MIP1α, and TGFβ at 6 hours post-injury or 4 DPI (data not shown).

DISCUSSION

Axons have the inherent ability to regrow following injury and altering the spinal cord environment may support this regeneration. The data from the current study demonstrates that 810 nm light, at a dosage of 1,589 J/cm^2, significantly improves axonal regrowth and functional improvement. Additionally, this study has shown that PBM, which penetrated to the depth of the spinal cord with 6% of the incident power, induced a statistically significant suppression of immune cell invasion and pro-inflammatory cytokine and chemokine gene expression.

The current study found a significant increase in mini-ruby labeled axons ($P < 0.01$) and double labeled (HM and Fast Blue; $P < 0.05$) neurons in the PBM group after CST lesion. The mini-ruby labeled axons quantified in this study were found only in the area of the dorsal funiculus normally occupied by the CST, suggesting axonal regeneration of the appropriate tract. Pettigrew and Crutcher [74] demonstrated that despite the inhibitory molecules present in white matter, neurite outgrowth is supported in directions parallel to white matter tracts and previous reports have demonstrated that treatment of the lesion site does allow for long distance regeneration of tracts [2,19,21,54].

Double labeled neurons, representing those whose axons were transected during the initial lesion and had regrown to vertebral level L3, were found only in the PBM group, with an average of 70.5 neurons counted, accounting for approximately 0.3% of all counted neurons. While this is a small percentage, it was found to be significantly greater than the control group ($P < 0.05$). This small percentage suggests a number of different interpretations, including that additional therapies in combination with light therapy or alteration of the applied light treatment parameters will increase axonal regeneration. However, it should be noted that the second tracer, fast blue, was injected into the gray matter of vertebral level L3, 24 mm caudal to the lesion, and was expected to label only those axons terminating in this area. Greater percentages of double-labeling have been reported following injection of the second tracer closer to the lesion site (within 6–7 mm distal to the lesion; [55,75]). The current study revealed that double labeled neurons accounted for approximately 30% of the number of mini-ruby labeled axons observed at 5 weeks post-lesion in the PBM group, further supporting the theory that a greater number of neurons had regenerated but were not counted with our labeling technique.

To date, no study has evaluated axonal regrowth of specific tracts using retrograde or anterograde tracing after PBM of SCI. A number of studies by Rochkind et al. [35,36] found that PBM at similar dosages in combination with transplantation increased axonal sprouting and axonal myelination within the graft, in comparison to transplantation alone. However, the source of these axons was not determined, nor were they found to extend beyond the graft.

Despite the small percentage of regeneration found in this study, studies have shown that functional improvement can be found with very small amounts of axonal regrowth [2,21,76,77]. This is supported by the current

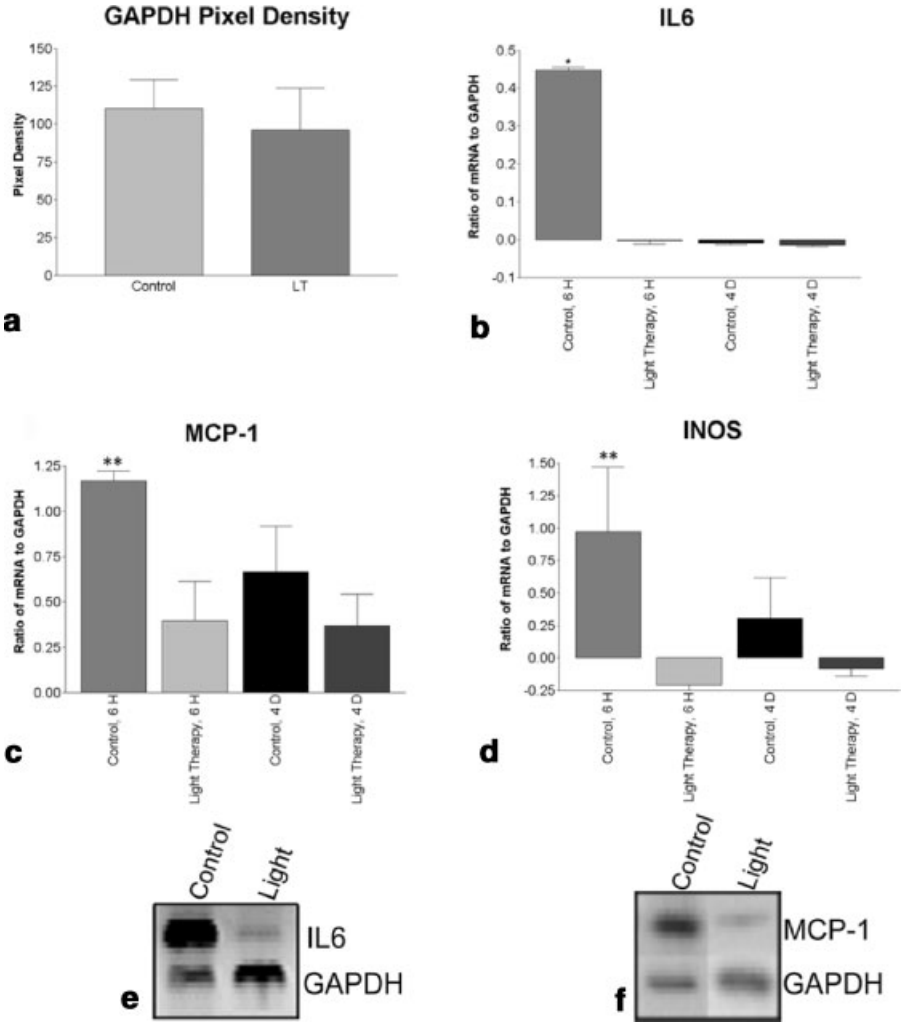


Fig. 6. Light suppresses gene expression. **a**: GAPDH expression; $P > 0.05$. **b**: IL6 expression at 6 hours and 4 days post-injury. **c**: MCP-1 expression at 6 hours and 4 days after SCI. **d**: iNOS expression at 6 hours and 4 days post-injury. $^{*}P < 0.001$; $^{**}P < 0.01$. Bars represent ratio of gene of interest to internal control mean ± SEM ($n = 5$/group/time point). Reverse color ethidium bromide–DNA complex fluorescence for IL6 (**e**) and MCP-1 (**f**) from the control and PBM groups, as well as their corresponding GAPDH band, at 6 hours post-injury.

study, in which functional recovery measurements, including angle of hindlimb rotation during locomotion and duration of time necessary to cross a ladder beam, were found to return to pre-injury values by at least 9 weeks post-injury following PBM. Both of these activities are associated with CST function and are significantly increased after CST lesion [78,79]. It was unexpected that angle of rotation data displayed a recovery to normal values at 1 week post-injury. It is possible that this early recovery is due to local sprouting, enhanced compensation or sparing of white matter induced by the PBM. Similar results have been observed with anti-inflammatory treatments or cell transplantations after injury, with an early return to normal values in BBB scores and inclined plane measurements [80,81]. While there was a significant increase in footfalls in both control and PBM animals post-surgery without a significant difference between these two groups ($P < 0.05$, ANOVA; Fig. 4b), ladder crossing time is positively correlated with hindlimb errors in step placement [79]. Analysis of errors in ladder crossing, including correct placement of hindpaws and grasping of ladder rungs, was not assessed and may have been modified by PBM, leading to this crossing time improvement.

Previous studies have also shown improvement in gross motor function after SCI and PBM [35,36]. These studies investigated non-specific recovery of function, such as weight bearance, step taking, improvements in BBB score, and electrophysiological measurement in the musculature of the hindlimbs, and found that PBM in combination with transplantation improved functional recovery.

Previous studies employing anti-inflammatory treatments have successfully improved axonal growth and return of function [1,21], and it is possible that the decrease in the inflammatory response is one reason for the recovery

observed after SCI and PBM. Although the immunohistochemical and RT-PCR findings do not confirm that PBM improves axonal regeneration and functional recovery because of its immunomodulatory actions, the results do provide a basis for this theory.

The current study determined that PBM significantly altered the invasion of a number of cell types that play a substantial role after SCI. Immunolabeling for macrophages/activated microglia, T lymphocytes, and astrocytes was significantly decreased post-injury; these cell types are involved in secondary damage to the spinal cord after injury [23]. This result expands upon Rochkind et al.'s [35,36] findings of decreased degeneration of peripheral and embryonic grafts and decreased scar formation, proposed to be due to suppression of the immune response after SCI.

Macrophages/activated microglia secrete cytotoxic proteolytic enzymes and free radicals [82] and induce the production of proteoglycans, which inhibit neurite growth [24,38]. It has been shown that reduction of the macrophage response with anti-inflammatory treatment after SCI improves function and regeneration [23,24].

Neutrophil invasion was not altered by PBM in this study. However, the invasion was greater at 16 DPI than earlier, which was a surprising finding and may have been due to suppression of neutrophil activity by sodium pentobarbital administration to both control and PBM rats from days 1 to 14 post-injury [73]. This effect is restricted to neutrophils alone and has not been shown to affect macrophage/microglia responses or other immune responses. As the data in our study for macrophage/activated microglia and pro-inflammatory gene expression in control rats is comparable to that of similar studies [42,46,62,83], we do not believe the anesthetic effect on neutrophil invasion significantly altered other aspects of this study.

However, the finding that PBM had no effect on neutrophil invasion is important to note. Several studies have found that methylprednisolone (MP), currently the only treatment available for acute SCI, fails to block neutrophil infiltration and activity after injury, while inhibiting macrophage invasion [1,84,85]. The mechanism of MP's actions is still under investigation, although several studies have found that this drug has numerous effects within the injured spinal cord. For example, administration of MP decreases the activation of NF-κB and the resultant expression of TNFα, which in turn diminishes the intensity and duration of the inflammatory response [86]. While PBM had no significant effect on TNFα mRNA production, a significant suppression of other downstream NF-κB genes that normally peak at 6–24 hours post-injury, such as IL6 and MCP-1 [42,49,52,62,87–89], was found. These genes are integrally involved in the immune response, and are suggested to play an important role in secondary injury and/or the lack of regeneration after SCI [89–94]. IL6, MCP-1 and iNOS are normally down-regulated beyond 24 hours post-injury, and PBM was not found to decrease their values beyond this point any further. Previous study has shown that interfering with the effects of these genes, through receptor antagonists or knockouts, decreases macrophage invasion and secondary injury [91,93].

Interestingly, IL1β, TNFα, and MIP1α, which have maximum expression at 3 hours post-injury or earlier, were not found to be altered by PBM at 6 hours post-injury. This finding suggests that either an effect of PBM on IL1β, TNFα and MIP1α was not detected by 6 hours post-injury or that PBM has a slow-acting effect within the spinal cord that takes several hours to become apparent.

The mechanism of how PBM affects gene transcription or any other cellular activity is currently unknown. Research into the transduction of light energy into cellular activity is ongoing and components of the electron transport chain (ETC) of mitochondria and a variety of enzymes are under consideration as possible photon acceptors. The presence of several maxima in the action spectra of cells suggests that more than one of these mechanisms may play a role in PBM [95,96]. Several researchers have suggested that components of the ETC of mitochondria are the primary photon acceptors [97–100] and it has been postulated that about 50% of near-infrared light is absorbed by chromophores within mitochondria, such as cytochrome c oxidase [101]. It has also been shown that near-infrared light reverses the inhibiting effect of tetrodotoxin on cytochrome c oxidase, restoring enzyme activity to control levels [102]. Additionally, light was found to induce changes in membrane permeability to calcium [103] and cellular oxidation state, potentially through light absorption by NADPH [104]. These PBM induced alterations can, potentially, lead to changes in cellular activity levels, which, in turn, leads to alterations in cellular processes including transcription and translation, cell proliferation and phagocytosis. These alterations have been demonstrated to be dosage dependent [33], with low dosages 0.001–10 J/cm^2 stimulating cellular activity while dosages greater than 10 J/cm^2 inhibit activity, as is the case in the current study. In this study, the dosage of 1,589 J/cm^2 is theorized to be inhibiting inflammatory cell activity, thus, altering the extracellular milieu and providing a potential mechanism for improved axonal regeneration through the lesion site. Unfortunately, a reason for this dose dependency is currently unknown.

Despite the lack of a defined mechanism, several significant changes have been shown after PBM of the injured spinal cord. These results demonstrate that PBM is a novel and non-invasive treatment for acute SCI that potentially acts through an immunomodulatory mechanism and suggest that light will be a useful treatment for humans.

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Bjordal:
Systemic Review – Tennis Elbow

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A systematic review with procedural assessments and meta-analysis of Low Level Laser Therapy in lateral elbow tendinopathy (tennis elbow)

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Abstract

Background: Recent reviews have indicated that low level level laser therapy (LLLT) is ineffective in lateral elbow tendinopathy (LET) without assessing validity of treatment procedures and doses or the influence of prior steroid injections.

Methods: Systematic review with meta-analysis, with primary outcome measures of pain relief and/or global improvement and subgroup analyses of methodological quality, wavelengths and treatment procedures.

Results: 18 randomised placebo-controlled trials (RCTs) were identified with 13 RCTs (730 patients) meeting the criteria for meta-analysis. 12 RCTs satisfied half or more of the methodological criteria. Publication bias was detected by Egger's graphical test, which showed a negative direction of bias. Ten of the trials included patients with poor prognosis caused by failed steroid injections or other treatment failures, or long symptom duration or severe baseline pain. The weighted mean difference (WMD) for pain relief was 10.2 mm [95% CI: 3.0 to 17.5] and the RR for global improvement was 1.36 [1.16 to 1.60]. Trials which targeted acupuncture points reported negative results, as did trials with wavelengths 820, 830 and 1064 nm. In a subgroup of five trials with 904 nm lasers and one trial with 632 nm wavelength where the lateral elbow tendon insertions were directly irradiated, WMD for pain relief was 17.2 mm [95% CI: 8.5 to 25.9] and 14.0 mm [95% CI: 7.4 to 20.6] respectively, while RR for global pain improvement was only reported for 904 nm at 1.53 [95% CI: 1.28 to 1.83]. LLLT doses in this subgroup ranged between 0.5 and 7.2 Joules. Secondary outcome measures of painfree grip strength, pain pressure threshold, sick leave and follow-up data from 3 to 8 weeks after the end of treatment, showed consistently

significant results in favour of the same LLLT subgroup ($p < 0.02$). No serious side-effects were reported.

Conclusion: LLLT administered with optimal doses of 904 nm and possibly 632 nm wavelengths directly to the lateral elbow tendon insertions, seem to offer short-term pain relief and less disability in LET, both alone and in conjunction with an exercise regimen. This finding contradicts the conclusions of previous reviews which failed to assess treatment procedures, wavelengths and optimal doses.

Background

Lateral elbow tendinopathy (LET) or "tennis elbow" is a common disorder with a prevalence of at least 1.7% [1], and occuring most often between the third and sixth decades of life. Physical strain may play a part in the development of LET, as the dominant arm is significantly more often affected than the non-dominant arm. The condition is largely self-limiting, and symptoms seem to resolve between 6 and 24 months in most patients [2].

A number of interventions have been suggested for LET. Steroid injections, non-steroidal anti-inflammatory drugs or a regimen of physiotherapy with various modalities, seem to be the most commonly applied treatments [3]. However, treatment effect sizes seem to be rather small, and recommendations have varied over the years. In several systematic reviews over the last decade [4,5], glucocorticoid steroid injections have been deemed effective, at least in the short-term. But in later well-designed trials evidence is found that intermediate and long-term effects of steroid injections groups yield consistently and significantly poorer outcomes than placebo injection groups, and physiotherapy or wait-and-see groups [6,7]. Nevertheless, steroid injections have been considered as the most thoroughly investigated intervention, with 13 randomized controlled trials comparing steroid injections to either placebo/local anaesthetic or another type of intervention [5]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been found to achieve smaller short-term effect sizes than steroid injections [8], and topical application seems to be the best medication administration route [8] For oral administration of NSAIDs for LET, evidence is inconclusive from two heterogeneous trials only [9]. The positive short-term results of anti-inflammatory therapies in LET appear to partly contradict the recent paradigm in tendinopathy research, where LET is thought to be mainly a degenerative disorder with minimal inflammation [10,11].

Exercise therapy and stretching exercises have been used either alone or in conjunction with manipulation techniques or physical interventions. Although the sparse evidence makes it difficult to assess the separate effect of active exercises or stretching [12], four studies have found that either exercises alone [13], or in conjunction with a physiotherapy package, are more effective than placebo ultrasound therapy or wait-and-see controls. Also exercise therapy, particularly eccentric exercises, have been found effective in the intermediate term in tendinopathies of the Achilles, patellar or shoulder tendons [14-17]. There is some evidence suggesting that joint manipulation or mobilisation techniques either of the wrist, elbow or cervical spine may contribute to short-term effects in LET [18-20].

Among the physical interventions, ultrasound therapy has been considered to offer a small benefit over placebo from two small trials [12], but a well-designed and more recent trial did not find significant effects of ultrasound therapy in LET [21]. Reviewers have arrived at different conclusions for the effect of acupuncture [22,23]. In reviews of physical interventions for LET, conclusions may vary between reviews because of differences in the treatment procedures. A good example of this is the negative conclusion of the LET review for extracorporeal shockwave therapy (ESWT) by Buchbinder et al. [24], where a later review with in-depth assessments of treatment intervention protocols [25], found that a subgroup of trials with proper treatment procedures and adequate timing of outcomes gave a positive result.

Low level laser therapy (LLLT) has been available for nearly three decades, and scattered positive results have been countered by numerous negative trial results. Several systematic reviews have found no significant effects from LLLT, in musculoskeletal disorders in general [26], and in LET in particular [12,23,27]. In this perspective it may seem futile to perform yet another systematic review in this area. But none of these reviews evaluated the results separately for the different LLLT treatment procedures, laser wavelengths or doses involved. Neither did they implement evidence of the newly discovered biomodulatory mechanisms which are involved when LLLT is applied. During the last 5–6 years the annual number of published LLLT reports in Medline has increased from 25 to around 200. We recently made a review of this literature, and concluded that LLLT has an anti-inflammatory effect in 21 out of 24 controlled laboratory trials, and a biostimulatory effect on collagen production in 31 out of 36 trials [28]. Both of these effects were dose-dependent

and could be induced by all wavelengths between 630 and 1064 nm with slight variations in therapeutic dose-ranges according to the wavelength used. The anti-inflammatory effect was seen in higher therapeutic dose-ranges than the biomodulatory effect on fibroblast cells and collagen fibre production. Diagnostic ultrasonography of tendinopathies has revealed that partial ruptures and tendon matrix degeneration are underdiagnosed if only physical examinations are made. Consequently, the stimulatory LLLT-effect on collagen fibre production should probably be beneficial for tendon repair. Another interesting feature was that LLLT with too high power densities or doses (above 100 mW/cm^2), seemed to inhibit fibroblast activity [29] and collagen fibre production [30]. Six years ago we showed in a systematic review of tendinopathy, that the effect of LLLT is dose-dependent [31]. At the time, the accompanying editoral suggested that the advanced review design could become the new standard for reviewing empirical therapies with unknown optimal doses and procedural differences [32]. Steroids induce a down-regulation of cortisol receptors, and we recently discovered that the cortisol antagonist mifepristone completely diminished the anti-inflammatory effect of LLLT [33]. All these recent findings from the LLLT literature, prompted the World Association for Laser Therapy (WALT) to publish dosage recommendations and standards for the conductance of systematic reviews and meta-analyses last year [34]. One of the issues that has lacked attention is the validity of LLLT-application procedures in tendinopathy. To our knowledge there are only three valid irradiation techniques for LLLT in tendinopathies: a) direct irradiation of the tendon, b) irradiation of trigger points and c) irradiation of acupuncture points.

In this perspective and as our previous tendinopathy review [31] is becoming outdated, there seems to be a need for a new in-depth review of the effects of LLLT in LET where possible confounders are analyzed and subgroup analyses are performed.

Methods

Literature search

A literature search was performed on Medline, Embase, Cinahl, PedRo and the Cochrane Controlled Trial Register as advised by Dickersin et al. [35] for randomised controlled clinical trials. Key words were: Low level laser therapy OR low intensity laser therapy OR low energy laser therapy OR phototherapy OR HeNe laser OR IR laser OR GaAlAs OR GaAs OR diode laser OR NdYag, AND tendonitis OR lateral epicondylitis OR lateral epicondylopathy OR tennis elbow OR elbow tendonitis OR lateral epicondylalgia OR extensor carpi radialis tendonitis. Handsearching was also performed in national physiotherapy and medical journals from Norway, Denmark, Sweden, Holland, England, Canada and Australia. Additional information was gathered from researchers in the field.

Inclusion criteria

The randomised controlled trials were subjected to the following seven inclusion criteria:

1) Diagnosis: Lateral elbow tendinopathy, operationalised as pain from the lateral elbow epicondyle upon finger or wrist extension

2) Treatment: LLLT with wavelengths in the range 632 – 1064 nm, irradiating either the tendon pathology, acupuncture points or trigger points

3) Design: Randomised parallel group design or crossover design

4) Blinding: Outcome assessors should be blinded

5) Control group: Placebo control groups or control groups receiving other non-laser interventions with at least 10 persons per group

6) Specific endpoints for pain intensity or global improvement of health measured within 1 – 52 weeks after inclusion.

Outcome measures

Primary outcome measures

measured after the end of treatment, either as:

a) pain intensity on a 100 mm visual analogue scale (VAS) defined as the pooled estimate of the difference in change between the means of the treatment and the placebo control groups, weighted by the inverse of the pooled standard deviation of change for each study, i.e. weighted mean difference (WMD) of change between groups. The variance was calculated from the trial data and given as 95% confidence intervals [95% CI] in mm on VAS, or

b) improved global health status. This was defined as any one of the following categories: "improved", "good", "better", "much improved", "pain-free", "excellent". The numbers of "improved" patients were then pooled to calculate the relative risk for change in health status. A statistical software package (Revman 4.2) was used for calculations.

Secondary outcome measures

c) painfree grip strength (dynamometer, vigorimeter)

d) pain pressure threshold (algometer)

e) sick leave (days)

f) follow-up results at more than 1 week after the end of treatment for pain intensity (WMD) and/or improved global health status (RR) as described for the primary outcome measures

Due to possibility of measurement by different scales, the results for outcomes c) and d) are defined as the unitless pooled estimate of the difference in change between the mean of the treatment and the placebo control groups, weighted by the inverse of the pooled standard deviation of change for each study, i.e. standardised mean difference (SMD) of change between groups. The variance are calculated from the trial data and given as 95% confidence intervals.

Analysis of bias, including methodological quality, funding source and patient selection

Positive bias direction, caused by flaws in trial methodology, funding source

Trials were subjected to methodological assessments by the 10 point Delphi/PedRo checklists [36]. as trials of weaker methodology have been found to exaggerate results in a positive direction [37]. As profit funding has been shown to affect trial conclusions in a positive direction [38], analysis of funding sources was also performed.

Negative bias direction, caused by poor prognosis or effective co-interventions

LET patients with long symptom duration and high baseline pain intensity are found to have significantly poorer prognosis in a trial with symptom durations of 8 to 21 weeks [2]. Recent steroid injections have been reported to negatively affect prognosis in LET over a period of 3–12 months after injections [6]. Patient selection of known responders only has been shown to inflate trial results with 38% [39], and consequently the inclusion of non-responders to treatments is likely to deflate effect sizes. Exercise therapy has been found effective in LET [13] and other tendinopathies [17], and the use of exercise therapy as a co-intervention may also deflate effect sizes or erase positive effects of LLLT. Consequently, we decided to analyze the included trials for presence of long symptom duration, treatment and treatment failures prior to inclusion, and effective co-interventions.

Results

Literature search results

The literature search identified 1299 potentially relevant articles that were assessed by their abstracts. 1119 abstracts were excluded as irrelevant, 180 full trial reports were evaluated, and 18 trials met the inclusion criterion for randomisation (Figure 1).

However a further three randomised trials had to be excluded for not meeting the *a priori* trial design criteria for sample size in control group, specific endpoints or blinding. The results of this assessment are summarised in Table 1.

Analysis of treatment procedures

The remaining 15 trials were then evaluated for adequacy of their treatment procedures for active laser and placebo laser for adherence to either of the three valid application techniques (inclusion criterion 2). This resulted in the exclusion of 2 trials (Table 2, Figure 2).

Publication bias

The five excluded RCTs [40-44] were taken into the publication bias analysis by a graphical plot as advised by Egger [45]. Four [40-42,44] out of the five excluded trials with grave methodological and procedural flaws, were small and reported negative results. Three trials with negative results for LLLT were performed by the same research group [40,46,47] although this group also reported a positive outcome [50]. Three of these trials met the eligibility criteria for this review and were included in the meta-analysis [46,47,50]. The five largest trials [43,48-51] all presented positive results, although Simunovic et al. [43] was excluded from our meta-analyses for variable timing of endpoints as stated above. Significant asymmetry was noted in the funnel plot, indicating a considerable degree of negative publication bias (Figure 3).

Bias analysis of 13 included trials

Positive bias detection – poor methodological quality and for-profit funding sources

The final study sample consisted of 730 patients in 13 trials. The mean and median methodological score was 6.5, and only one trial did not satisfy half or more methodological criteria [52]. Two trials used the acupoints application technique [46,47], while the remaining eleven trials used the tendon application technique. None of the trials stated funding from laser manufacturing companies or had authors with affiliations to laser manufacturers. The trial characteristics and the sum methodological scores are listed in Table 3.

Subgroup analysis for methodological quality

The pre-planned subgroup analysis by methodological quality was not performed as all but a single low quality trial were rated fairly similarly with 6–8 criteria fulfilled out of 10 possible criteria. Minor inter-observer differences have been reported for methodological scorings by the Pedro criteria list [36], and the variance could be within the range of measurement error for this methodological criteria list [53]. In addition, fulfilment of more than 50% of methodological criteria is often considered as a threshold for acceptable quality [54], and all but one trial with negative results were assessed with scores above this threshold. Consequently, we considered a separate

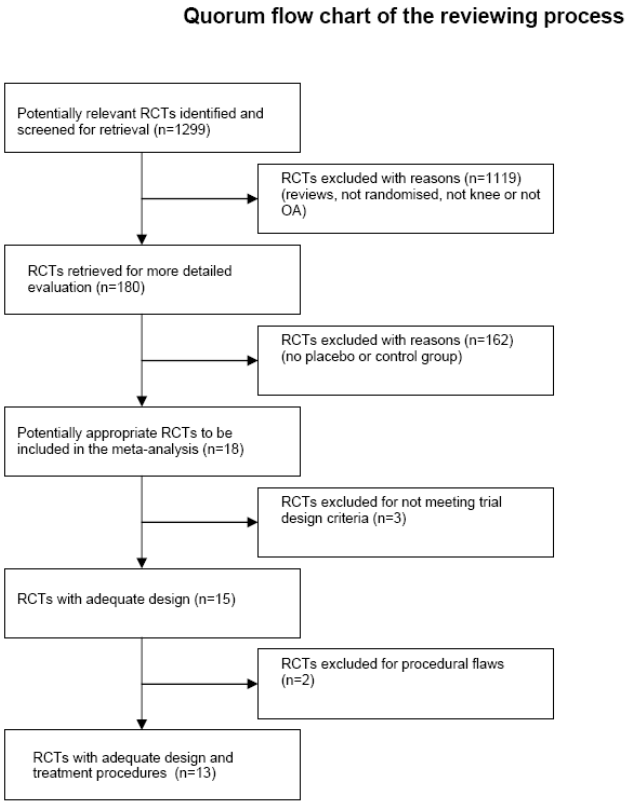

Figure 1
Quorum flow chart. Quorum flow chart of the steps in the reviewing process.

subgroup analysis by methodological quality to be unnecessary to perform.

Negative bias detection – inclusion of patients with poor prognostic factors and effective co-interventions

Three trials reported details confirming enrolment of patients without poor prognosis [48,55,56]. In two of these trials [55,56], both active and placebo groups received concurrent exercise therapy, which may have deflated effect size. Seven trials reported demographic data affirmative on the inclusion of LET patients with poor prognosis, which are likely to deflate effect sizes. Results for possible confounding factors which may deflate effect sizes are summarized in Table S4, Additional file 1.

Assessment of LLLT procedures and treatment variables

There was considerable heterogeneity in the treatment procedures and LLLT doses used in the included trials. Treatment characteristics for the 11 trials which used direct irradiation of tendon pathology are listed in Table S5, Additional file 1.

Treatment characteristics for trials which used acupoint irradiation are listed in Table S6, Additional file 1.

Outcomes and effect sizes

Dichotomized trial results

Eight out of thirteen trials (62%) reported one or more outcome measures in favour of LLLT over placebo. Eleven trials used the tendon application technique, and eight (73%) of these trials reported positive results for one or more outcome measures (Table 3). All seven trials using 904 nm wavelength and the tendon application technique yielded positive results [48-51,55-57], whereas three trials using lasers with 820/30 nm [58,52]and 1064 nm [59] wavelengths found no significant effect of LLLT. A single trial administering LLLT with a wavelength of 632 nm [60], also found significantly better results for the LLLT group. In the two trials where LLLT was administered to acupuncture points [46,47], no significant differences between LLLT and placebo were found for any of the outcome measures.

Meta-analyses of effects

Primary outcomes

Continuous data for pain relief was available from 10 trials in a way which made statistical pooling possible. At the first observation after the end of the treatment period, LLLT was significantly better than controls with a WMD of 10. 2 mm [95% CI: 3.0 to 17.5] in favour of LLLT on a 100 mm VAS (p = 0.005). In a subgroup of five trials [48,50,55-57] where 904 nm LLLT was administered directly to the tendon, LLLT reduced pain by 17.2 mm [95% CI: 8.5 to 25.9] more than placebo (p = 0.0001). One trial [60] with 632 nm LLLT, showed significantly better results for LLLT than a wrist brace and ultrasound therapy, but none of the results from trials with wavelengths of 820 nm or 1064 nm, or acupoint application technique were significantly different from placebo. The results are summarized in Figure 4.

Seven trials [46,49-51,55,57,58] presented data in a way which allowed us to pool data for global improvement. LLLT was significantly better than placebo with an overall relative risk for improvement at 1.36 [95% CI: 1.16 to 1.60] (p = 0.002). In a subgroup of five trials [49-51,55,57] where 904 nm LLLT was used to irradiate the symptomatic tendon, the relative risk for global improvement was significantly better than placebo at 1.53 [95% CI 1.28 to 1.83] (p < 0.0001). In the remaining two trials [46,58] where LLLT was administered to acupoints or with 820 nm wavelength, the relative risk for global improvement was not significantly different from placebo at 0.80

Table 1: Randomised LLLT-trials excluded for not meeting trial design criteria for diagnosis, blinding or specific endpoints.

Study by first author	Year	Method score	Laser wavelength	Application technique	Result	Reason for exclusion
Mulcahy [40]	1995	5	904	Not stated	No significant differences between active and placebo LLLT	Does not satisfy control group criterion: Lacks sufficient patient numbers in placebo control group as only 3 patients had tendinopathy
Simunovic [41]	1998	3	830	Tendon + Trigger Points	LLLT significantly better than placebo	Does not satisfy criterion for specific endpoint and standard number of treatments: Only bilateral conditions were given placebo treatment, but data for this group were not presented
Vasseljen [42]	1992	5	904	Tendon	Traditional physiotherapy significantly better than LLLT	Does not satisfy blinding criterion: Neither therapist, patients or observers were blinded in the traditional physiotherapy group

Trial characteristics by first author, method score, laser wavelength in nanometer, laser application technique, trial results and reason for exclusion.

[95% CI 0.50 to 1.22]. The results are summarized in Figure 5.

Secondary outcomes

Painfree grip strength showed significantly better results after LLLT than placebo with SMDs of 0.66 [95% CI: 0.42 to 0.90] [$p < 0.0001$). When trials were subgrouped by application technique and wavelengths, only trials with irradiation of tendons and wavelengths 632 nm [60] or 904 nm [48,49,56,57], showed positive results versus control with SMDs at 1.09 [95% CI: 0.42 to 1.76] and 1.30 [95% CI: 0.91 to 1.68], respectively. The results are summarized in Figure 6.

Two trials with 904 nm wavelength using application technique with tendon irradiation [50,56] reported a small, but significantly elevated pain pressure threshold with SMD at 0.34 [95% CI: 0.04 to 0.63] ($p = 0.02$), The results are summarized in Figure 7.

Sick leave

One trial with 904 nm LLLT administered directly over the tendon insertion, presented sick leave data [51]. The relative risk for not being sicklisted after treatment was significantly in favour of LLLT at 2.25 [95% CI: 1.25 to 4.06] ($p = 0.0005$).

Follow-up

Six of the trials provided continuous follow-up data on a 100 mm VAS measured between 3 and 8 weeks after the end of treatment [47,48,56,57,59,60]. The combined WMD was 11.30 mm [95% CI: 7.5 to 16.1] in favour of LLLT. For global improvement, three trials [46,51,57] provided data suitable for statistical pooling, and the RR was calculated to 1.68 [95% CI: 1.32 to 2.13] in favour of LLLT. Subgroup analyses showed that three trials [48,56,57] administering 904 nm LLLT directly over the tendon, WMD improved to 14.3 [95% CI: 7.3 to 21.3] and RR for improvement to 2.01 [95%CI: 1.48 to 2.73] in favour of LLLT, while a single trial [60] with 632 nm wavelength and the same application procedure reported WMD of 14.0 [95%CI: 7.0 to 20.6]. The results are summarized in Figures 8 and 9.

Only two trials using the tendon application technique with 904 nm wavelengths reported follow-up results beyond 8 weeks. They reported persisting significant improvement after LLLT for PFS at 3 months (SMD 0.40 [95%CI: 0.05 to 0.75]) [49], and significantly less patients with no or minor pain at work at 5.5 months (RR = 2.1 [95%CI: 1 to 4.3]) [57], respectively. Other outcomes were not significantly different beyond 8 weeks. For the two trials using acupoint irradiation [46,47], no significant differences were found at any of the follow-up sessions.

Side-effects and compliance

Treatment was generally well tolerated and no adverse events were reported. Compliance was high ranging from 100% to 91% in all but two trials [48,58]. One of these trials [48] had a considerably longer treatment period (8 weeks) than the other trials (median 3 weeks), and all withdrawals were caused by lack of effects. In another trial [58] using 830 nm wavelength, an exceptionally high

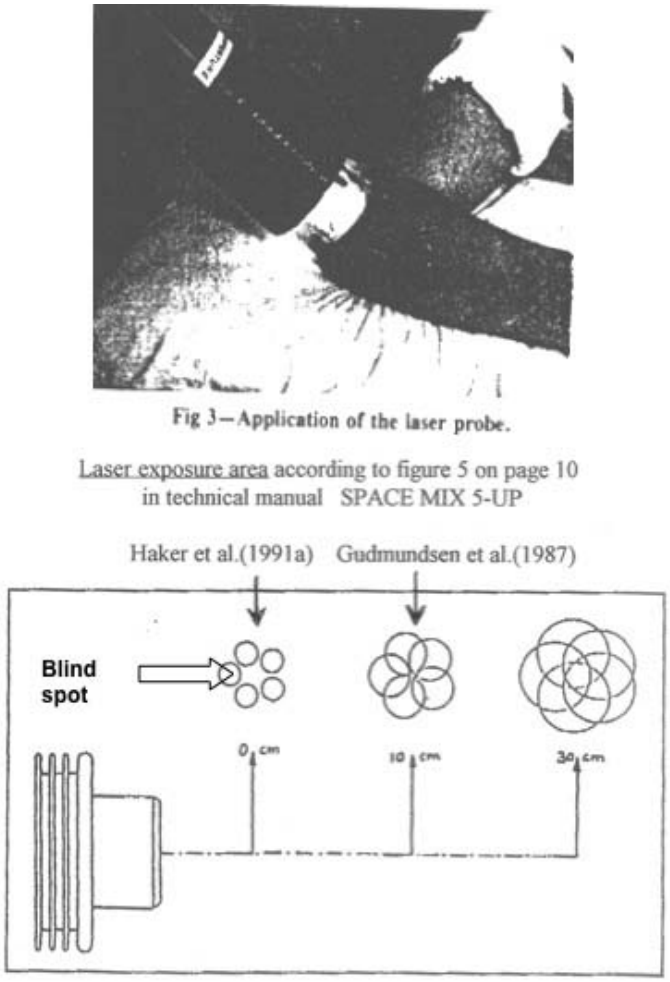

Figure 2
Photograph showing laser therapy procedure with laser head in skin contact in trial by Haker et al. The photograph is taken the trial report in from Archives of Physical Medicine 1991. The drawing of the laser spot sizes at different distances is taken from the manual of Space Mix 5 Mid-Laser (Space s.r.l, Italy).

withdrawal/dropout rate of 15% occurred after a single treatment session without any given reason.

Discussion

In this review, we found that most RCTs of LLLT for LET were of acceptable methodological quality. This finding is in line with previous reviews [12,23,27], although there were some differences between reviewers in methodological scores for individual trials. RCTs of LLLT are of similar methodological quality and include similar sample sizes as RCTS included in recent reviews of corticosteroid injections [5] and topical or oral NSAIDs [8]. Two of the previous reviews of LLLT for LET found only six RCTs [12,23], whereas an earlier review found ten RCTs [27], and excluded one RCT for methodological shortcomings [43]. We used broader searching criteria in our review and had no language restrictions. This resulted in 18 potentially eligible RCTs. We excluded one RCT for not meeting the inclusion criteria of specific endpoints [43] and another two RCTs for complete lack of blinding [44] and a lack of an LET control group [42]. None of the previous LET reviews assessed the LLLT regimen for procedural errors, while our procedural assessments resulted in exclusion of another two RCTs with grave procedural errors, such as leaving the tendon insertion and acupoints unirradiated [40] and giving adequate LLLT to the placebo group [61]. These exclusions resulted in 13 RCTs being eligible for our review which is twice the number of RCTs included in two of the previously published reviews[12,23].

Previous LET-reviews of LLLT [12,23,27] and pharmacological interventions like NSAID [8] or corticosteroid injections [5] have not assessed possible bias from for-profit funding sources or publication bias. Our analysis

Table 2: Randomised LLLT-trials excluded for not meeting criteria of valid procedures for active laser and placebo laser treatment.

Study by first author	Method score	Wave-length	Application technique	Result	Reason for exclusion
Haker [43]	6	904	Tendon	No significant differences	Photograph in trial report shows that the laser probe was kept in skin contact and thereby violated the manufacturers' recommendation of a keeping the laser head at a distance of 10 cm. This violation caused a central blind spot of ca 3 cm^2 which left the tendon pathology unexposed to LLLT (See Figure 2)
Siebert [44]	6	904 + 632	Tendon	No significant differences	Active laser treatment to the placebo group received red 632 nm LLLT, which we calculated to be (2.25J), which again is an adequate LLLT dose. Consequently this trials lacks a placebo or non-laser control group

Trial characteristics given by first author, method score, laser wavelength, laser application technique, trial results and reason for exclusion.

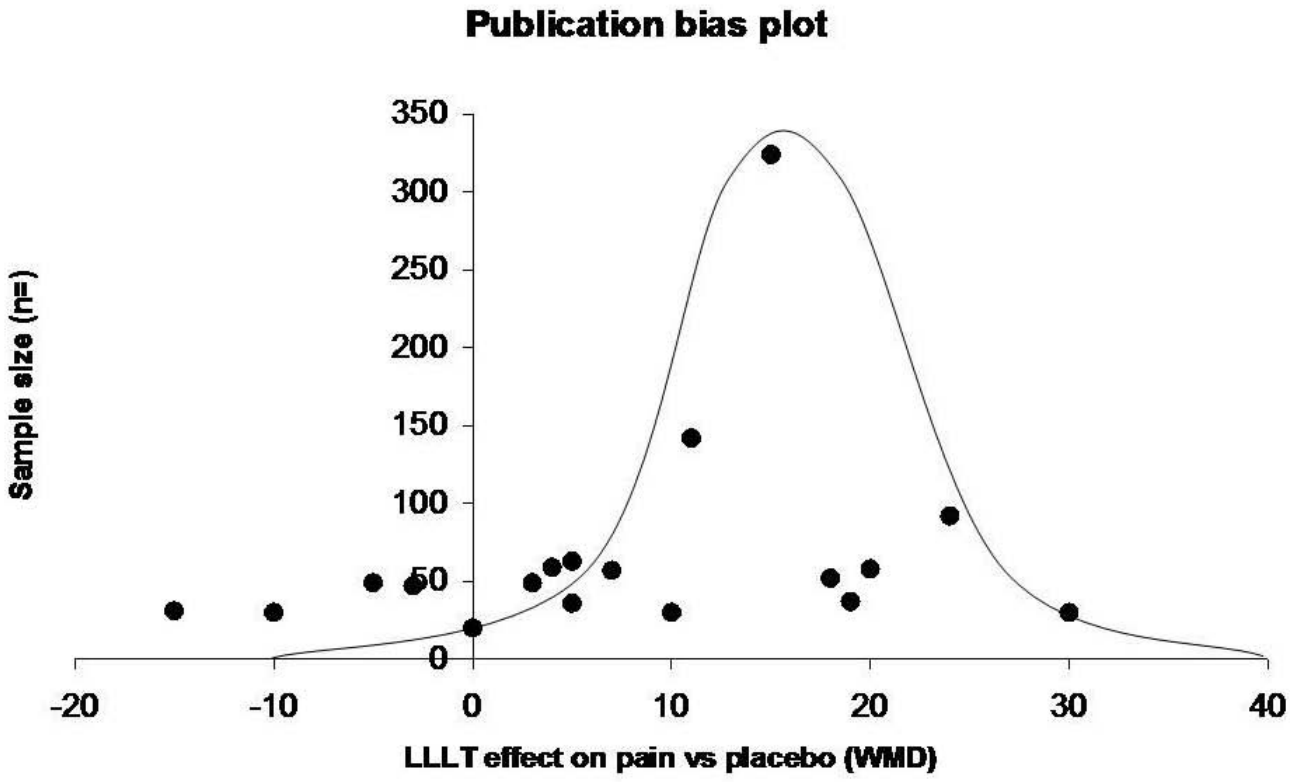

Figure 3
Funnel plot of published trial results given by WMD for pain relief over placebo measured on 100 mm VAS (x-axis), and sample size (y-axis).

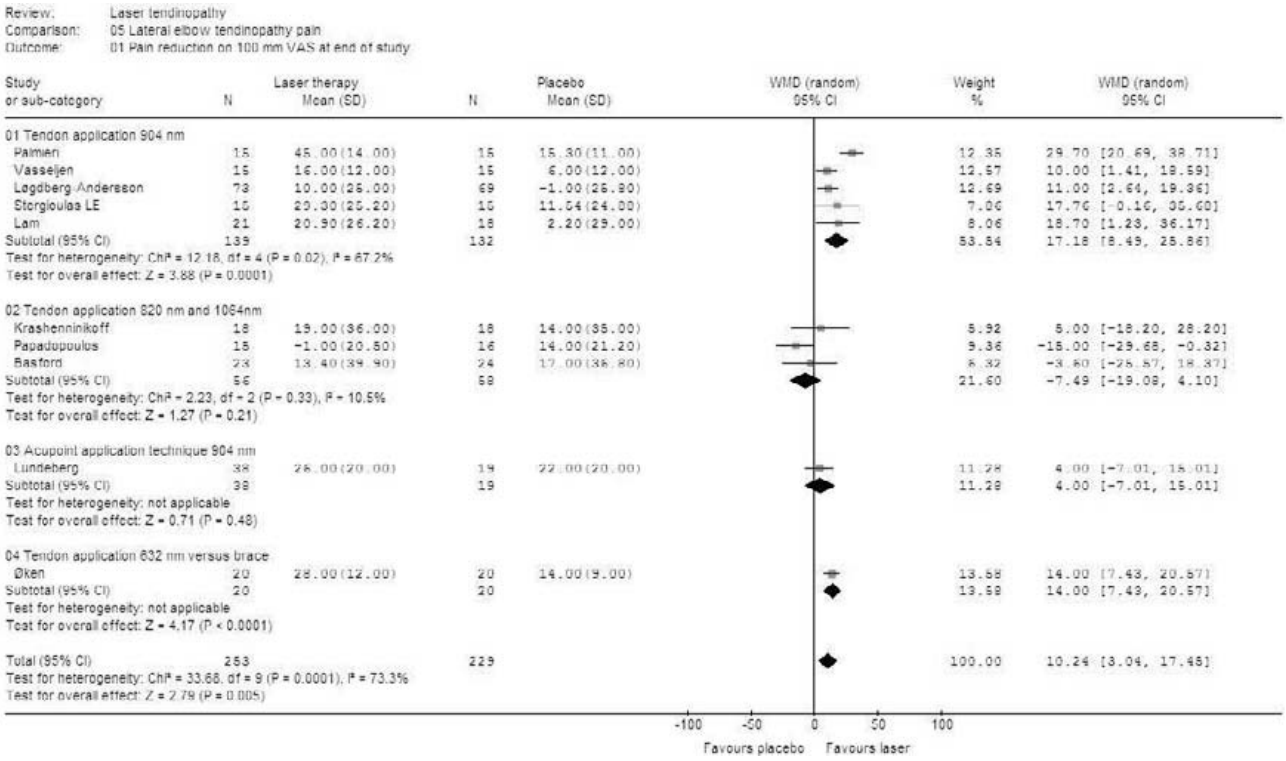

Figure 4

End of treatment results for LLLT measured as the WMD pain reduction on 100 mm VAS. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

Table 3: Included randomised LLLT-trials.

Study by first author	Method score	Patient numbers	Application technique	Control	Trial results
Basford [53]	8	47	Tendon	Placebo	0
Gudmundsen [51]	6	92	Tendon	Placebo	++
Haker [46]	7	49	Acupoints	Placebo	0
Haker [50]	6	58	Tendon	Placebo	+
Krashenninikoff [54]	6	36	Tendon	Placebo	0
Lam [55]	7	37	Tendon	Placebo	++
Løgdberg-Anderson [49]	7	142	Tendon	Placebo	++
Lundeberg [47]	6	57	Acupoints	Placebo	0
Oken [56]	7	59	Tendon	UL, Brace	++
Palmieri [57]	6	30	Tendon	Placebo	++
Papadoupolos [52]	4	31	Tendon	Placebo	-
Stergioulas [48]	7	62	Tendon	Placebo	++
Vasseljen [58]	8	30	Tendon	Placebo	+
Total	6.5(Mean)	730			

Trial characteristics by first author, method score, laser application technique, control group type, trial results. The abbreviations used are determined by the following categories: (-) means a result in favour of the control group, (0) means a non-significant result, (+) means a positive result for LLLT in at least one outcome measure, and (++) means a consistent positive results for more than one outcome measure.

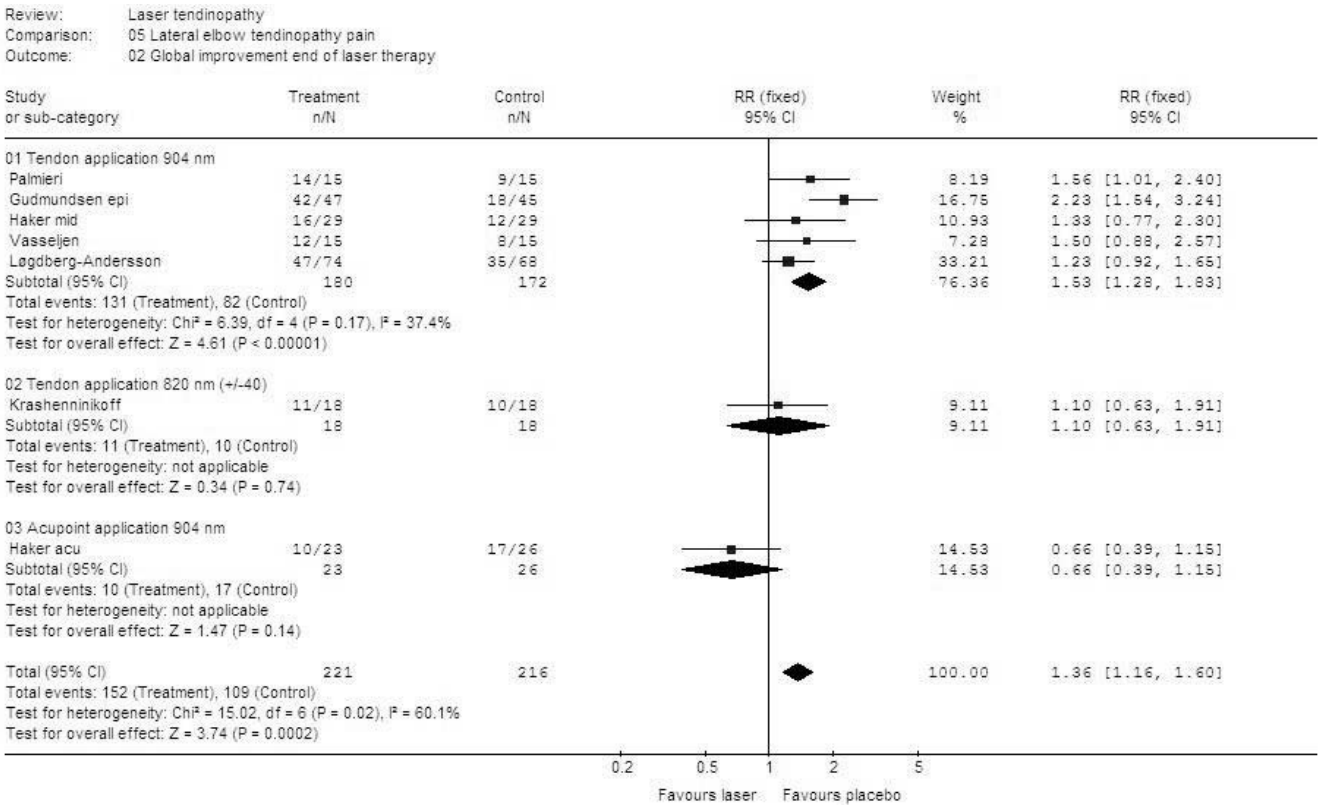

Figure 5

End of treatment results for LLLT measured as global improvement. Trials are subgrouped by application technique and wavelengths, and their combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

revealed that bias from for-profit funding was largely absent in the available LLLT material and that trials were performed by independent research groups receiving funding from internal sources or non-profit organisations. This feature of the LLLT literature is definitely different from pharmacological pain treatments where up to 83% of trials may be industry-funded [62]. A second feature of the LLLT-literature is that publication bias seems to go in a negative direction. This is distinctly different from the drug trials [63,64] where positive results have been found to account for up to 85% of the published trials in single journals [63], although this bias seems to be lesser or absent in high impact journals [64]. Our review suggests that LLLT trials reporting negative results are more likely to be published than trials with positive results. To our knowledge we are the first to demonstrate such bias, but such negative publishing bias is probably not unique to LLLT, and it may also be present for other electrophysical agents including TENS and acupuncture. We were surprised to see how large well-designed positive trials of LLLT [51,50] were published in unlisted journals or journals with low-impact factor, and how small negative trials [46], often with grave methodological [42] or procedural flaws [40] were published in higher ranking journals. This may reflect a predominance of RCTs designed using drug-research methodology paradigms without due consideration given to adequacy of the technique used in delivering LLLT, leading to under dosing and negative outcome bias [65]. In addition, it has been that documented drug sponsorship of research activities may influence guideline panels, journal editors and referees [66,67] leading to negative views on non-drug treatments such as LLLT as reflected in editorials in pain journals [68] and national medical journals [69].

Despite these concerns, we believe that the positive overall results of this review need to interpreted with some caution. They arise from a subgroup of 7 out of the 13 included trials [48-51,55-57]. These 7 trials had a narrowly defined LLLT regimen where lasers of 904 nm wavelength with low output (5–50 mW) were used to irradiate the tendon insertion at the lateral elbow using 2–6 points or an area of 5 cm^2 and doses of 0.25–1.2 Joules per point/area. The positive results for this subgroup of trials were

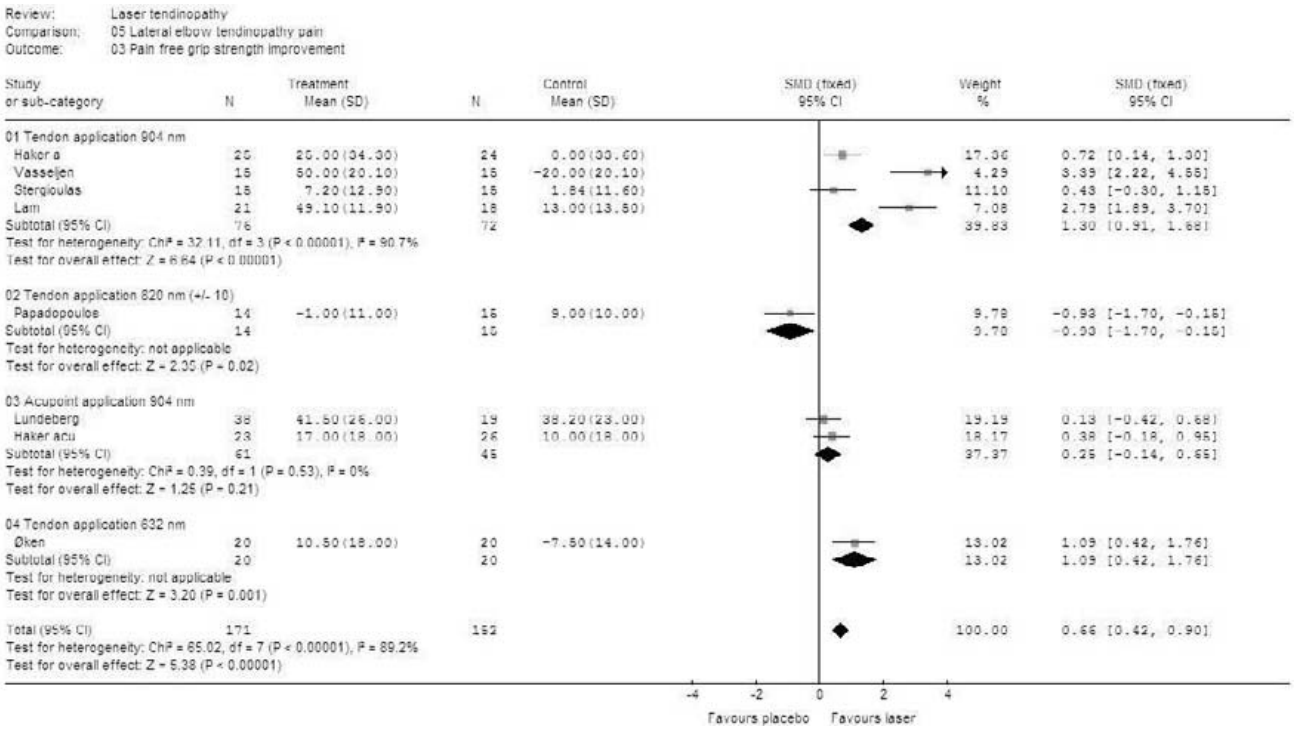

Figure 6

End of treatment results for LLLT measured as the SMD for pain-free grip strength. Trials are subgrouped by application technique and wavelengths, and their combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

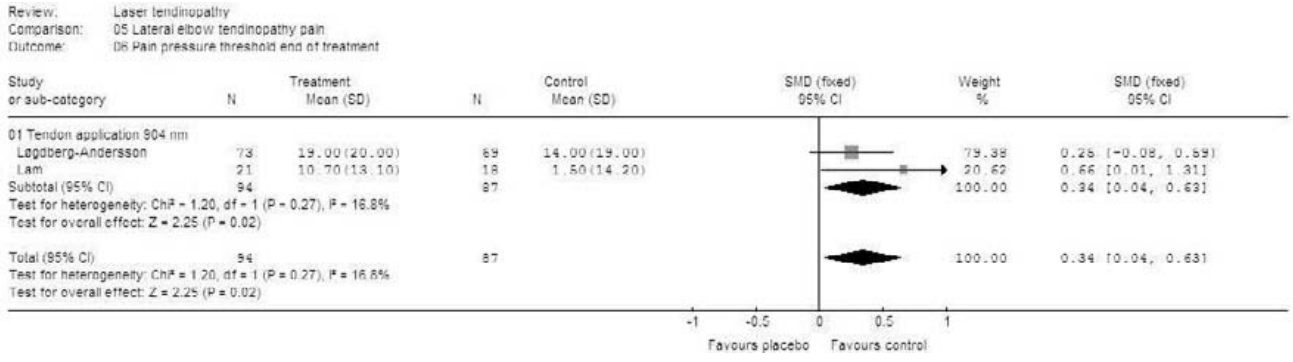

Figure 7

End of treatment results for LLLT measured as the SMD for pain pressure threshold. Only trials using the tendon application technique and 904 nm wavelength were available, and their combined results are shown as the total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

consistent across outcomes of pain and function, and significance persisted for at least 3–8 weeks after the end of treatment, in spite of several factors which may have deflated effect sizes.

For the red 632 nm wavelength which has a poorer skin penetration ability [70], a single trial [60] with a higher dose (6 Joules) seemed to be equally effective as the lower doses of 904 nm used in the seven positive trials. These

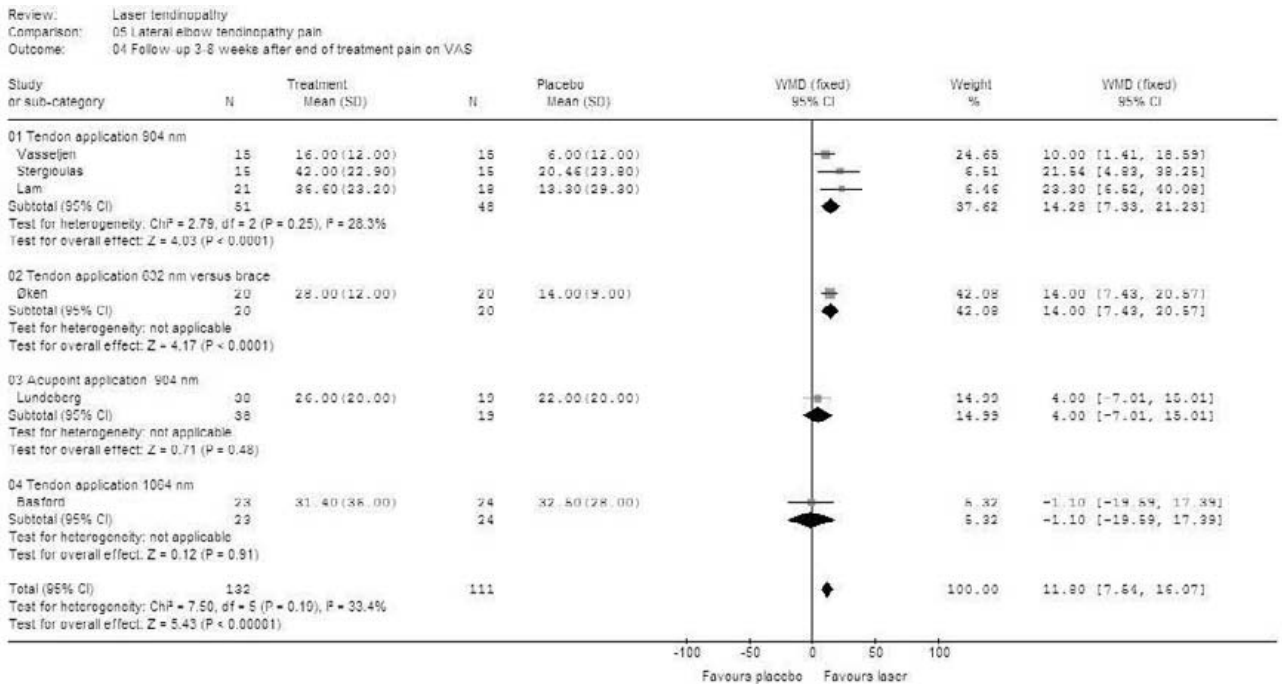

Figure 8

Follow-up results at 3–8 weeks after end of treatment for LLLT measured as the WMD for pain reduction on 100 mm VAS. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

LLLT-doses are well within the therapeutic windows for reducing inflammation, increasing fibroblast activity and collagen fibre synthesis, and the dosage recommendations suggested by WALT [71].

The negative results for the 830 nm GaAlAs and 1064 nm NdYag lasers can be attributed to several factors such as too high doses, too high power density or the inclusion of patients with poor prognosis from long symptom duration and prior steroid injections. These wavelengths have previously been found effective in some tendon animal studies and in other locations such as shoulder tendinopathies [72,73]. At this time it is not possible to draw firm conclusions about the clinical suitability of wavelengths 820, 830 and 1064 nm in LET treatment, but the lack of evidence of effects indicates that they cannot be recommended as LET treatment before new research findings have established their possible effectiveness. The lack of effect for these lasers may also serve as a reminder that higher doses is not always best. We have been witnessing a tendency where newly developed lasers with these wavelengths are being marketed with ever-increasing power and power densities. This may be inappropriate because current knowledge about LLLT mechanisms and dose-response patterns at higher powers is inconsistent or lacking.

The positive results for combining LLLT of 904 nm wavelength with an exercise regimen, are encouraging. We would have thought that exercise therapy could have erased possible positive effects of LLLT, but the results showed an added value in terms of a more rapid recovery when LLLT was used in conjunction with an exercise regimen. This may indicate that exercise therapy can be more effective when inflammation is kept under control. Adding LLLT to regimens with eccentric and stretching exercises reduced recovery time by 4 and 8 weeks in two trials [48,56]. For this reason, LLLT should be considered as an adjunct, not an alternative, to exercise therapy and stretching.

Based on the above findings, LLLT should be considered as an alternative therapy to commonly used pharmacological agents in LET management. Cochrane-based reviews of NSAIDs [8] and corticosteroid injections [5] have found evidence of short-term effects within 4 and 6 weeks, respectively. The short-term reduction in pain intensity after corticosteroid injections may appear to have a more

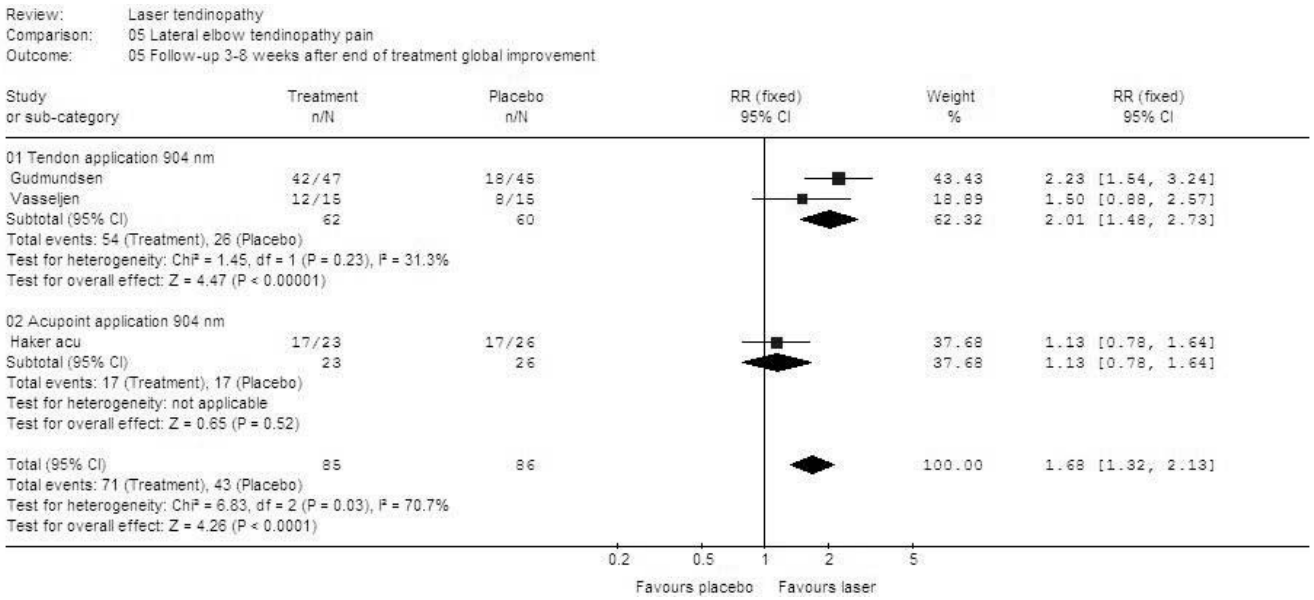

Figure 9

Follow-up results at 3–8 weeks after the end of treatment measured as the relative risk for global improvement for LLLT compared to placebo. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

rapid onset and may also be larger in effect size than after LLLT. But on the other hand, the available LLLT-material is confounded by factors capable of deflating effect sizes. In this perspective, there is a need for more high quality trials with head-to-head comparison of short-term effects between LLLT and corticosteroid injections. In the longer term, NSAIDs seems to be ineffective and corticosteroid injections seem to be harmful both at 26 and at 52 weeks [6]. For LLLT there are some significant long-term effects found at 8, 12 and 24 weeks after the end of treatment.

Conclusion

The available material suggests that LLLT is safe and effective, and that LLLT acts in a dose-dependent manner by biological mechanisms which modulate both tendon inflammation and tendon repair processes. With the recent discovery that long-term prognosis is significantly worse for corticosteroid injections than placebo in LET, LLLT irradiation with 904 nm wavelength aimed at the tendon insertion at the lateral elbow is emerging as a safe and effective alternative to corticosteroid injections and NSAIDs. LLLT also seems to work well when added to exercise and stretching regimens. There is a need for future trials to compare adjunctive pain treatments such as LLLT with commonly used pharmacological agents.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JMB had the original idea, which was developed through lengthy discussions with contributions from RABL-M, JJ, CC, AEL, AS and MIJ. The literature search, including handsearching, was performed by all members of the author team. The first draft was written by JMB, RABL-M and JJ, and revised by AS and MIJ. Methodological assessments of trials were performed by JMB, AEL, CC, AS. The statistical analysis was performed by JMB, RABL-M, JJ and MIJ. The final linguistic revision was performed by MIJ and all members of the author team read and commented on the manuscript before submission.

Additional material

Additional file 1

Tables 4-6.

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Chow:
Management of Neck Pain

Efficacy of low-level laser therapy in the management of neck pain: a systematic review and meta-analysis of randomised placebo or active-treatment controlled trials

Roberta T Chow, Mark I Johnson, Rodrigo A B Lopes-Martins, Jan M Bjordal

Summary

Background **Neck pain is a common and costly condition for which pharmacological management has limited evidence of efficacy and side-effects. Low-level laser therapy (LLLT) is a relatively uncommon, non-invasive treatment for neck pain, in which non-thermal laser irradiation is applied to sites of pain. We did a systematic review and meta-analysis of randomised controlled trials to assess the efficacy of LLLT in neck pain.**

Methods **We searched computerised databases comparing efficacy of LLLT using any wavelength with placebo or with active control in acute or chronic neck pain. Effect size for the primary outcome, pain intensity, was defined as a pooled estimate of mean difference in change in mm on 100 mm visual analogue scale.**

Findings **We identified 16 randomised controlled trials including a total of 820 patients. In acute neck pain, results of two trials showed a relative risk (RR) of 1·69 (95% CI 1·22–2·33) for pain improvement of LLLT versus placebo. Five trials of chronic neck pain reporting categorical data showed an RR for pain improvement of 4·05 (2·74–5·98) of LLLT. Patients in 11 trials reporting changes in visual analogue scale had pain intensity reduced by 19·86 mm (10·04–29·68). Seven trials provided follow-up data for 1–22 weeks after completion of treatment, with short-term pain relief persisting in the medium term with a reduction of 22·07 mm (17·42–26·72). Side-effects from LLLT were mild and not different from those of placebo.**

Interpretation **We show that LLLT reduces pain immediately after treatment in acute neck pain and up to 22 weeks after completion of treatment in patients with chronic neck pain.**

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Introduction

Chronic pain is predicted to reach epidemic proportions in developed countries with ageing populations in the next 30 years.[1] Chronic neck pain is a highly prevalent condition, affecting 10–24% of the population.[2–5] Economic costs of this condition are estimated at hundreds of millions of dollars,[2] creating an imperative for evidence-based, cost-effective treatments. Low-level laser therapy (LLLT) uses laser to aid tissue repair,[6] relieve pain,[7] and stimulate acupuncture points.[8] Laser is light that is generated by high-intensity electrical stimulation of a medium, which can be a gas, liquid, crystal, dye, or semiconductor.[9] The light produced consists of coherent beams of single wavelengths in the visible to infrared spectrum, which can be emitted in a continuous wave or pulsed mode. Surgical applications of laser ablate tissue by intense heat and are different from LLLT, which uses light energy to modulate cell and tissue physiology to achieve therapeutic benefit without a macroscopic thermal effect (sometimes termed cold laser). LLLT is non-invasive, painless, and can be easily administered in primary-care settings. Incidence of adverse effects is low and similar to that of placebo, with no reports of serious events.[10,11]

Research into the use of LLLT for pain reduction[12,13] and tissue repair[14,15] spans more than 30 years. However, reports do not identify this therapy as a potential treatment option,[16] possibly because of scepticism about its mechanism of action and effectiveness.[17] Research from the past decade suggests that LLLT produces anti-inflammatory effects,[18–21] contributing to pain relief. Cochrane reviews of the efficacy of LLLT in low-back pain[22] and rheumatoid arthritis[23] have been unable to make firm conclusions because of insufficient data or conflicting findings. However, effectiveness depends on factors such as wavelength, site, duration, and dose of LLLT treatment. Adequate dose and appropriate procedural technique are rarely considered in systematic reviews of electrophysical agents. Research into the dose-response profile of LLLT suggests that different wavelengths have specific penetration abilities through human skin.[17,24,25] Thus, clinical effects could vary with depth of target tissue. We have shown the importance of accounting for dose and technique in systematic reviews of transcutaneous electrical nerve stimulation[26] and LLLT,[11,21] and our approach is an acknowledged means of establishing efficacy.[27]

The only systematic review focusing solely on LLLT in treatment of neck pain included four randomised controlled trials, and concluded that there was evidence of short-term benefit of LLLT at infrared wavelengths of 780, 810–830, and 904 nm.[28] A Cochrane review of physical medicine for mechanical neck disorders, since

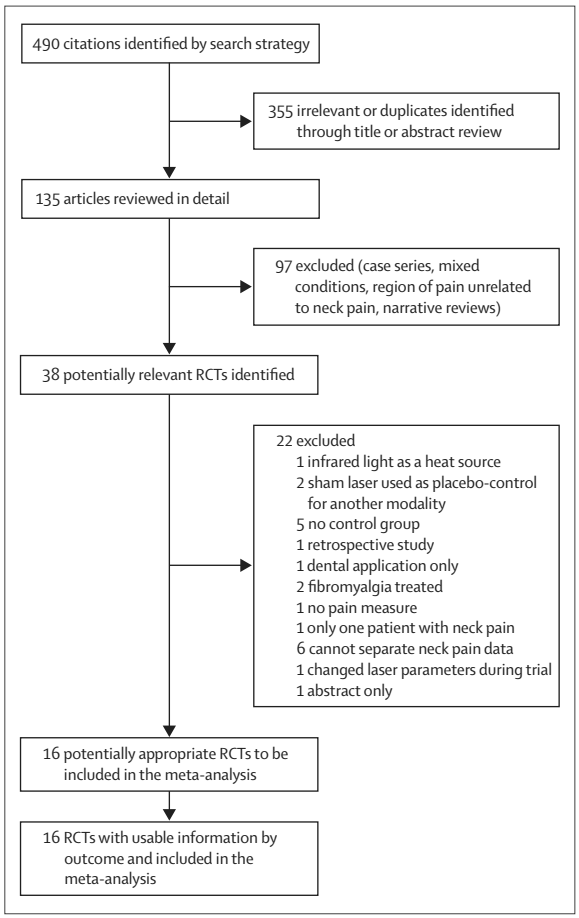

Figure 1: **Selection process**
RCT=randomised controlled trial.

withdrawn because much time had passed without an update, included three LLLT trials, for which outcomes did not differ from those of placebo.[29] The same investigators did a meta-analysis[30] of 88 randomised controlled trials of conservative treatments for acute, subacute, and chronic mechanical neck disorders, which included eight trials using LLLT. They concluded that LLLT has intermediate and long-term benefits.

These reviews did not identify treatment variables associated with positive outcomes, include non-English language publications, or quantitatively assess data.[28,30] We have therefore undertaken a new systematic review and meta-analysis of LLLT in neck pain to establish whether LLLT relieves acute and chronic neck pain and to systematically assess parameters of laser therapy to identify treatment protocols and dose ranges (therapeutic windows) associated with positive outcomes.

Methods

Search strategy and selection criteria

We did a search of published work without language restriction using Medline (January, 1966, to July, 2008), Embase (January, 1980, to July, 2008), Cinahl (January, 1982, to July, 2008), the Physiotherapy Evidence Database (January, 1929, to July, 2008), Biosis (January, 1926, to July, 2008), Allied and Complementary Medicine (January, 1985, to July, 2008), and the Cochrane Central Register of Controlled Trials (second quarter of 2008). Keywords used for neck pain and related conditions were: "neck pain/strain", "cervical pain/strain/syndrome", "cervical spondylosis/itis", "cervicobrachial (pain/disorder/syndrome)", "myofascial (pain/disorder/syndrome)", "trigger points", "fibromyalgia", "whiplash/WAD", "osteoarthritis/arthritis", and "zygaphophyseal/ZG joints". We combined these keywords with synonyms for LLLT: "low-level/low-energy/low reactive-level/low-intensity/low-incident/low-output/infrared/diode/semiconductor/soft or cold or mid/visible"; "laser therapy", "(ir)radiation", "treatment"; "low-energy photon therapy"; "low output laser"; "LLLT"; "LILT"; "LEPT"; "LELT"; "LILI"; "LELI"; "LPLI"; "biostimulation"; "photobio/stimulation/activation/modulation"; "light therapy"; "phototherapy"; "narrow band light therapy"; "904 nm"; "830 nm"; "632 nm"; "1064 nm"; "GaAs"; "GaAlAs"; "HeNe"; and "defocused CO_2". We consulted experts and searched reference lists of retrieved reports and textbooks for additional references.

Citations were screened and full reports of potentially relevant studies obtained. We applied inclusion and exclusion criteria, assessed methodological criteria, and extracted data including trial characteristics, demographic data, laser parameters, pain outcome measures, and co-interventions. Non-English language studies were translated by JMB.

We included randomised or quasi-randomised controlled trials of LLLT for acute or chronic neck pain as defined by trial investigators, and identified by various clinical descriptors included under the term non-specific neck pain.[31] These diagnostic labels included neck strain, neck sprain, mechanical neck disorders, whiplash, neck disorders, and neck and shoulder pain. We also used surrogate terms for neck pain, such as myofascial pain and trigger points.[32,33] Study participants were restricted to those aged 16 years and older. We excluded studies in which specific pathological changes could be identified, such as systemic inflammatory conditions—eg, rheumatoid arthritis, localised or generalised fibromyalgia, neck pain with radiculopathy, and neck pain related to neurological disease. We excluded abstracts and studies for which outcome measures for neck pain could not be separated from data for other regions of the body. Two reviewers (RTC, JMB) independently undertook the search of published work, screened studies, and extracted data. Any disagreements between reviewers were resolved by consensus with other team members acting as arbiters (RABL-M, MIJ).

Investigators had to have used a laser device that delivered irradiation to points in the neck identified by tenderness, local acupuncture points, or on a grid at predetermined points overlying the neck. Control groups had to have been given either placebo laser in which an

	n	Design	Diagnosis	Jadad score	Control	Sites treated	Cointerventions	Primary pain outcome measure
Ceccherelli et al (1989)[43]	27	DB RCT	Cervical myofascial pain	3	Placebo	Tender points in neck and distal acupuncture points	NR	VAS
Flöter et al (1990)[45]	60	DB, RCT	Cervical osteoarthritis	3	Placebo	Tender points in neck	NR	VAS
Taverna et al (1990)[52]	40	DB, RCT	Chronic myofascial pain	3	Placebo	Tender points in neck	NR	Graded subjective assessment: no change to optimum
Toya et al (1994)[53]	39	DB, RCT	Cervical pain complex	5	Placebo	Site not specified	No physical or medical therapy allowed	Graded subjective assessment: exacerbation to excellent
Soriano et al (1996)[39]	71	DB, RCT	Acute cervical pain	3	Placebo	Site not specified	No NSAIDs or other medical or physical therapy allowed	Graded subjective assessment: exacerbation to excellent
Laakso et al (1997)[49]	41	DB, RCT	Neck pain with trigger points in neck	3	Placebo	Three most painful trigger points	Simple analgesic drugs allowed as needed; NSAIDs, corticosteroids, tricyclic antidepressants excluded; no physical therapies	VAS
Özdemir et al (2001)[50]	60	DB, RCT	Neck pain related to neck osteoarthritis	3	Placebo	Six arbitrary points over neck muscles	NR	VAS
Seidel and Uhlemann (2002)[51]	48	DB, RCT	Chronic cervical syndrome	3	Placebo	Local neck points and distal acupuncture points	Acupuncture not allowed less than 6 months before inclusion; drug therapy unchanged during trial	VAS
Hakgüder et al (2003)[47]	62	DB, RCT	Neck pain with one trigger point	3	Exercise with LLLT and exercise alone	One active trigger point in levator scapulae or trapezius	NR	VAS
Chow et al (2004)[42]	20	DB, RCT	Neck pain (non-specific)	5	Placebo	Multiple tender points in cervical spine and attachments	Simple analgesic drugs allowed; no physical therapies	VAS
Gur et al (2004)[46]	60	DB, RCT	Chronic myofascial pain in the neck	5	Placebo	Up to ten trigger points	NR	VAS
Ilbuldu et al (2004)[48]	40	DB, RCT	Myofascial pain syndrome	2	Placebo and needling	Trigger points in upper trapezius	Simple analgesic drugs as needed; exercise to all groups	VAS
Altan et al (2005)[41]	53	DB, RCT	Cervical myofascial pain syndrome	3	Placebo	Three trigger points bilaterally and one trigger point in trapezius	No NSAIDs or analgesic drugs; exercise in both groups	VAS and graded assessment
Aigner et al (2006)[40]	45	SB, RCT	Acute whiplash injury	0	Placebo	Local and distal acupuncture points	Both groups wore cervical collar; paracetamol and chlormezanone	Assessment of subjective pain symptoms
Chow et al (2006)[13]	90	DB, RCT	Non-specific neck pain	5	Placebo	Local tender points	Simple analgesic drugs allowed; no physical therapies	VAS
Dundar et al (2007)[44]	64	DB, RCT	Cervical myofascial pain syndrome	3	Placebo	Three trigger points bilaterally	No NSAIDs or analgesic drugs	VAS

n=number of patients. DB=double blind. RCT=randomised controlled trial. NR=not reported. VAS=visual analogue scale. NSAIDs=non-steroidal anti-inflammatory drugs. SB=single blind.

Table 1: **Study design and outcome measures**

identical laser device had an active operating panel with the laser emission deactivated or an active treatment control (eg, exercise). We also included trials in which an active control was used as a co-intervention in placebo and real laser groups.

To be eligible for inclusion, a study had to compare pain relief along a 0–100 mm visual analogue scale, a numerical rating scale, or by patient-reported improvement (eg, categorical report of no change to complete relief of pain) as a primary outcome measure before and after laser therapy. Functional measures of disability (eg, neck pain disability questionnaire) were assessed as secondary outcome measures. We also examined adverse events where reported, although did not specify these a priori. Duration of follow-up was assessed and defined as short term (<1 month), medium-term (1–6 months), and long term (>6 months).

Assessment of methodological quality and heterogeneity

Reviewers assessed all studies for methodological quality on the basis of Jadad criteria (maximum score 5).[34] Jadad criteria allocate a point each for randomisation, double-blind design, and description of dropouts. If randomisation and double-blind concealment are assured, an additional 2 points are added. If randomisation or double-blind concealment is not assured, a point is deducted for each. A trial with a score of 3 or more is regarded as high quality. Data from trials with scores of 3 or more were grouped and analysed separately from those scoring less than 3.

We assessed clinical heterogeneity by considering population difference in age, sex, duration of symptoms, and outcomes. Clinical judgment was used to establish whether trials were sufficiently similar to allow pooling

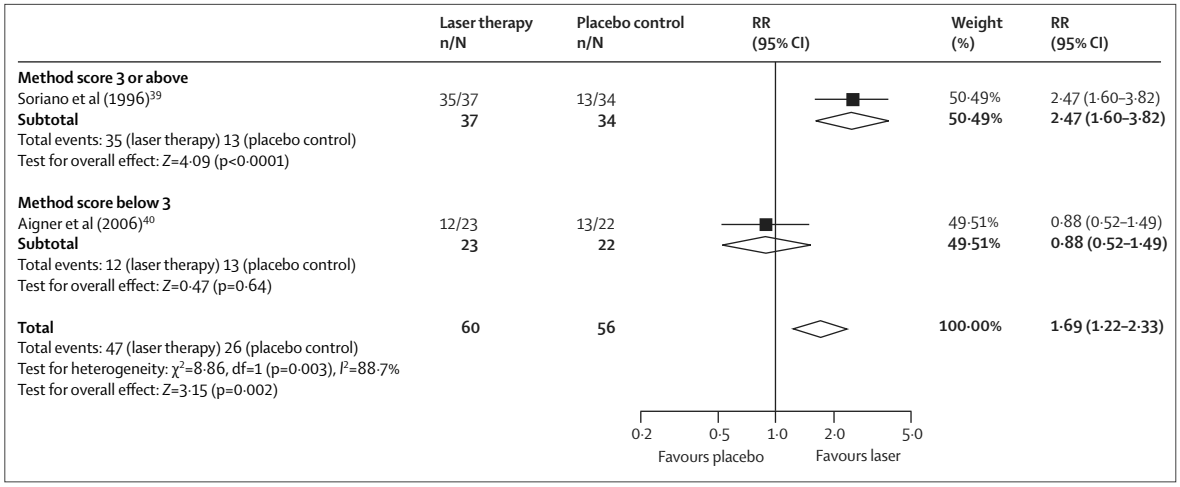

Figure 2: **Relative risk of improvement in acute neck pain in laser-treated versus control groups in two randomised trials reporting categorical data**
RR=relative risk.

of data. The specific parameters of laser devices, application techniques, and treatment protocols were extracted and tabulated by laser wavelength. Details for power output, duration of laser irradiation, number of points irradiated, and frequency and number of treatments were listed. When specific details were not reported, calculations were made from those described in the report when possible. When crucial parameters were not reported, we contacted manufacturers of laser devices and trial investigators to obtain missing information. Not all data were available because of the time elapsed since publication of some studies. Heterogeneity was qualitatively assessed for these factors by an expert in laser therapy (JMB).

We used five levels of evidence to describe whether treatment was beneficial: strong evidence (consistent findings in several high-quality randomised controlled trials); moderate evidence (findings from one high-quality randomised controlled trial or consistent findings in several low-quality trials); limited evidence (one low-quality randomised trial); unclear evidence (inconsistent or contradictory results in several randomised trials); and no evidence (no studies identified).[35]

Statistical analysis

Effect size for the primary outcome, pain intensity, was defined as a pooled estimate of the mean difference in change in mm on a 100 mm visual analogue scale between the mean of the treatment and the placebo groups, weighted by the inverse of the SD for every study—ie, weighted mean difference of change between groups. Variance was calculated from the trial data and given, with 95% CI, in mm on visual analogue scale. For categorical data, reported pain relief was dichotomised into two categories (improvement or no improvement), and we calculated relative risk (RR) of improvement, with 95% CI. For the secondary outcome, disability, effect size was defined as the standardised mean difference, which was a combined outcome measure without units—ie, the standardised mean difference in change between active laser groups and placebo groups for all included trials, weighted by the inverse of the variance for each study.[36]

Mean differences of change for laser-treated and control groups and their respective SDs were included in the statistical pooling. If variance data were not reported as SDs, they were calculated from the trial data of sample size and other variance data values such as p values, *t* values, SE, or 95% CI. Results were presented as weighted mean difference between laser-treated and control with 95% CI in mm on visual analogue scale—ie, as a pooled estimate of the mean difference in change between the laser-treated and control groups, weighted by the inverse of the variance for each study.[37] Statistical heterogeneity was assessed for significance ($p<0\cdot05$) with Revman 4.2, and χ^2 and *F* values greater than 50%. For categorical data, we calculated combined RRs for improvement, with 95% CI. A fixed effect model was used unless statistical heterogeneity was significant ($p<0\cdot05$), after which a random effects model was used. Publication bias was assessed by graphical plot.[38] Revman 4.2 was used for statistical analysis and Microsoft Excel 2003 (version 11) to plot dose-response curves.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

We identified 16 randomised controlled trials of a possible 38 that were suitable for inclusion, and that included 820 patients (figure 1). Two trials[39,40] provided data for laser therapy of acute neck pain, one treating acute whiplash-associated disorders and one treating acute neck pain of no defined cause. The other 14 trials reported response of chronic non-specific neck pain without radiculopathy to

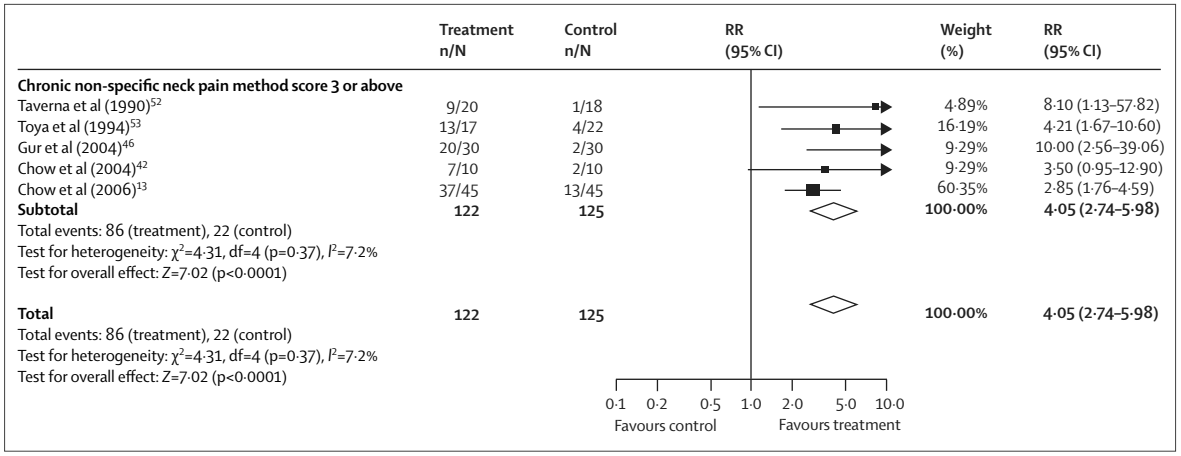

Figure 3: **Relative risk of global improvement in laser-treated versus control groups in five trials reporting categorical data for improvement in chronic pain**
RR=relative risk.

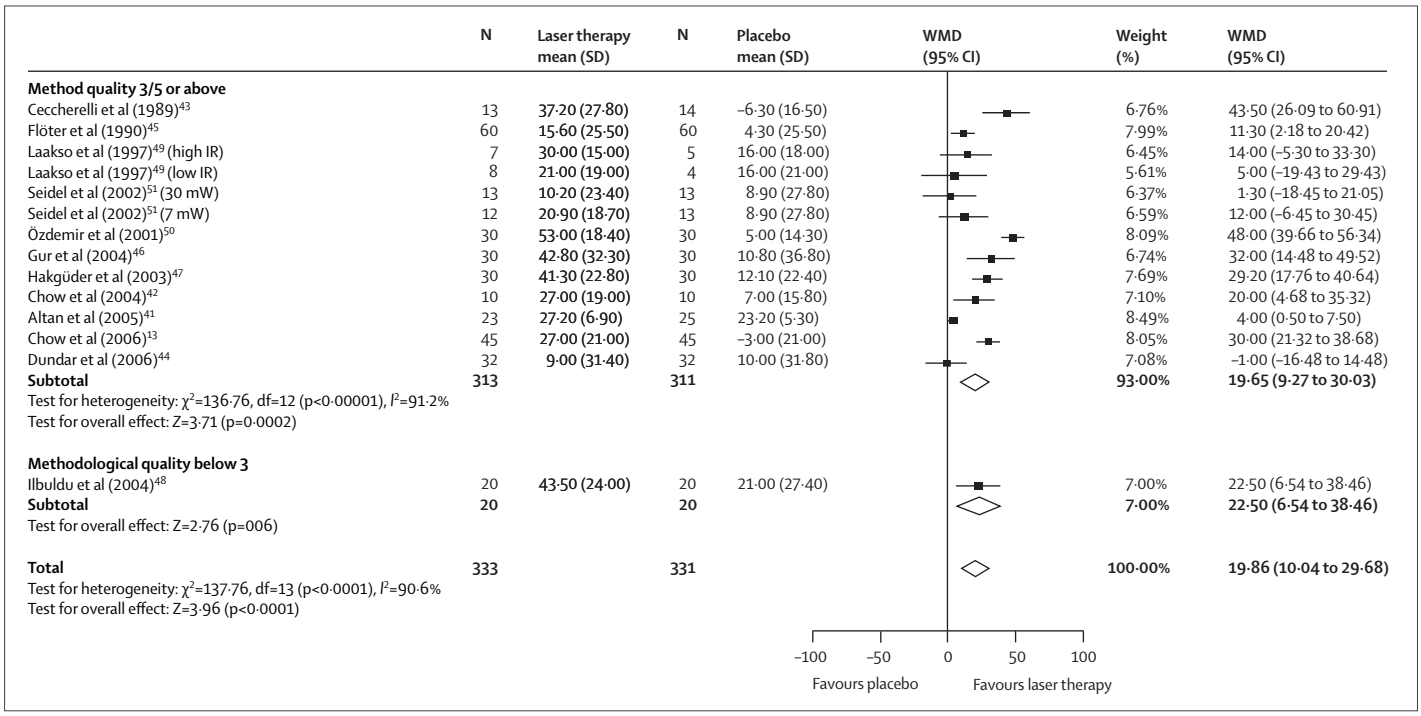

Figure 4: **Weighted mean difference in chronic pain reduction on 100 mm visual analogue scale between laser-treated and placebo-treated groups from 11 randomised trials grouped according to Jadad criteria**
WMD= weighted mean difference. IR=infrared.

laser therapy.[13,41–53] Of the studies included, 648 (79%) of the sample of patients with chronic neck pain were women, and patients had a mean age of 43 years (SD 9·8), mean symptom duration of 90 months (SD 36·9), and mean baseline pain of 56·9 mm (SD 7·5) on a 100 mm visual analogue scale in any trial. Co-interventions were inconsistently reported (table 1). Ten trials reported co-interventions, and six studies did not report or limit co-interventions. Of the studies reporting co-interventions, five groups of investigators explicitly excluded use of concurrent physical therapies, and four excluded use of non-steroidal anti-inflammatory drugs. Four studies allowed use of simple analgesic drugs as needed. Methodological quality assessment values for the trials by Jadad scoring ranged from 0 to 5 (table 1).

Analysis of categorical data for immediate before and after LLLT effects showed that LLLT groups in the two trials[39,40] of acute neck pain had a significant RR of 1·69 (95% CI 1·22–2·33) for improvement immediately after treatment versus placebo (figure 2). Methodological quality varied between these two studies. Five trials of chronic neck pain reported categorical data, and all were

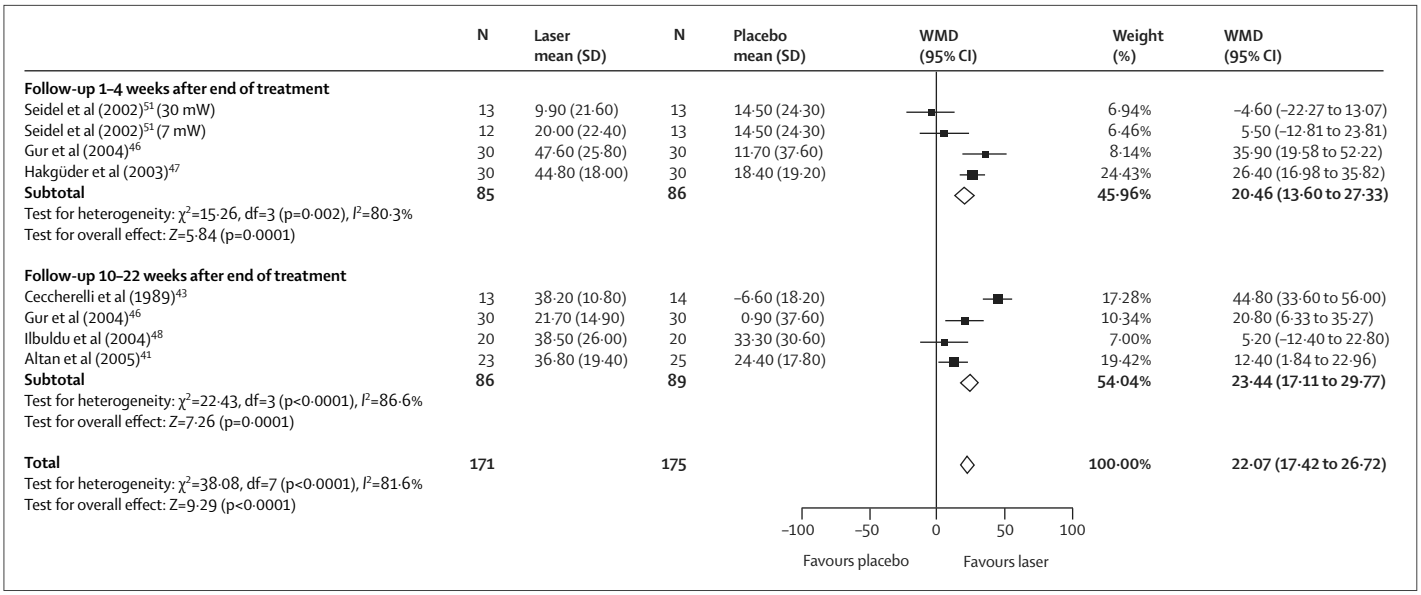

Figure 5: **Weighted mean difference in pain reduction on 100 mm visual analogue scale between placebo-treated and laser-treated groups in seven trials reporting follow-up data**
WMD= weighted mean difference.

high-quality trials with methodological scores of 3 or more. RR of pain improvement with LLLT was 4·05 (2·74–5·98) compared with placebo at the end of treatment (figure 3).

Analysis of data from visual analogue scale showed that in patients in 13 groups in 11 trials, irrespective of methodological quality, pain intensity was reduced by a mean value of 19·86 mm (10·04–29·68) compared with placebo groups (figure 4). Seven trials with eight LLLT groups provided follow-up data for 1–22 weeks after end of treatment (figure 5). The pain-relieving effect in the short term (<1 month) persisted into the medium term (up to 6 months). Five studies provided evidence for improvement in disability at end the of LLLT treatment (figure 6). Several questionnaire-based outcome measures were used—specifically, the neck pain and disability scale,[54] Northwick Park neck pain questionnaire,[55] short form 36,[56] Nottingham health profile,[57] and neck disability index.[58]

Positive publication bias, which tends to exclude negative studies, was not apparent on testing (figure 7).[38] The plot has an aggregation in the lower left quadrant of several small studies with results showing no or only small changes in visual analogue scale.[59] If publication bias towards only positive studies was present, few studies would lie in this position and small studies would have exaggerated positive outcomes. The slight asymmetry might be partly due to a negative publication bias, the small number of studies, and because we have included the most reported studies so far.

We subgrouped trials according to a-priori protocol in acute and chronic categories for the statistical analyses. Within these categories, we noted small variations between trials in patient characteristics such as baseline pain, symptom duration, age, and sex, and we did not detect any clinical heterogeneity (data not shown). Laser parameters and application techniques, including treatment protocols, were heterogeneous (table 2). Laser irradiation was applied to an average of 11 points (range 3–25) in the neck. Energy delivered per point ranged from 0·06 to 54·00 J, with irradiation durations of 1–600 s. Patterns of treatment ranged from a one-off treatment to a course of 15 treatments, which were administered daily to twice a week. On average, participants received a course of ten treatments. Visible (632·8 and 670·0 nm) and infrared (820–830, 780, and 904 nm) wavelengths were used at average power outputs ranging from 4 to 450 mW, in pulsed and continuous wave mode.

When trials with significant results in favour of LLLT were subgrouped by wavelength, doses and irradiation times seemed fairly homogeneous within narrow ranges (table 3). We noted a distinct dose-response pattern for each wavelength for which LLLT is effective within a narrow therapeutic window. For 820–830 nm, mean dose per point ranged from 0·8 to 9·0 J, with irradiation times of 15–180 s. For 904 nm doses, mean dose per point was 0·8–4·2 J, with irradiation times of 100–600 s. Investigators who used doses outside the minimum (0·075 J and 0·06 J)[40,49] and maximum (54 J)[44] limits of these ranges did not show any effect of LLLT, lending further support to a dose-dependent response for LLLT in neck pain.

Significant heterogeneity exists in categorical data for improvement from two studies[39,40] of acute neck pain (p=0·003, χ^2=8·86, I^2=88·7%). This finding could be attributable to the low dose per point used in one study.[40,62] We noted no heterogeneity between trials of chronic neck

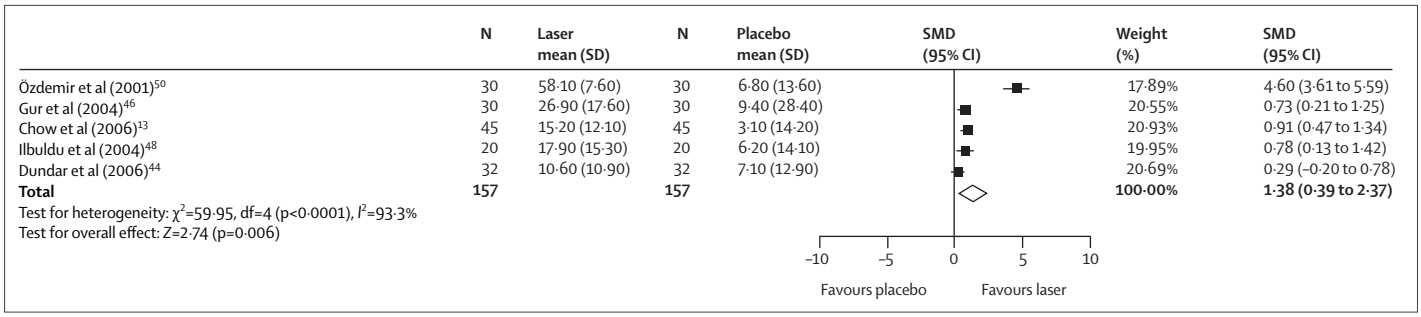

Figure 6: **Standardised mean difference in disability scores between placebo-treated and laser-treated groups from five trials**
SMD=standardised mean difference.

pain reporting on categorical data (p=0·37, χ^2=4·31, I^2=7·2%).

For continuous data from 100 mm visual analogue scale in chronic neck pain, we detected significant heterogeneity across all wavelengths (p<0·0001, χ^2=137·76, I^2=90·6%). However, when heterogeneity was addressed separately by wavelengths, most heterogeneity could be accounted for by variations in doses and application procedures. Removal of the study[44] that used a very high dose from the disability analysis eliminated statistical heterogeneity (p=0·31, χ^2=3·61, I^2=16·9%). For pain intensity on 100 mm visual analogue scale for 820–830 nm wavelength, this study caused heterogeneity together with results of a second study[50] that showed a highly significant effect, without obvious reasons for heterogeneity. After removal of both studies from the 820–830 nm analysis, statistical heterogeneity was eliminated (p=0·12, χ^2=10·20, I^2=41·2%), but the overall effect remained similar, with narrower confidence intervals after (22·0 mm [14·5–29·6]) than before (21·6 mm [10·3–32·9]) removal.

For 904 nm wavelength, statistical heterogeneity was evident for analysis of pain intensity on 100 mm visual analogue scale (p=0·00001, χ^2=28·37, I^2=89·4%). The only study in the review using a scanning application procedure in contact with the skin had weaker than average results.[45] Contrary to other laser application procedures, this method irradiates the target area intermittently. Few studies compare scanning irradiation with stationary irradiation, and most LLLT studies have used a stationary laser beam. Another study using 904 nm wavelength[41] with non-significant results has been criticised for absence of laser testing and calibration, and the actual dose used remains uncertain.[63] Removal of these two trials from the 904 nm analysis of pain reduction on 100 mm visual analogue scale increased the overall effect from 20·6 mm (95% CI 5·2–36·2) to 37·8 mm (25·4–50·1).

50% of trials did not report side-effect data. Side-effects reported included tiredness, nausea, headache, and increased pain, but were mild and, apart from one study in which unusual tiredness occurred more in the laser group than in the placebo group (p>0·01),[42] did not differ from those of placebo.

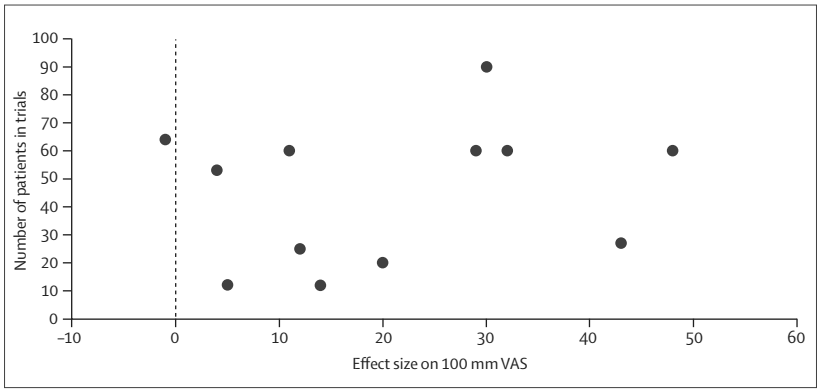

Figure 7: **Publication bias plot**
Plot of effect size between placebo and real laser groups within each trial versus their respective sample sizes. Red circles show one trial. VAS=visual analogue scale.

Discussion

Our results show moderate statistical evidence for efficacy of LLLT in treatment of acute and chronic neck pain in the short and medium term. For chronic pain, we recorded an average reduction in visual analogue scale of 19·86 mm across all studies, which is a clinically important change.[64,65] Categorical data for global improvement also significantly favoured LLLT. From our analysis, 820–830 nm doses are most effective in the range of 0·8–9·0 J per point, with irradiation times of 15–180 s. At 904 nm, doses are slightly smaller (0·8–4·2 J per point), with slightly longer irradiation times (100–600 s) than at 820–830 nm.

Our findings build on those of previous reviews of LLLT[28,30] by including non-English language studies, laser acupuncture studies in which local points were treated, and a quantitative analysis. Our search strategy has identified a greater number of studies than have previous reviews, and draws attention to the intrinsic difficulties in searching the topic of LLLT. Specifically, no accepted terminology exists for laser therapy. We have overcome this limitation by using as wide a range of synonyms as possible.

Moreover, many apparently disparate diagnostic terms are applied to patients presenting with neck pain. These terms suggest distinct clinical entities; however, there is strong evidence that a definitive diagnosis of the causes of neck pain is not possible in a clinical

	Wavelength (nm [mode])	Average output (mW)	J per point	Total time per point (s)	Frequency of treatment	Number of repetitions
Ceccherelli et al (1989)[43]	904 (p)	~25	1	~40	Three times per week on alternate days for 4 weeks	12
Flöter et al (1990)[45]	904 (p); 632·8 (cw)	20·5 (9·5 IR; 11·0 red HeNe)	1	600	Twice per week for 3 weeks	6
Taverna et al (1990)[52]	904 (p)	24	2	180–300	Six times per week for 2·5 weeks	15
Toya et al (1994)[53]	830 (cw)	60	NR	NR	One application only	1
Soriano et al (1996)[39]	904 (p)	40	4	100	Five times per week for 2 weeks	10
Laakso et al (1997)[49]	820 (p)	25	0·06; 0·40	1; 6	Three alternate days per week for 1·5 weeks	5
Laakso et al (1997)[49]	670 (p)	10	NR	4; 18	Three alternate days per week for 1·5 weeks	5
Özdemir et al (2001)[50]	830 (cw)	50	0·75	15	Five times per week for 2 weeks	10
Seidel and Uhlemann (2002)[51]	830 (cw)	7	0·42	60	Twice per week for 4 weeks	8
Seidel and Uhlemann (2002)[51]	830 (cw)	30	1·8	60	Twice per week for 4 weeks	8
Hakgüder et al (2003)[47]	780 (cw)	5	1	196	Five times for week for 2 weeks	10
Chow et al (2004)[42]	830 (cw)	300	9	30	Twice per week for 7 weeks	14
Gur et al (2004)[46]	904 (p)	11·2	0·18–1·80	180	Five times per week for 2 weeks	10
Ilbuldu et al (2004)[48]	632·8 (cw)	NR	2	NR	Three alternate days per week for 4 weeks	12
Altan et al (2005)[41]	904 (p)	4	0·5	120	Five times per week for 2 weeks	10
Aigner et al (2006)[40]	632·8 (cw)	5	0·075	15	Three times per week for 3 weeks	9
Chow et al (2006)[13]	830 (cw)	300	9	30	Twice per week for 7 weeks	14
Dundar et al (2006)[44]	830 (cw)	450	54	120	Five times per week for 3 weeks	15

p=pulsed. cw=continuous wave. IR=infrared. HeNe=helium-neon. NR=not reported.

Table 2: **Laser parameters and treatment regimen**

setting.[66,67] By using the term non-specific neck pain, which encompasses many descriptors,[31] we have addressed the clinical reality that patients presenting with neck pain can have several concurrent sources of pain from joints, muscles, and ligaments.

In addition to aggregating all included studies, irrespective of diagnostic label, we also combined data irrespective of the intended rationale for treatment, as long as neck muscles and spinal joints were exposed to laser irradiation. Transcutaneous application results in laser-energy scattering and spreading into a three-dimensional volume of tissue, up to 5 cm for infrared laser.[68] Since the same effect would be achieved with application of laser energy to acupuncture points, we also included data from studies in which local points in the neck were treated as part of the protocol. Evidence suggests that trigger points in the neck coincide with the location of acupuncture points in 70–90% of patients (eg, BL10, GB 20, GB21, and Ah Shi points).[69,70] Since trigger points and acupuncture points are characterised by tenderness, the treatment effect of laser irradiation to tender points, trigger points, or acupuncture points is likely to be the same. We did not distinguish any differences in subgroup analyses between these techniques. Thus, when treating neck pain with LLLT, irradiation of known trigger points, acupuncture points, tender points, and symptomatic zygapophyseal joints is advisable.

Dose assessment is crucial for interpretation of outcomes of LLLT studies, for which failure to achieve a dose in the recommended range has been identified as a major factor for negative outcomes.[71] The direct relation between positive outcomes of trials with adequate doses of laser irradiation for the appropriate condition has been shown in acute injury and soft-tissue inflammation,[21] tendinopathies,[72] rheumatoid arthritis,[73] lateral epicondylitis,[11] and osteoarthritis.[10]

Several crucial parameters of laser devices are needed to assess dose of laser irradiation, but these doses were inconsistently reported in the studies that we reviewed. No study provided all parameters identified as important by the Scientific Committee of the World Association of Laser Therapy.[74] In neck pain, however, there is little reason to believe that factors other than a plausible anatomical target, dose per point, and irradiation times are essential for efficacy of class 3B lasers (5–500 mW). We had sufficient data relating to each of these components of therapy, when combined with manufacturers' specifications, to identify a dose-response pattern for the number of joules per point and wavelength used and positive outcome. Subgrouping of studies by wavelength and ascending doses reduced apparent heterogeneity in treatment protocols and laser parameters, and showed a dose-response pattern with distinct wavelength-specific therapeutic windows. Most statistical heterogeneity disappeared when we excluded trials with small doses or flaws in treatment procedure from efficacy analyses. Additionally, a very high dose (54 J) of 830 nm LLLT used in one trial did not cause beneficial nor harmful effects.[44] This finding suggests not only that doses of this magnitude are higher than the therapeutic window, but also that LLLT is safe even if such an overdose is delivered. Frequency of treatments varied from daily to twice a week, raising questions about optimum treatment frequency.

Our analysis suggests that the optimum mean dose per point for 820–830 nm was 5·9 J, with an irradiation time of 39·8 s, and for 904 nm, 2·2 J delivered with an irradiation time of 238 s. We recommend a multicentre, pragmatic trial in an appropriately powered study to test the effectiveness of parameters of this order, with both pain intensity and functional improvement as outcome measures.

Data from seven trials were available for up to 22 weeks after the end of treatment, suggesting that positive effects were maintained for up to 3 months after treatment ended. Trials of knee osteoarthritis,[75] tendinopathies,[61,76] and low back pain reported similar longlasting effects of LLLT.[77,78] These results contrast with those for non-steroidal anti-inflammatory drugs in arthritis and spinal disorders, for which the effect ends rapidly when drug use is discontinued.[71] Reduction of chronic neck pain at the end of treatment of 19·86 mm and at follow-up of 23·44 mm on a visual analogue scale of 100 mm represents clinically significant pain relief.[64,65] This result compares favourably with those of pharmacological therapies that are widely used in treatment of neck pain, for which investigators have shown no conclusive evidence of benefit.[32] Intake of oral analgesic drugs was not systematically reported; however, randomisation within trials would keep the confounding effect of this factor to a minimum.

Half the studies obtained data for side-effects,[39,42,44–46,49,52,53] with tiredness reported in the laser-treated group in three studies,[42,46,49] which was significant in one study.[42] Since LLLT does not generate destructive heat, safety relates mainly to potential eye damage, dependent on class of laser device (classes 1–4), which is defined by analysis of several parameters. Safety glasses are required for classes 3B and 4 to eliminate this risk, and would be required for use in all studies. Systematic reporting of side-effects in future studies would also be recommended to clarify short-term and long-term safety aspects of LLLT.

Mechanisms for LLLT-mediated pain relief are not fully understood. Several investigations exploring the pleiomorphic tissue effects of laser irradiation provide plausible explanations for the clinical effects of LLLT. Anti-inflammatory effects of red and infrared laser irradiation have been shown by reduction in specific inflammatory markers (prostaglandin E_2, interleukin 1β, tumour necrosis factor α), in in-vitro and in-vivo animal studies and in man.[79] In animal studies, the anti-inflammatory effects of LLLT are similar to those of pharmacological agents such as celecoxib,[80] meloxicam,[81] diclofenac,[82] and dexamethasone.[80] Chronic neck pain is often associated with osteoarthritis of zygapophyseal joints,[83] which is manifested by pain, swelling, and restricted movement as clinical markers of local inflammation. Laser-mediated anti-inflammatory effects at this joint could result in decreased pain and increased mobility. The distance between skin surface and lateral aspect of the facet joint is typically 1·5–3·0 cm without pressure, and less with contact pressure (measured with ultrasonography [unpublished data, JMB]). Since 830 nm and 904 nm lasers penetrate to several centimetres,[24,84] anti-inflammatory effects at zygapophyseal joints are a plausible mechanism of pain relief.

Another possible mechanism of LLLT action on muscle tissue is a newly discovered ability to reduce oxidative stress and skeletal muscle fatigue with doses similar to those delivering anti-inflammatory effects. This effect has been reported in an animal study[85] and in human studies with biceps humeri contractions and different wavelengths.[86,87] Because muscle fatigue is usually a precursor of muscle pain, and chronic trapezius myalgia is associated with increased electromyograph activity during contractions and impaired microcirculation,[88] reduction of oxidative stress and muscular fatigue could be beneficial in patients with acute or chronic neck pain.

	Mean dose per point (J)	Mean irradiation time per point (s)
632·8 nm[48]	2	200
780 nm[47]	1	196
820–830 nm[13,42,50,53]	5·9 (3·4)	39·8 (30·3)
904 nm[39,41,43,45,46,52]	2·2 (1·6)	238 (184)

Data are mean (SD, when applicable). LLLT=low-level laser therapy.

Table 3: **Mean dose per point and irradiation times for wavelengths of LLLT used in studies with statistically significant results**

Inhibition of transmission at the neuromuscular junction could provide yet another mechanism for LLLT effects on myofascial pain and trigger points.[89,90] Such effects could mediate the clinical finding that LLLT decreases tenderness in trigger points within 15 min of application.[91] Laser-induced neural blockade is a further potential mechanism for the pain-relieving effects of LLLT.[92,93] Selective inhibition of nerve conduction has been shown in Aδ and C fibres, which convey nociceptive stimulation.[94,95] These inhibitory effects could be mediated by disruption to fast axonal flow in neurons[93] or inhibition of neural enzymes.[96]

These tissue effects of laser irradiation might account for the broad range of conditions that are amenable to LLLT treatment. Whether specific treatment protocols are necessary to elicit different biological mechanisms is unknown. Heterogeneity of treatment protocols might be due partly to variation in LLLT parameters and protocols, eliciting different effects. Whatever the mechanism of action, clinical benefits of LLLT occur both when LLLT is used as monotherapy[13,43] and in the context of a regular exercise and stretching programme.[46,47] In clinical settings, combination with an exercise programme is probably preferable. The results of LLLT in this review compare favourably with other widely used therapies, and especially with pharmacological interventions, for which evidence is sparse and side-effects are common.[16,32]

Contributors

RTC participated in the literature search, development of inclusion and exclusion criteria, selection of trials for inclusion in the analysis, methodological assessment, data extraction and interpretation, and writing of the report. MIJ participated in data analysis and interpretation, critically reviewed the report with special expertise in pain management, and contributed to writing of the report. RABL-M participated in data interpretation and analysis, and critically reviewed the report with respect

to the mechanism of action of laser, and relevance to neck pain. JMB participated in development of inclusion and exclusion criteria, translation of non-English language articles, methodological assessment, data analysis and interpretation, writing of the results section of the report, and supervised writing of the report as a whole.

Conflicts of interest

RTC is a member of the World Association for Laser Therapy (WALT), the Australian Medical Acupuncture College, the British Medical Acupuncture Society, the Australian Pain Society, the Australian Medical Association, and the Royal Australian College of General Practitioners. MIJ is a member of the International Association of the Study of Pain. RABL-M is funded by Fundação de Amparo do Estado de São Paulo (FAPESP, Brazil) and is scientific secretary of WALT, from which he has never received funding, grants, or fees. JMB is a member of the Norwegian Physiotherapy Association, Norwegian Sports Physiotherapy Society, Norwegian Society for Rheumatological and Orthopedic Physiotherapy, and has received research awards and grants from the Norwegian Manual Therapy Association, the Norwegian Neck and Back Congress, the Norwegian Research Council, the Norwegian Fund for Postgraduate Training in Physiotherapy, and the Grieg Foundation. He is also president of WALT, a position for which he has never received funding, grants, or fees.

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Houreld:
Effects on Diabetic Wounded Cells

Effect of Laser Irradiation on Diabetic Wounded Fibroblast Cells in Vitro

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Summary

Diabetes is known to be associated with impaired wound healing, and loss of collagen related to diabetes may be due to decreased levels of synthesis or enhanced metabolism of newly synthesized collagen or both. This study aims to determine if laser irradiation stimulates cellular proliferation, nitric oxide (NO) and collagen synthesis in diabetic fibroblast cells. Induced diabetic wounded human fibroblast cells were irradiated at 830 nm with 5 J/cm^2. Post-laser irradiation there was a significant increase in proliferation at 24 and 48 h and reactive oxygen species and NO generation at 15 min. There was no change in collagen type I. Laser irradiation at 830 nm with 5 J/cm^2 significantly stimulates cellular proliferation and NO synthesis in diabetic wounded fibroblast cells.

Introduction

Normal wound healing requires both destructive and reparative processes in controlled balance aimed at reversing the loss of structural integrity. During wound healing, collagen synthesis is important during the remodeling phase, where new extracellular matrix (ECM) is synthesized. This fine balance is regulated by matrix metalloproteinases (MMPs), which destroy collagen, and their inhibitors tissue inhibitor metalloproteinase (TIMPs). Collagen synthesis begins on the rough endoplasmic reticulum (RER) with the production of three pro-α-chains, which are then hydroxylated, and glycosylated in the Golgi. Formation of 4-hydroxylproline in these procollagen chains is catalyzed by prolyl-4-hydroxylase (P4H, EC 1.14.11.2). Procollagen is formed from the á-chains that fold into a triple-helical conformation, is secreted from vesicles, and undergoes proteolysis at its ends in the extracellular space. Collagen

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molecules are then cross linked into fibrils which then self-assemble into fibres.[1]

Nitric oxide (NO) has been considered to have a biphasic effect in pathological conditions being both beneficial and detrimental depending on the concentration. NO has been shown to down-regulate ECM proteins, such as type I collagen,[2,3] at the same time, in early wound healing, NO favours collagen synthesis and the formation of granulation tissue. Fibroblasts isolated from healing wounds synthesize NO spontaneously and inhibition of NO synthesis decreases collagen synthesis.[4-6] Diabetes is known to be associated with impaired wound healing, and is associated with a variety of alterations in connective tissue metabolism. Loss of collagen related to diabetes may be due to decreased levels of synthesis or enhanced metabolism of newly synthesized collagen or both.[7] NO is significantly reduced in chronic ulcers and impaired healing of diabetic wounds is thought to be related to this decrease.[8,9] Burrow *et al.,* demonstrated that normal skin fibroblasts produce more NO than diabetic human skin fibroblasts, and that there was a direct relationship between NO levels and MMP expression.[10]

Various studies show that phototherapy modulates collagen and NO synthesis both *in vitro* and *in vivo*. Gavish *et al.,* found an increase in collagen synthesis in porcine aortic smooth muscle cells at 780 nm.[11] Maiya *et al.,* demonstrated an increase in collagen in diabetic rats (632.8 nm),[12] which corresponded with the work of Carvalho and colleagues.[13] Zhu *et al.,* and Chi *et al.,* showed direct evidence of NO generation in illuminated cells.[14,15] Since NO has been linked to ECM synthesis, it would appear plausible that laser phototherapy may influence collagen synthesis via NO.

Materials and Methods

Human skin fibroblast cells (WS1, ATCC CRL1502) were grown according to standard culture techniques. A diabetic model was achieved by growing cells in minimal essential media (basal glucose of 5.6 mMol/L) containing an additional 17 mMol/L glucose.[16-18] A wound was simulated whereby the monolayer of cells was scratched using a sterile pipette.[17,19] Approximately $6x10^5$ cells in 3.3 cm diameter culture plates were irradiated in the dark using a 830 nm diode laser with a dose of 5 J/cm^2 (spot size 9.08 cm^2). Unirradiated normal wounded and diabetic wounded cells were used as controls. The study design is summarized in Table 1. Cellular proliferation was examined using

Table 1 Study design (n=4).

	Proliferation	**Collagen I**	**NO[a]**	**ROS[b]**
Incubation Time	24 or 48 h	24 or 48 h	15 min or 1 h	15 min
Method	Fluorescence	ELISA	Griess reagent system	Immunofluorescent staining
Data Collection	Fluorescent spectroscopy	Spectroscopy	Spectroscopy	Fluorescence microscopy

NO[a] Nitric oxide ROS[b] Reactive oxygen species

the VisionBlue™ Fluorescent Cell Viability Assay (BioVision, K303-500), NO by the Griess Reagent System (Promega, G2930), collagen type I by ELISA and reactive oxygen species (ROS) using the Image-iT Live Green Reactive Oxygen Species Detection Kit (Invitrogen, I36007).

Results

Irradiated diabetic wounded cells showed a significant increase in proliferation at 24 ($p<0.001$) and 48 h ($p<0.01$) as compared to both normal wounded and diabetic wounded unirradiated control cells (Fig 1). ELISA did not reveal any significant changes in collagen type I at 24 or 48 h (Fig 2). Cells incubated for 48 h showed an increase in both proliferation and collagen compared to cells incubated for 24 h ($p<0.001$ and $p<0.01$ respectively). Staining of WS1 cells for ROS revealed an increase in staining in unirradiated and irradiated diabetic cells (Fig 3). Diabetic cells irradiated with 5 J/cm^2 showed positive ROS staining comparable to the positive control (100 μM *tert*-butyl hydroperoxide). Diabetic wounded cells incubated at 37°C for 15 min post-laser irradiation showed a significant increase in NO compared to both normal wounded and diabetic wounded unirradiated cells ($p<0.01$), (Fig 4). There was no significant change when cells were incubated for 1 h. There was a significant decrease in NO ($p<0.01$) in irradiated diabetic wounded cells incubated for 1 hour compared to cells incubated for 15 min.

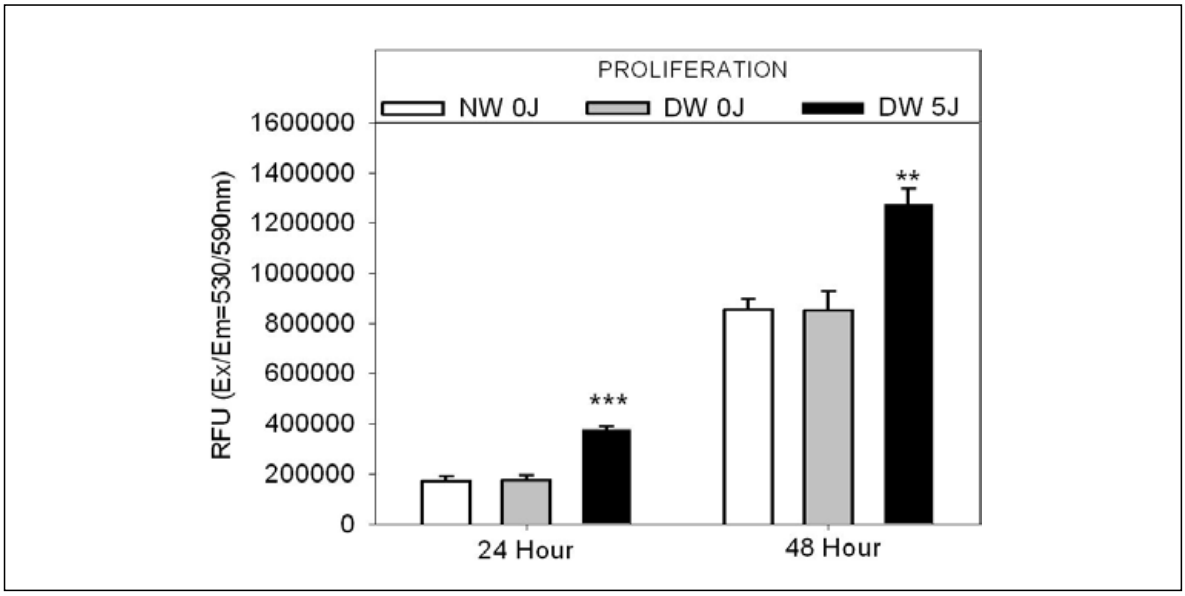

Fig 1.Cellular proliferation was determined in diabetic wounded human skin fibroblast cells 24 and 48 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. There was a significant increase in relative fluorescent units (RFU), and hence proliferation, in irradiated cells compared to control cells. Proliferation was increased at 48 h in all cell types ($p<0.001$).

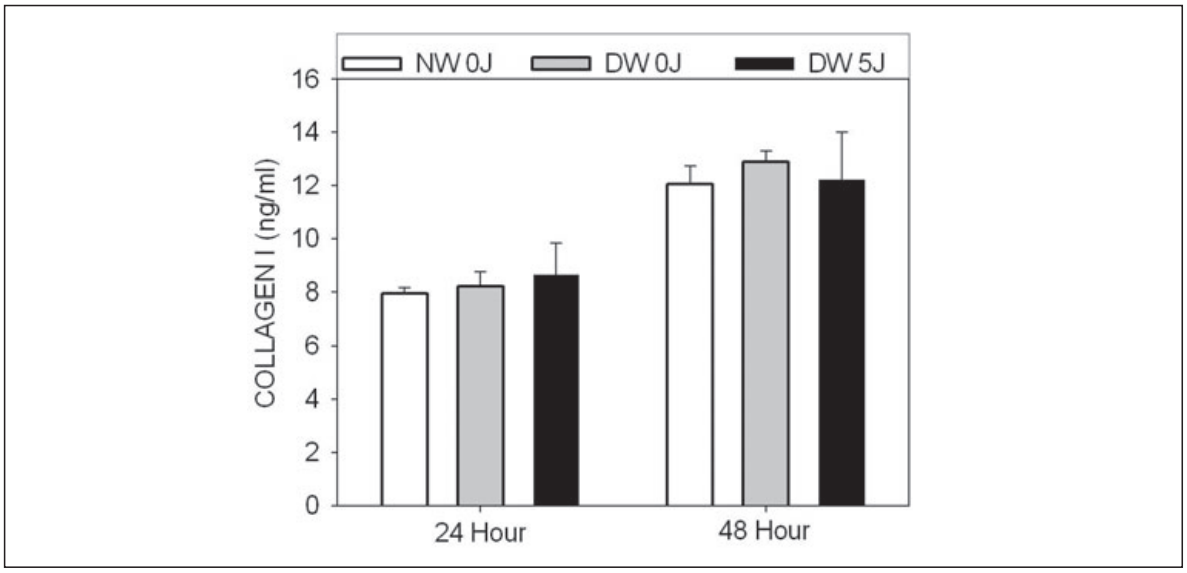

Fig 2.Collagen type I was determined by ELISA in diabetic wounded human skin fibroblast cells 24 and 48 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. Laser irradiation had no effect on collagen synthesis at 24 and 48 h.

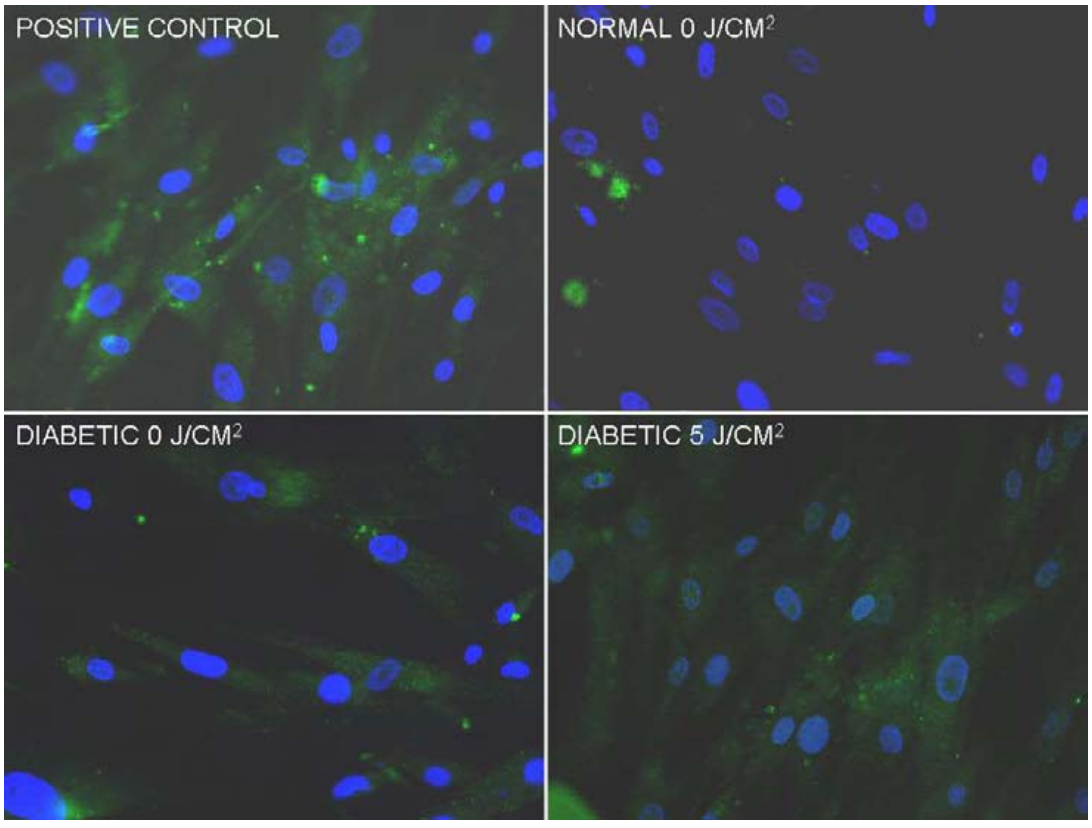

Fig 3.The generation of reactive oxygen species (ROS) was determined by immunofluorescent staining in diabetic fibroblast cells 15 min post-laser irradiation. Normal and diabetic unirradiated cells served as controls, and treatment with 100 μM tert*-butyl hydroperoxide served as a positive control. Cells irradiated with 5 J/cm² showed an increase in ROS generation. Diabetic unirradiated cells showed an increase in ROS compared to normal unirradiated cells, however the generation of ROS was not as much as in irradiated cells.*

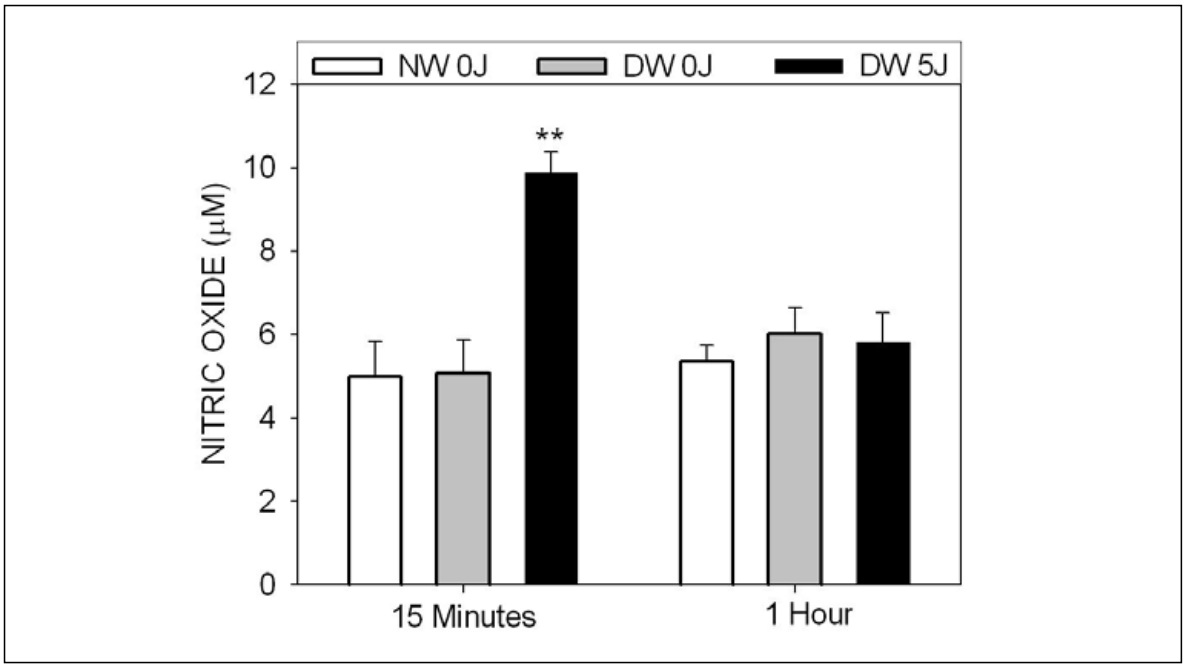

Fig 4.Nitric oxide (NO) was determined in diabetic wounded human skin fibroblast cells 15 min and 1 h post-laser irradiation (DW 5J). Normal wounded (NW 0J) and diabetic wounded (DW 0J) unirradiated cells served as controls. There was a significant increase at 15 min, while at 1 h there was no significant change. There was a significant decrease in irradiated cells at 1 h compared to 15 min ($p<0.01$).

Conclusion

In vitro irradiation of diabetic wounded fibroblast cells at a wavelength of 830 nm with 5 J/cm^2 stimulates wound healing. There was an increase in migration (results not shown), proliferation and NO generation. There was a significant increase in NO 15 min post-irradiation and no change at 1 h, suggesting NO is released directly by a photochemical mechanism.[20] Changes in the cellular redox state can modulate many biological processes, including proliferation. There was no significant changes between non-irradiated normal wounded and diabetic wounded cells, except in ROS staining. Despite literature showing an increase in collagen post-laser irradiation, this study could not show any differences at 24 or 48 h. However there was an increase at 48 h compared to 24 h. A longer incubation time (e.g. 72 h) or a change in irradiation parameters might produce a change in these results since there is strong evidence in the literature that laser irradiation stimulates collagen synthesis in a variety of cell types. This paper cannot link laser irradiation, NO generation and collagen synthesis, since there is an increase in ROS and NO, but no significant increase in collagen type I. Laser irradiation of diabetic wounded fibroblast cells has a positive effect on wound healing, cellular proliferation and ROS generation, including NO.

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Rochkind:

Nerve Cell Tissue and Nerve Repair

Laser Phototherapy: A New Modality for Nerve Cell Tissue Engineering Technology, Cell Therapy and Nerve Repair

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Basic Sciences And Clinical Trial

Studies, which evaluated the effects of 632.8nm and 780nm laser irradiation on Schwann[1] and nerve cell[2] cultures and injured peripheral nerves of animals[3-7] showed positive results. Laser phototherapy induces Schwann cell proliferation[1] and affects nerve cell metabolism and induces nerve processes sprouting[2].

I - Laser phototherapy for treatment of experimental peripheral nerve injury

Laser phototherapy significantly improves recovery of the injured peripheral nerve [3,4,6,7] and, in addition, decreases posttraumatic retrograde degeneration of the neurons in the corresponding segments of the spinal cord.[5]

Our previous studies investigating the effects of low power laser irradiation 632.8 and 780nm on injured peripheral nerves of rats have found:

1. Protective immediate effects which increase the functional activity of the injured peripheral nerve.[8]
2. Maintenance of functional activity of the injured nerve over time.[4]
3. Influence of the LPLI on scar tissue formation at the injured site (Fig.1).[6]
4. Prevention or decreased degeneration in corresponding motor neurons of the spinal cord (Fig.2)[5]
5. Influence on axonal growth and myelinization (Fig.3)[4,7]

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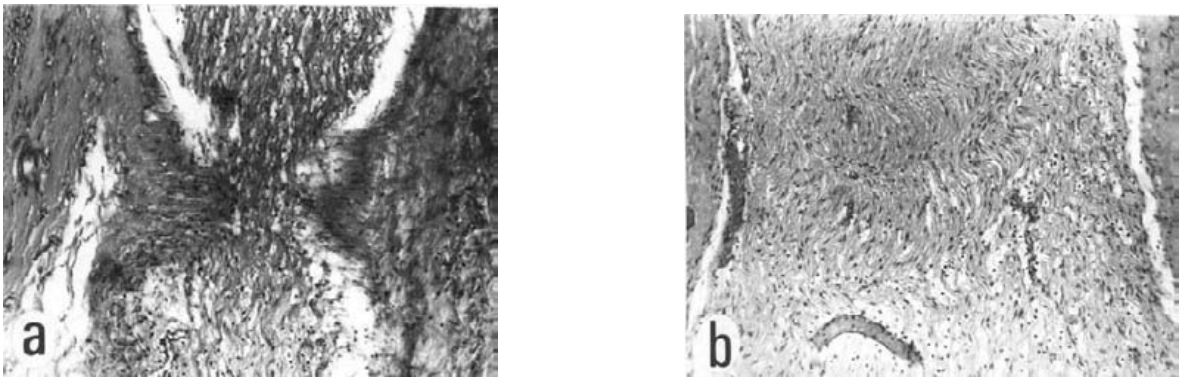


Fig.1. Decrease or prevention of scar tissue formation at the site of injury. (Lasers in Surgery and Medicine 7:441-443, 1987) A- Scar in the place of the injury in the non-laser treated nerve. B- Prevention of scar formation after laser treatment.

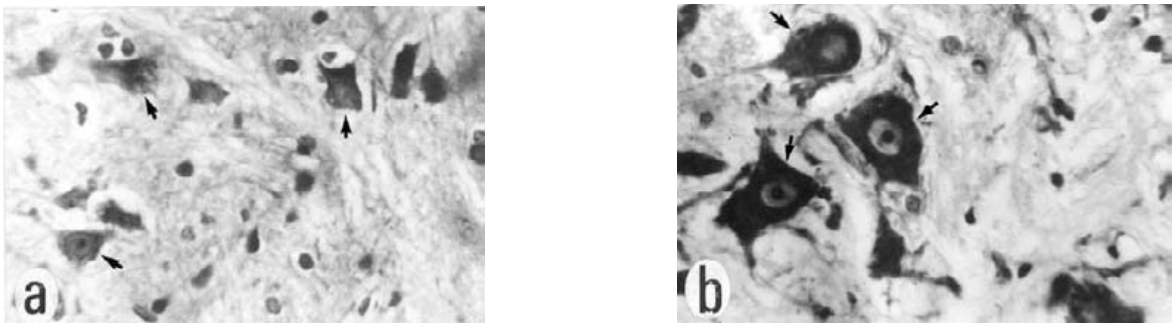


Fig.2. (Spine 15: 6-10, 1990). Progressive degeneration changes in the corresponding neurons of the spinal cord after peripheral nerve injury in the control non-irradiated group (A). Decrease of degeneration process after laser treatment (B).

Moreover, direct laser irradiation of the spinal cord improves recovery of the corresponding injured peripheral nerve.[7,9] Our results suggest that laser phototherapy accelerates and improves the regeneration of the injured peripheral nerve.

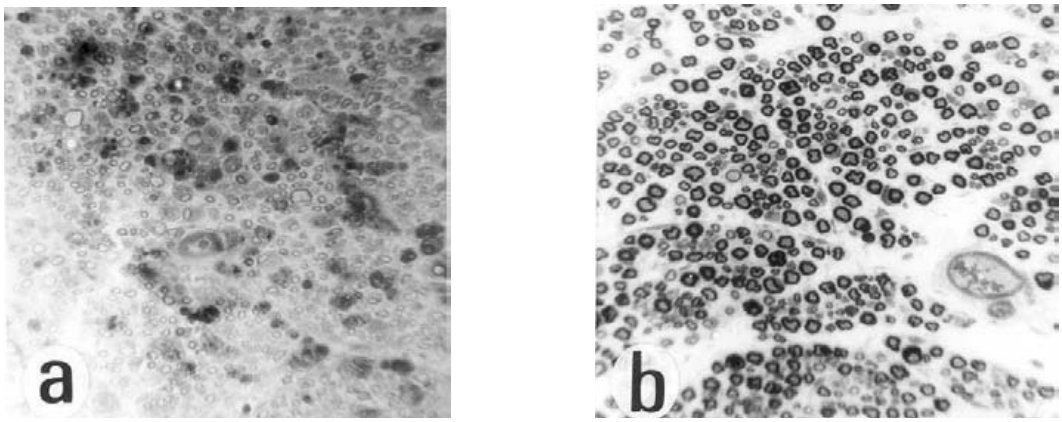


Fig.3. (Neurosurgery 20: 843-847, 1987) Increase in rate of axonal growth and myelinization: a- without treatment; b- laser treated nerve

780nm Laser Phototherapy in Clinical Study
II - Clinical double-blind, placebo-controlled randomized trial

Since our animal studies were positive, an evaluation of the response to 780nm laser phototherapy was in order. Therefore, a clinical double-blind, placebo-controlled randomized study was performed to measure the effectiveness of 780nm low power laser irradiation on patients who had been suffering from incomplete peripheral nerve and brachial plexus injuries for 6 months up to several years.[10] Most of these patients were discharged from initial orthopedics, neurosurgeons and plastic surgeons without further treatment.

In this study 18 patients with a history of traumatic peripheral nerve / brachial plexus injury (at least six months after the injury), with a stable neurological deficit and a significant weakness, were randomly divided to receive either 780nm laser or placebo (non-active light) irradiation. The analysis of the results of this trial in the laser-irradiated group showed statistically significant improvement in motor function in the previously partially paralyzed limbs, compared to the placebo group, where no statistical significance in neurological status was found (Fig.4).

Electrophysiological observation during the trial supplied us with important diagnostic information and helped to determine the degree of functional recovery in nerve-injured patients. The electrophysiological analysis also showed statistically significant improvement in recruitment of voluntary muscle activity in the laser-irradiated group, compared to the placebo group (Fig.5)

This study shows that in long-term peripheral nerve injured patients 780nm low power laser irradiation can progressively improve peripheral nerve function, which leads to significant functional recovery.

III - Further development in peripheral nerve reconstruction and role of 780nm laser phototherapy

This study was done to show the use of low power laser treatment enhances the regeneration and repair of a reconstructed injured peripheral nerve.[11]

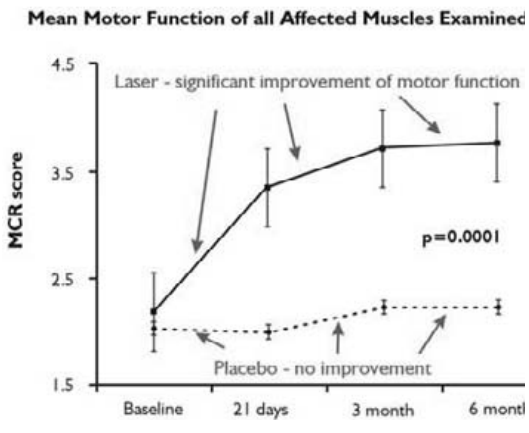


Fig.4

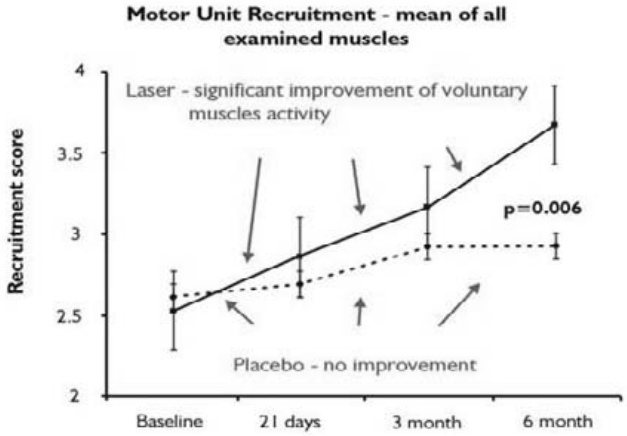


Fig.5

Photomedicine & Laser Surgery 25: 436-442, 2007)

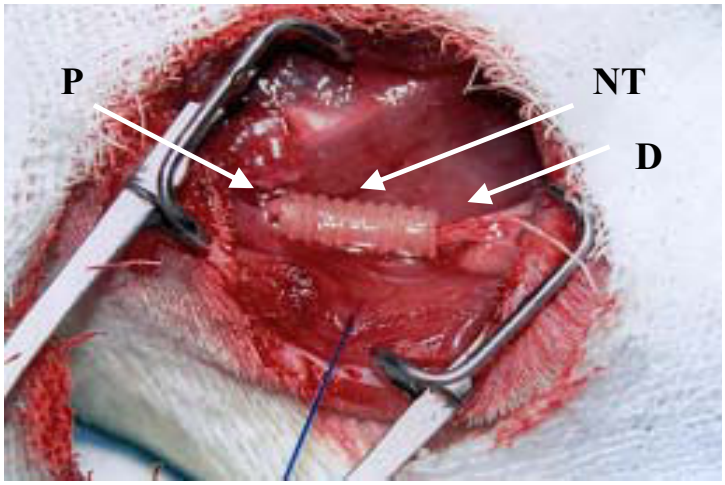


Fig.6 (Photomedicine & Laser Surgery: 25: 137-143, 2007) A neurotube (NT) placed between the proximal (P) and the distal (D) parts of the nerve for the reconnection of 0.5 cm nerve defect (arrows).

The 5mm segment of the right sciatic nerve was removed and proximal and distal parts were inserted into a bioabsorbable neurotube (Fig.6).

The rats were divided into two groups laser treated and non-laser treated. Postoperative low power laser irradiation was applied for 30 min. transcutaneously on the transplanted peripheral nerve area and corresponding segments of the spinal cord, during 14 consecutive days. Conductivity of the sciatic nerve was studied by stimulating the sciatic nerve and recording the somato-sensory evoked potentials (SSEP) from the scalp. Three months after surgery SSEP somato-sensory evoked potentials were found in 70% of the rats in the laser-treated group in comparison with 40% of the rats in the non-irradiated Group.[11] Morphologically, the previously transected nerve had good reconnection four months after surgery in both groups and the neurotube had dissolved (Fig.7).

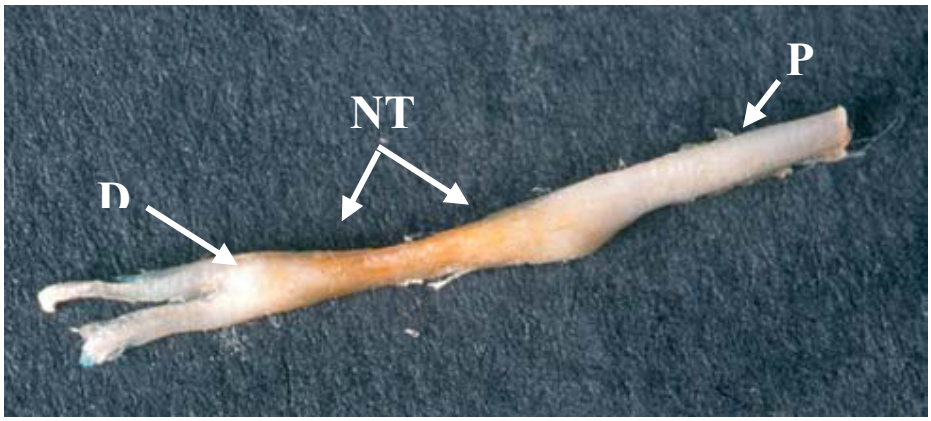


Fig.7 (Photomedicine & Laser Surgery: 25: 137-143, 2007) Sciatic nerve of adult rat which was reconstructed by the neurotube (see arrows: NT-neurotube area, D-distal part, P-proximal part).

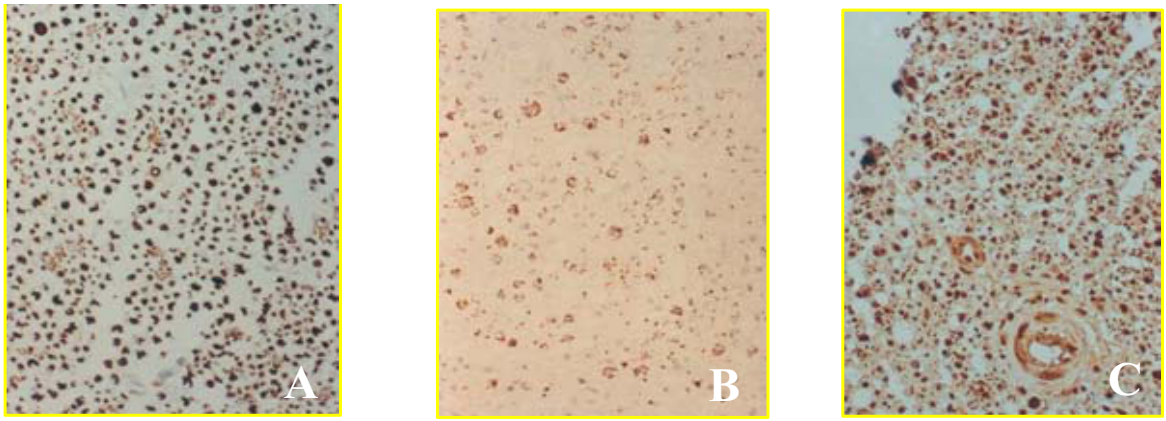


Fig.8.

The immuno-histochemical staining (Fig.8) using a monoclonal antibody-neurofilament showed more intensive axonal growth in neurotube-reconstructed and laser-treated rats (C) compared with the results of the non-laser treated group (B).

IV- Influence of 780nm low power laser irradiation on nerve cell growth in vitro

In this work the effect of 780nm laser phototherapy on sprouting and cell size of embryonic rat brain cells on microcarriers (MC) NVR-N-Gel in culture[12] was investigated. *Cell cultures:* Whole brains were dissected from 16-day old rat embryos (Sprague Dawley). After mechanical dissociation, cells were seeded directly in NVR-N-Gel, or suspended in positively charged cylindrical MC. Single cell-MC aggregates were either irradiated with LPLI within one hour after seeding, or cultured without irradiation. *NVR-N-Gel* (hyaluronic acid and laminin) was enriched with the following growth factors: BDNF and IGF-1.

780nm Low Power Laser irradiation: Laser powers were 10, 30, 50, 110, 160, 200 and 250 mw. Dissociated cells or cell-MC aggregates embedded in NVR-N-Gel, were irradiated for 1, 3, 4 or 7 min. A rapid sprouting of nerve processes from the irradiated cell-MC aggregates was detected already within 24h after seeding (Fig.9).

The extension of nerve fibers was followed by active neuronal migration. Differences between controls, and irradiated stationary dissociated brain cultures, became evident at about the end of the first week of cultivation - several neurons in the irradiated cultures exhibited large perikarya and thick elongated processes (Fig 10).

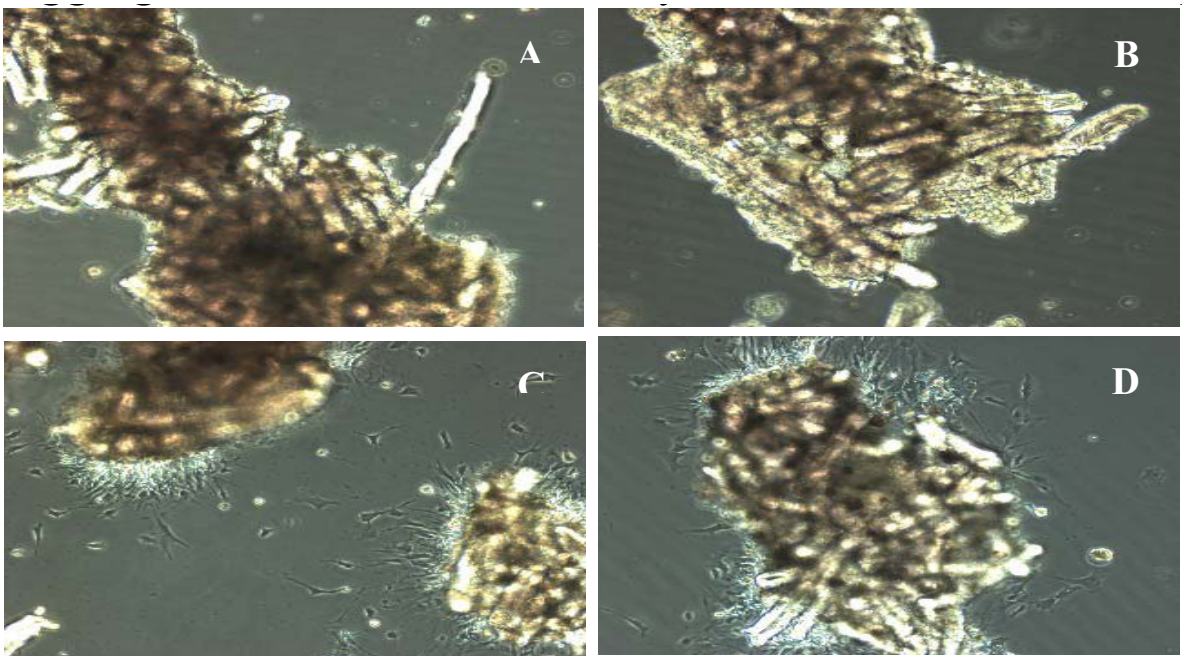


Fig 9. Effect of 780nm low power laser irradiation on initial sprouting and migration from DE-53 MCs in NVR-N-Gel. Initial sprouting and cellular migration is observed in irradiated cultures but not in non-irradiated control, already one day after the transfer to stationary cultures in NVR-N-Gel. A&B: Non-irradiated controls. C: Single irradiation of 250mW, for 1min. D: Single irradiation of 250mW for 3min. Original magnification: 200X.

V - Further development in spinal cord reconstruction and role of 780nm laser phototherapy

The following treatment method was developed recently in our laboratories to enhance regeneration and to repair traumatic paraplegia in rats, resulting from spinal cord transaction.[13,14] Embryonal spinal cord cells dissociated

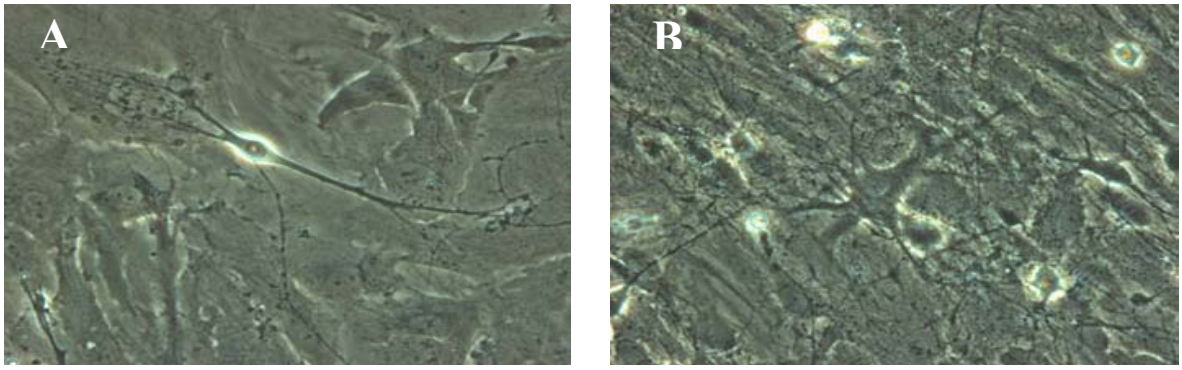
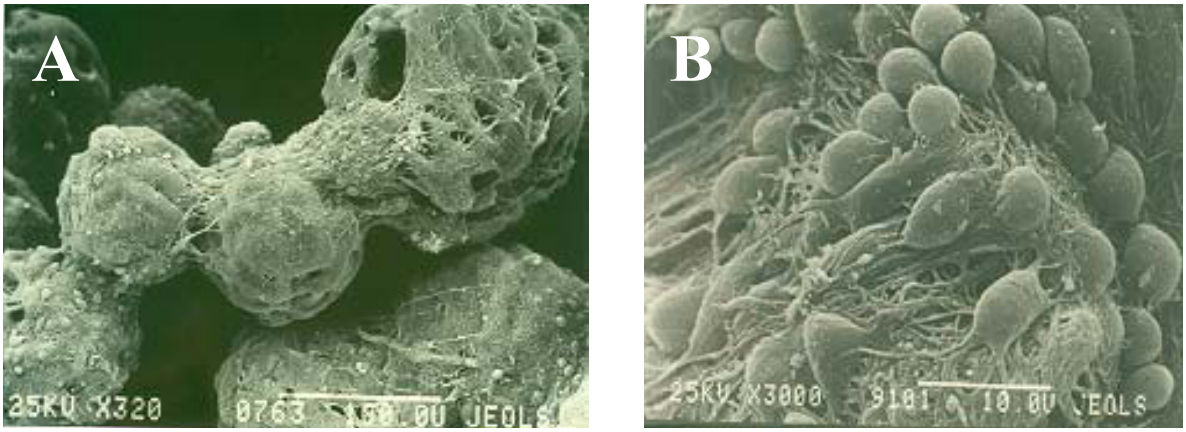


Fig 10. Effect of LPLI treatment on perikarya and fibers of nerve cells derived from rat embryonic brain. Dissociated brain cells were embedded in NVR-N-Gel and were either exposed to single irradiation of 160mW for 3min (B), or served as non-irradiated controls (A). Large neural cells exhibiting thick fibers were observed in 8 days in vitro (DIV) irradiated cultures. Original magnification: 200X.



Biodegradable microcarriers Embryonal spinal cord cells

Fig.11A (Neurological Research 24: 355-360, 2002). In vitro reconstructed composite implants containing neuronal cells attached to gelatinous microcarriers (MCs).
Fig.11B Embryonal spinal cord cells (B).

from rat fetuses were cultured on biodegradable microcarriers (MCs) (Fig.11A) and embedded in hyaluronic acid (HA) (Fig.11B).

The cell-MCs aggregates were implanted into sites of the completely transected spinal cord of adult rats. These implants served as regenerative and repair sources for reconstructing neuronal tissue. During the following 14 post-operative days, the implanted area of the spinal cord was irradiated transcutaneously, 30 minutes daily to enhance the neuro-regenerative repair process.

The post-operative follow-up (from 3 to 6 months) showed that the rats which underwent embryonic nerve cell transplantation and laser treatment showed that most effective re-establishment of limb function, gait performance and intensive axonal sprouting occurred and after nerve cell implantation and laser irradiation (Fig. 12A,B), compared to rats without treatment (Fig. 13A,B).

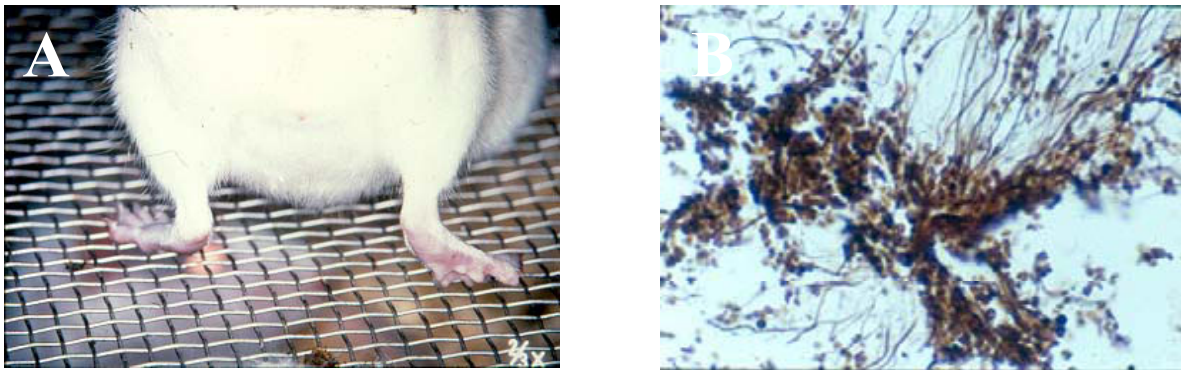


Fig. 12 (Neurol Res 24: 355-360, 2002) Active movement in both legs after embryonal nerve cell implantation in the transected spinal cord followed by low power laser treatment (A). Diffuse sprouting of axons at the site of nerve cell implantation followed by laser irradiation (modified Bodian's stain X 400) (B).

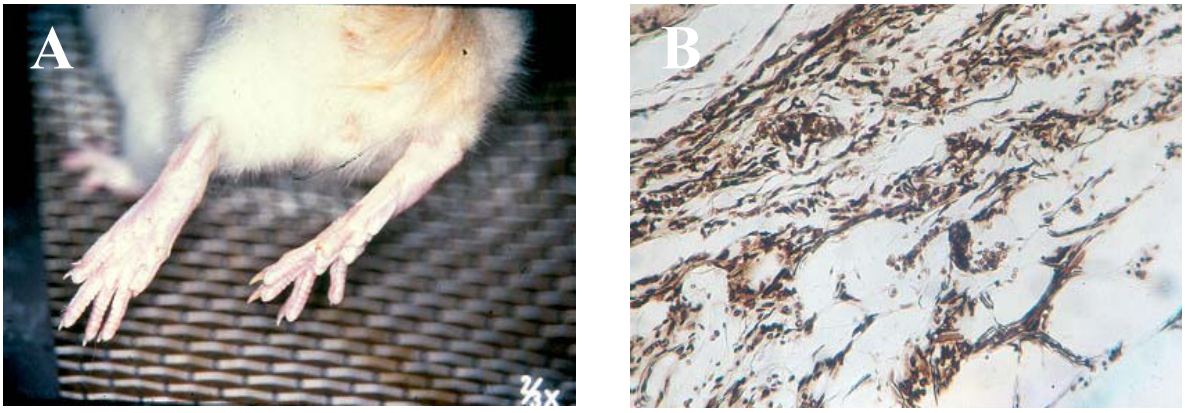


Fig. 13 (Neurol Res 24: 355-360, 2002) Complete paralysis of legs after spinal cord transection (A). Proliferating fibroblast and capillaties in a stansected spinal cord of a rat without treatment (modified Bodian's stain X 400) (B).

The rats which underwent spinal cord transection only remained completely paralyzed in the lower extremities (Fig. 14A)

This study suggests that nerve cell implants which contain embryonal spinal cord cells attached to microcarriers and embedded in hyaluronic acid are a regenerative and reparative source for the reconstruction of the transected spinal cord. In addition, low power laser irradiation accelerates axonal growth and spinal cord regeneration.

In conclusion: The extensive review article, which was published in *Muscle and Nerve* in 2005[15] revealed that most of experimental studies showed phototherapy to promote the recovery of the severely injured peripheral nerve. This review makes possible to suggest that time for broader clinical trials has come.

The significance of our experimental and clinical studies is the provision of new nerve tissue engineering technology and 780nm laser phototherapy for treatment of severe nerve injury.

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Laasko:
Pain Management

Dose Thresholds and Effect Mechanisms for Pain Management with LASER Phototherapy

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Summary

Arguably, the two most important aspects regarding laser phototherapy for pain management are those of effect mechanism (to determine the most appropriate application) and of dosing parameters. Herein is summarised the outcomes of more than 15 years of the author's research to date, to clarify some of these factors. Initial investigations demonstrated that the central descending inhibitory (endogenous opioid) system was involved in the effect mechanism of phototherapy and that doses below 5J/cm^2 in the wavelengths tested, had the best effects in a chronic pain clinical model. Subsequent research using a non-invasive clinical model in lateral epicondylalgia has established that the descending inhibitory system is not the sole likely origin of the treatment response; or, if it is, it plays this role selectively for only some combinations of wavelength, dose and power output. Repeated low doses of laser (at some wavelengths) are sufficient to stimulate physiological responses and reduce pain in subjects with lateral epicondylalgia. It is clear further information is required for dosing and dose threshold factors of laser phototherapy for clinical pain management.

Introduction

Substantial research has investigated possible mechanisms underlying the therapeutic actions attributed to LLLT. The underlying mechanism of laser-mediated analgesia remains unknown (Zinman, Ngo, New, Gogov, Ng & Bril, 2004). A number of clinical trials to assess the efficacy of laser therapy for musculoskeletal pain syndromes have been undertaken (Gan, Thorsen & Lønnberg, 1993), however research in the field continues to be hampered

perhaps as a consequence of methodological shortcomings and the extensive array of combinations of dosing parameters. The increasing number of trials with a result "*in favour of LLLT is by far too large to be explained by random chance alone*" (Gur, Sarac, Cevik, Altindag & Sarac, 2004, p.230) thereby justifying further investigations. The optimisation of laser-mediated analgesia requires a sound understanding of the underlying mechanism (Baxter, 1994), and the confirmation of such mechanisms necessitates consideration of the process of pain perception and modulation.

The Gate Control Theory of pain, proposed by Melzack and Wall in 1965, shifted the emphasis of pain mechanisms from the periphery to the central nervous system. The idea that the transmission of pain from the periphery could be modulated by controls descending from the brain required the brain to be recognised as an "*active system that filters, selects and modulates inputs*" (Melzack, 1999, p.123). Current investigations of descending pain modulation originated from the work of Reynolds (1969) who reported that abdominal surgery in the rat could be performed without a general anaesthetic, and instead with electrical stimulation of the midbrain periaqueductal gray (PAG) region (Gebhart, 2004). The PAG has retained its importance for endogenous analgesic mechanisms despite other regions within the brain since being recognised as areas from which analgesia can be elicited (Wright, 1995). There appear to be two forms of analgesia that originate from distinct regions within the PAG: stimulation of the dorsal system (dPAG) elicits non-opioid analgesia with concurrent excitation of the sympathetic nervous system (sympathoexcitation); and, stimulation of the ventral system (vPAG) results in an opioid form of analgesia and is characterised by sympathoinhibition (Wright, 1995). A clinical model of lateral epicondylalgia has been developed by others, and is one that we utilise for the purpose of testing this proposed laser effect mechanism. Moreover, the model can be used to test dose efficacy and thresholds of stimulation using different combinations of laser parameters. The development of our knowledge about opioid-based and descending pathways of pain mediation is discussed below.

Human chronic pain (trigger point) model:

There is evidence to support both opioid and non-opioid based analgesia following LLLT. Walker (1983) was the first to investigate the descending pain inhibitory systems as a factor implicated in laser-mediated analgesia after finding increased levels of urinary 5-hydroxyindolacetic acid (a by-product of serotonin metabolism) and concurrent hypoalgesia in subjects with chronic pain. We were able to demonstrate support for this assertion, observing decreased pain and increased levels of adrenocorticotrophic hormone (ACTH) and â-endorphin (BEP) following laser irradiation of myofascial trigger points (Laakso, Cramond, Richardson & Galligan, 1994; Laakso, Richardson and Cramond, 1997). In a randomised, double-blind placebo-controlled study, we compared the effect of two doses ($1J/cm^2$ and $5J/cm^2$) of 820 nm and 670 nm laser, and near-monochromatic light (660nm, 30 nm bandwidth) in 56 participants with trigger points of pain in the neck and shoulder region. Subjects

received treatment over a 2 week period. Outcome measures included subjective pain scores and measures of plasma BEP and ACTH to assess the opioid response.

The results of the above study demonstrated that 820 nm laser at 1J/cm^2 and 5J/cm^2 resulted in significant reductions in pain ($p<0.001$). Of interest was that only those participants who received laser phototherapy complained of side effects from the treatment. ACTH increased cumulatively to treatment with 820 nm laser at 1J/cm^2 ($p<0.001$); and with 820 nm and 670 nm laser at 5J/cm^2 ($p<0.05$). Plasma BEP levels were noted to increase significantly between Days One and Four ($p<0.05$) in subjects who received 820 nm laser at 5J/cm^2 but this increase plateaued after this time. We concluded that laser hypoalgesia was dependent on dose, or power output. Moreover, we suggested that ongoing treatment at the higher dose (5J/cm^2) had no further beneficial effects beyond a few treatments.

Although the relationship between peripherally circulating BEP and ACTH and central analgesia could not be established at the time, we hypothesised that inflammatory mediators such as lymphokines (in particular interleukin-1: IL-1) might be stimulated by the application of laser phototherapy. Furthermore, we hypothesised that IL-1 (or other cytokines) might be capable of causing central release of endogenous factors through the stress-immune system (the hypothalamic-pituitary-adrenal axis) in close relationship with the sympathetic nervous system. The alternative hypothesis that we proposed at this time, was that local inflammatory factors (e.g., corticotropin-releasing hormone - CRF) at the site of laser application may have had a direct effect on circulating opioids. This factor remained unresolved. It was not until Stein (1995) and Machelska, Cabot et al (1998) established the presence of peripheral immune-cell derived opioid and opioid receptors, and the preferential homing of immune cells to inflamed sites where they secreted opioids to reduce nociception, that a method became available to test these hypotheses. We went on to study this possible effect in an animal model.

Animal inflammatory model:

In an attempt to determine how local pain relief is mediated by laser phototherapy and how dose affects the relationship, we tested the hypothesis that peripheral opioids are involved in inflammatory pain in an animal model. The model entailed induction of inflammation in the hind-paws of male Wistar rats, and comparison of paw volume, temperature and pressure threshold in non-inflamed, and laser-treated and untreated inflamed hind-paws. Over a number of pilot trials, we tested a range of dose and wavelength combinations to learn more about this factor. The initial unpublished pilot results using 780nm laser at a dose of 5J/cm^2 (the chosen dose was designed to reflect the outcomes of the human chronic pain trial results above) demonstrated no significant effects on the outcome measures when assessed at 5 min after intervention. A further unpublished pilot trial using the same wavelength at 4J/cm^2 demonstrated no significant effects on the same outcomes measures when assessed at 1 hr and 6 hr post-intervention. In a further pilot trial using

820 nm laser at 5J/cm^2 (reflecting the dose and wavelength used in the human chronic pain trial) we noted that repeated treatment at 1hr and 6 hr post-laser had no effect on outcome measures. We repeated the trial of 780nm laser @ 1 J/cm^2 and 2.5J/cm^2 (Laakso and Cabot, 2005) and found that 1 J/cm^2 had no significant effect on anti-nociceptive responses (paw pressure and paw thermal thresholds) but 2.5 J/cm^2 resulted in selective significant improvement in paw pressure threshold at 30 minutes after laser phototherapy but not in paw thermal threshold. Immunohistochemistry of paw tissues demonstrated normal BEP-containing lymphocytes in the hind-paws of control animals but no BEP-containing lymphocytes after 336 h in the hind-paws of animals that received laser at 2.5 J/cm^2. We were led to conclude that the dose/wavelength combination differentiated selectively via the pressure-sensitive rather than the thermal-sensitive neural pathways. Subsequent research by Rittner and Stein (2005) suggests that efficient central analgesia signals a reduced need for recruitment of opioid-containing immune cells to the injured site perhaps suggesting that laser phototherapy may stimulate neural pathways (eg, descending pathways or the sympathetic nervous system - SNS) requiring no local opioid response.

It is interesting to note that subsequent to the above studies, in a study that investigated the effect of 830nm laser @ 200.7 J/cm^2 on peripheral endogenous opioid analgesia in rats, Hagiwara et al (2007) have established that proopiomelanocortin (POMC – a precursor molecule to ACTH and BEP) and CRF demonstrated significantly increased levels at 24 h after laser (compared to controls). In the same study, paw thermal threshold increased at 24 h after laser phototherapy, with the effect being transiently reversed under the influence of naloxone. Paw pressure threshold was not measured. The authors also found that there was a larger accumulation of BEP positive cells in harvested paw tissue at 48 h after laser phototherapy compared to controls.

We have gone on to examine further the effect of laser phototherapy in this model (Kingston, Cabot and Laakso, 2008), and found that anti-nociceptive responses in rats are not evident at 10 minutes after laser phototherapy, confirming the time-dependent nature or threshold for stimulation effects. Furthermore, we have also examined the effect of laser phototherapy on BEP content in regional lymph nodes in response to 780nm laser at 2.5 J/cm^2. The conclusion to be drawn from these results is that there is indeed an opioid-based analgesic effect selectively based on dose and/or wavelength; and on timing of laser application with a probable peak physiologic threshold for effect. The challenge is to identify the specific dose and wavelength combinations which provoke the effects; when it is most efficacious to apply the laser phototherapy; and to confirm these effects in humans. We have gone on to test an innovative, non-invasive method for doing so in a clinical model of pain, in the construct that the mechanism of effect is regulated through the SNS. A summary of the outcomes of our clinical research to date follow.

Human chronic pain model to test sympathetic nervous system outflow:

Sympathoinhibition following laser therapy was demonstrated in a study

investigating the effect of laser phototherapy on sympathetic activity in individuals with myofascial trigger points (Snyder-Mackler, Barry, Perkins & Soucek, 1989). The finding provides support for an opioid-based (sympathoinhibitory) effect of laser-mediated analgesia. Conversely, early animal studies demonstrated that analgesia elicited by irradiation to the tails of experimental rats was only partially reversed by naloxone, a potent opioid antagonist, suggesting the analgesia was not opioid-dependent (Jacob & Ramabadran, 1978). As noted above, there is some evidence to the contrary (Hagiwara et al, 2007), and the effect may be transient or dose/wavelength-dependent, or time-dependent.

To further investigate the role of the SNS to laser stimulation, a pilot study using pain-free subjects was undertaken to test the feasibility of the model (described in Graham and Laakso, 2008). It is possible to measure physiological responses such as heart rate (HR), blood pressure (BP), skin temperature (ST) and skin conductance (SC) which are reflective of SNS outflow. The direction of change in sympathetic activity (either sympathoexcitation or sympathoinhibition) occurring following the intervention, concurrent with analgesia, may provide support for an analgesic effect mediated by the dPAG (causing a non-opioid response) or the vPAG resulting in an opioid-response. A change in pain levels without observing changes in SNS measures supports an alternative mechanism not involving the SNS. In the pilot study, no significant changes in SNS measures were found in pain-free subjects. Following the work of Karu (1989) in which she concluded that the intensity of effect is determined by a cell's physiologic state prior to irradiation, this result was not unexpected. The pilot trial resulted in establishing the procedure as a non-invasive method by which to investigate the effect of laser phototherapy on SNS activity using symptomatic subjects.

Subsequent to the above studies, we have gone on to test the effect of laser phototherapy in a clinical model of pain, i.e., lateral epicondylalgia (LE – tennis elbow). The model is convenient as the incidence of LE is between 1-3% of the general population (Shiri et al, 2006); the elbow is easily accessed in affected individuals; the procedure is non-invasive; and the methodology (as a reflection of central hypoalgesia) has been established in research investigating other interventions (Paungmali et al, 2003; Simon, Vicenzino & Wright, 1997; Chiu & Wright, 1996; Vicenzino, Collins & Wright, 1996).

In the first (thus far unpublished) study (McKirdy and Laakso, 2005), we conducted a repeated measures, randomised, placebo-controlled, double-blind trial in 21 subjects with chronic LE. Participants received 3 interventions on 3 separate days in random order: (1) control - no intervention (2) placebo (deactivated) laser, and (3) laser at 780nm (Compu-Lase SM 2000, Spectra-Medics Pty. Ltd.) at 2.5 J/cm^2 to the 3 most tender points at the lateral epicondyle. Participants acted as their own controls. Subjective pain scores, pain-free grip strength, pain pressure threshold, HR, BP, mean arterial pressure (MAP), blood flux (BF), SC and ST were measured during baseline, intervention and post-intervention periods on each experimental day. The results demonstrated a statistically significant treatment effect on cutaneous BF ($p=0.036$) and MAP ($p=0.032$). The change in BF (increase of 2.69%) and

MAP (decrease of 1.75 mmHg) indicated a sympathoinhibitory (opioid) response to LLLT. No statistically significant treatment effects were noted for other sympathetic outcome measures or for the pain-related measures.

In a separate follow-up single case study, we applied laser (780nm) at 3 J/cm^2 to each of 11 tender points at the lateral epicondyle and insertion of the common extensor tendon. The single case study (of a 49 year old female) demonstrated a statistically significant treatment effect on BF, glabrous ST, pileous ST, and ulnar SC ($p=0.01$). In contrast to the group comparison study described above, the change in BF (decrease of 62.41%), glabrous ST (decrease of 1.20%), pileous ST (increase of 1.88%), and ulnar SC (decrease of 8.34%) indicated a sympathoexcitatory (non-opioid) response to laser phototherapy.

To clarify the disparate nature of the above results, we conducted another single case study (of a 39 year old male) using 780nm laser at 2.5 J/cm^2 to each of 13 tender points at the lateral epicondyle and insertion of the common extensor tendon (Laakso, Meppem et al, 2006). On this occasion, grip strength and pain pressure threshold improved after laser phototherapy; BF decreased by 30% and glabrous ST also decreased after treatment. The results reflected a mixed sympathetic nervous system response with likely bias towards sympathoinhibition indicating opioid-based analgesia.

In a further attempt to clarify the nature of the effect as well as identify whether a dose threshold is apparent, we have recently replicated some of the methodologies described above. In a repeated measures, randomised, placebo-controlled, double-blind study of 19 participants with chronic LE, we investigated the effect of 830nm laser (OmniLase, Laserdyne Technologies, N. Stenning & Co., Pty Ltd) at 3 J/cm^2 applied repeatedly to the 3 most tender points for a total of 13 exposures (Barnes and Laakso, 2008). Participants acted as their own controls. The same outcome measures were included as used in the above study by McKirdy and Laakso (2005). The findings demonstrated no measurable effects on immediate post-treatment pain scores or on sympathetically-mediated outcome measures. However, all participants reported improved pain scores at 24 h after the laser intervention.

The above study (Barnes and Laakso, 2008) was designed to identify the minimum laser dose threshold required to gain an immediate treatment effect. Despite the improved pain scores at 24 h, the decision to use a different wavelength in this study (compared to the wavelength used in the previous study by McKirdy and Laakso, 2005) confirmed the wavelength-dependent nature and the time-dependent nature of laser hypoalgesia, and partly confirms the WALT guidelines which recommend only wavelengths between 780-820nm or 904nm for LE.

Conclusions

The studies described above, outline the continuum of work which we have pursued over a number of years, in order to understand the nature of dosing, timing of treatment responses, and the effects of wavelength on possible descending pathways of pain. Much work is still required to elucidate

the effect mechanisms / pathways for laser hypoalgesia, and the effective laser and dose parameters required. At this point in time, it is reasonable to conclude that some wavelength/dose combinations have an effect through opioid-dependent pathways, and other such combinations do not. Beyond a better understanding of the specific conditions in which laser is likely to be most efficacious, knowledge of effect mechanisms is unlikely to have a significant bearing on those who are 'laser converts'. However, this knowledge is important in convincing those who remain yet to be convinced of the efficacy of laser. Most importantly, knowledge of the minimum effective dose threshold is important to understand, if we wish to optimise the way in which we utilise laser phototherapy.

Acknowledgements

Some results presented herein have been from studies with research students Julia McKirdy, Erin Kingston and Allison Barnes (at Griffith University); Dr David Graham (at The University of Queensland and the Gold Coast Hospital); or in collaboration with Dr Peter Cabot (at The University of Queensland).

[Human and animal research ethics approval was gained for all studies described above].

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Alameida-Lopes:
Bone Repair Using Different Energy Doses

Effects of Continuous and Pulsed Infrared Laser Application on Bone Repair Using Different Energy Doses. Study in Rats

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Summary

The Laser Therapy effects on the cellular proliferation are extensively searched and widely known. However, there are controversies on the best out put power used in the applications, the ideal fluency and irradiance, better emission mode and the adequate number of sessions in order to obtain the best results. The aim of this paper was to search for the best application fluency and emission mode, using an infrared laser in the repair of bone defects in the rat tibia. Thus, the histological quality of the neo-formed bone was evaluated by analysis using common optic microscopy and polarized light. Application Parameters: 100 mW, 830 nm, spot diameter = 0,06 nm, CW and 10 Hz, 3 sessions with 72 h of interval, energies and respective fluencies: 2 J =70 J/cm^{2,} 4 J =140 J/cm^2, 6 J =210 J/cm^2, 8 J =160 J/cm^2, 10 J =200 J/cm^2. Conclusions: Laser Therapy has increased and accelerated the time bone repairing process (in the initial period of 10 days). This laser effect showed to be dose-dependent with the presence of an effective therapeutic window presenting biostimulation of the bone tissue between 4J and 8 J of total energy for both emission mode. The use of the laser with 10 J of energy generated, characterized by the bioinhibition of the tissues (in the initial period of 10 days). This inhibition took place at the exact irradiation spot).

 KX19C0006

Introduction

The Laser Therapy effects on the cellular proliferation are extensively researched and widely known. However there are controversies on the best out put power used in the applications, the ideal fluency and irradiance, and the adequate number of sessions in order to obtain the best results (ALMEIDA-LOPES, et. al. 2001; BAXTER, 1997; PRETEL, et al. 2007). An incorrect use of Laser Therapy may provoke inhibitory effects however a small number of papers in the literature has proven this effect (GIMENEZ, 1985; BOLTON, 1995). The aim of this paper was to search for the best application fluency, using a 830nm pulsed diode laser in the repair of bone defects in the Rat tibia. Thus, the histological quality of the neo-formed bone was evaluated by analysis using common optic microscopy and polarized light.

Methods

The sample consisted by 72 *Holtzman* rats, weight was 300 g on average were used in this study, obtained from the Dentistry School of Araraquara – UNESP – Brazil)

The research project was reviewed and approved by the Ethics in Animal Research Committee of the Dentistry School of Araraquara, UNIARA, Brazil (process number 462/06).

After shaving and asepsis of the tibia with 2% chlorhexidine, a incision was made, skin and periosteal flaps were elevated, the underlying bone tissue was exposed and a trephin cylindrical blade was prepared using stainless steel bur at low speed under constant sterile saline coolant (Fig.1). Thereafter, the animals were randomly assigned to two groups (n=36) according to the treatment of the bone defects.

The histomorphological analysis was performed under light microscopy. Each specimen was independently examined by two trained examiners blinded to the treatment of each group. The followed histomorphological event tissue repair were evaluated.

Table 1 – Aplicattion parameters and distribution of the animals

Application Parameters	**Periods (10th and 30th)**
Potency: 100 mW	
Wavelength: 830 nm	**Continuons laser**
laser beam diameter = 0,06 mm	**Pulsed laser**
laser operation: CW and 10 Hz	
Treatment: 3 sessions with 72 h of interval	Three rats for period and group

Treatment groups

Fluency (J/cm^2)	0	70	140	210	280	350
Energy (J)	0	2	4	6	8	10

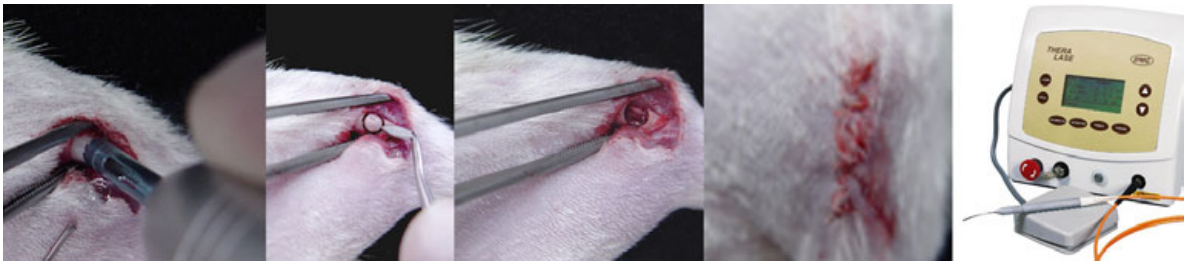

Figure 1 – Surgery procedure and treatment of the animals

Results

The mesoscopic and histological results showed by the slides.

Continuous Laser

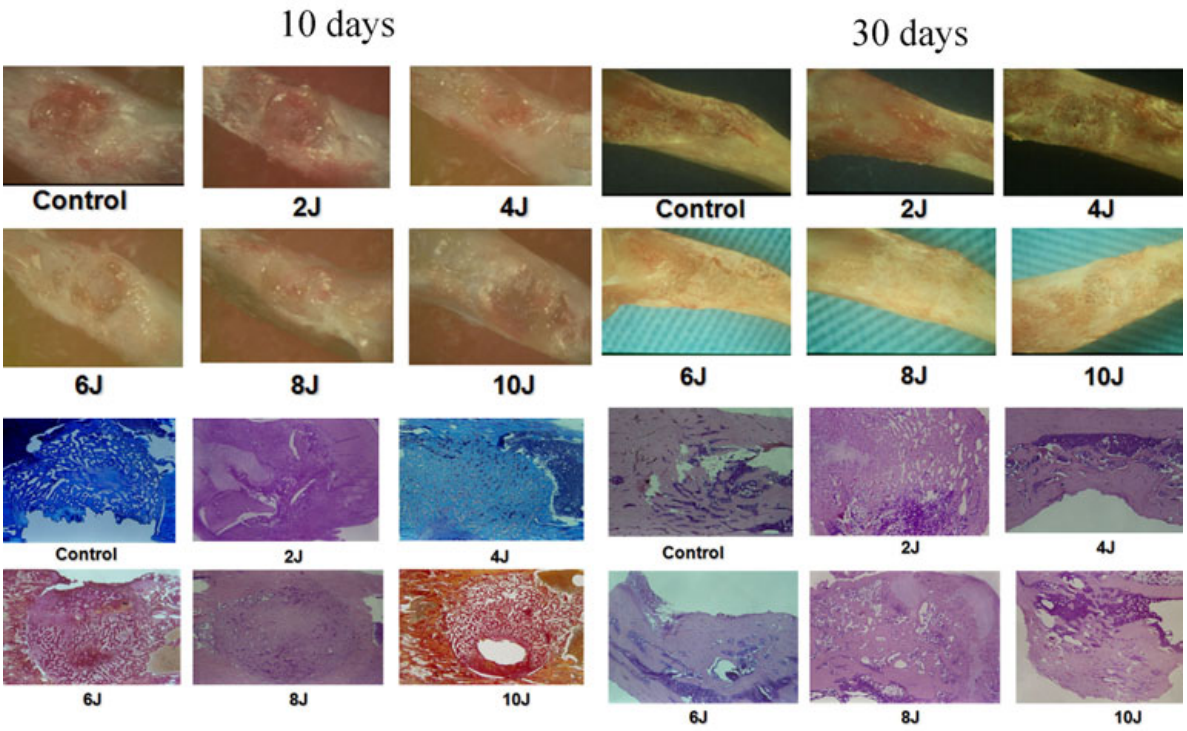

Figure 2 - Mesoscopic and histological results by continuons laser at 10th and 30th

Pulsed Laser

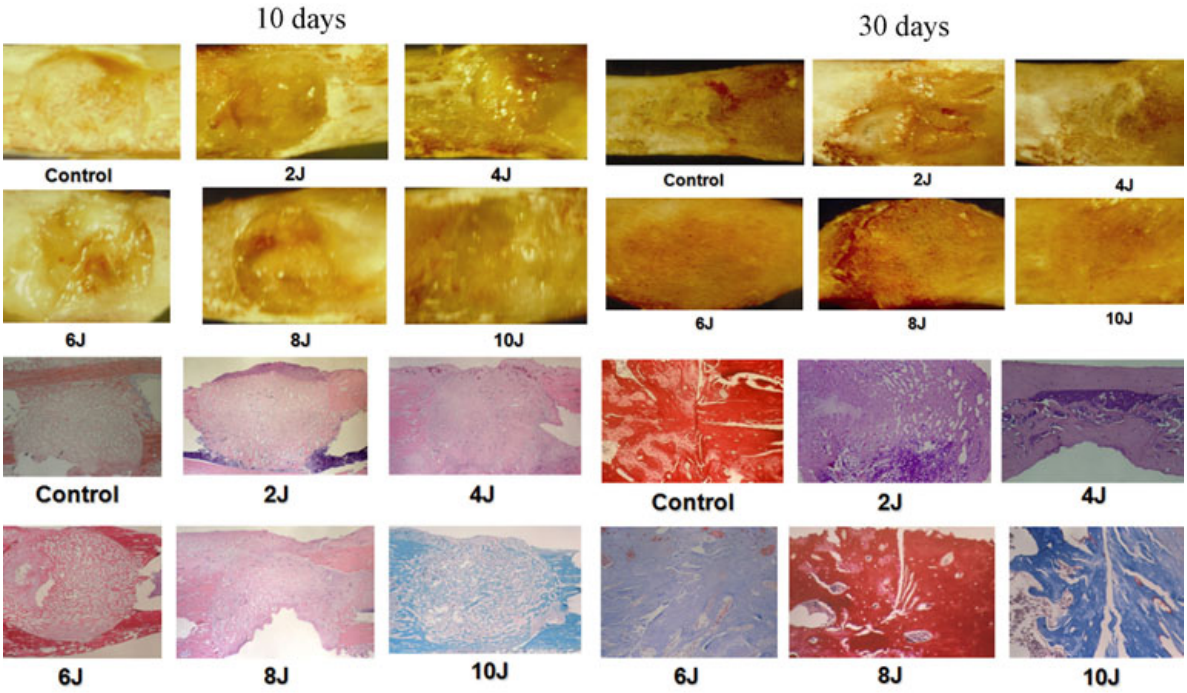

Figure 3 - Mesoscopic and histological results by pulsed laser at 10th and 30th

Conclusions

– Laser Therapy continuous and pulsed has increased and accelerated the tissue bone repairing process (in the initial period of 10 days).

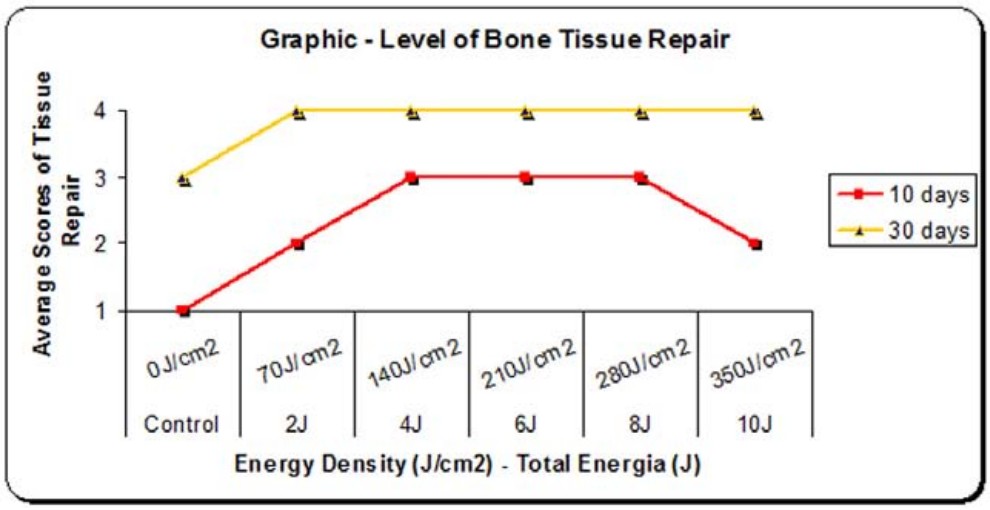

Figure 4 - showing average scores of tissue repair in function of the energy density with continuous laser mode.

– This Laser effect showed to be dose-dependent with the presence of an effective therapeutic window presenting biostimulation of the bone tissue between 4J and 8J of total Energy in both modes.
– The use of the Laser with 10J of Energy, CW generated local damage, characterized by the bioinhibition of the tissues (in the initial period of 10 days). This inhibition took place at the exact irradiation spot.

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Matsumoto:

Cyclo-oxygenase-2 Bone Repair

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ORIGINAL ARTICLE

Low-level laser therapy modulates cyclo-oxygenase-2 expression during bone repair in rats

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Abstract The goal of this study was to analyze the role of cyclo-oxygenase-2 following bone repair in rats submitted to low-level laser therapy. A total of 48 rats underwent surgery to inflict bone defects in their tibias having been randomly distributed into two groups: negative control and laser exposed group, i.e., the animals were treated with low-level laser therapy by means of gallium arsenide laser at 16 J/cm^2. The animals were killed after 48 h, 7 days, 14 days, or 21 days. The tibias were removed for morphological, morphometric, and immunohistochemistry analysis for cyclo-oxygenase-2. Statistical significant differences ($P<0.05$) were observed in the quality of bone repair and quantity of formed bone between groups 14 days after surgery in the laser exposed group. In the same way, cyclo-oxygenase-2 immunoreactivity was more intense in bone cells for intermediate periods evaluated in this group. Taken together, such results suggest that low-level laser therapy is able to improve bone repair in the tibia of rats after 14 days of surgery as a result of an up-regulation for cyclo-oxygenase-2 expression in bone cells.

Keywords Cyclo-oxygenase-2 · Immunohistochemistry · Rats · Bone repair

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Introduction

In past decades, laser has been indicated for several therapeutic purposes [1–4]. There are many laser types available in the global market, with different modes of action. Independent of its mode of presentation, a plethora of beneficial effects has been demonstrated for many in vitro and in vivo test systems, including antibacterial, antiviral, anti-tumor, cell differentiation, immuno-potentiating and repair activities [5–10]. Particularly, low-level laser therapy has been increasingly used to treat hard tissue injuries by promoting wound healing and reducing pain [11]. This is because this type of laser has been demonstrated as a noninvasive method for the stimulation of osteogenesis and to reduce the time of fracture consolidation through bioenergetic, bioelectrical, biochemical and biostimulatory effects on cells [12].

The tibia is the long bone most often fractured, and it is associated with a high incidence of nonunion and delayed healing [13]. With the aim of reducing the substantial incapacity associated with bone fracture and the high socioeconomic costs, a variety of interventions has been studied, including the use of low-level laser therapy [14]. However, few reports have been published with low-level laser therapy focusing on bone repair after mechanical damage in the tibias up to now [15].

Cyclo-oxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins, and two isoforms, cyclo-oxygenase-1 and cyclo-oxygenase-2, have been identified. Cyclo-oxygenase-1 is constitutively expressed in many tissues and mediates the synthesis of prostaglandins required for normal physiological function. Cyclo-oxygenase-2 is normally undetectable in most tissues but is rapidly induced by pro-inflammatory or mitogenic stimuli [16]. Recently, cyclo-oxygenase-2 has been implicated

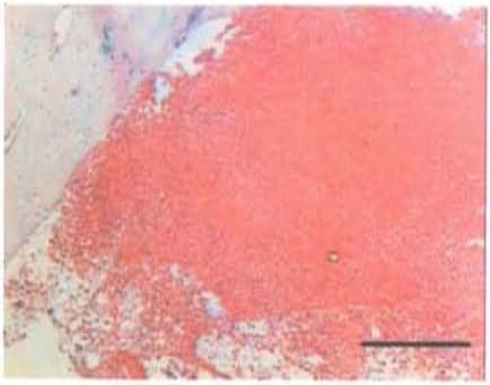

Fig. 1 Bone defect after 48 h. H&E. The *bar* represents 34 μm

in several different cellular mechanisms, such as angiogenesis, proliferation and the prevention of apoptosis [17]. To date, there are no reports that have investigated cyclo-oxygenase-2 expression during bone repair submitted to low laser therapy. This justifies this study and others as well.

Therefore, the aim of this study was to analyze the role of cyclo-oxygenase-2 following bone repair in rats submitted to low-level laser therapy. Certainly, such data will contribute to a better understanding of laser outcomes upon cellular systems during bone repair.

Material and methods

Animals and experimental design

All experimental protocols used in this study were approved by the Ethics Committee for Animal Research, Universidade do Sagrado Coração (USC), Bauru, SP, Brazil.

A total of 48 male rats (*Rattus norvegicus albinus*, Wistar), 8 weeks old and weighing approximately 250 g, were randomly distributed into two groups containing 24 animals each, with four different periods in which they were killed: (a) 2 days, (b) 7 days, (c) 14 days, and (d) 21 days. The groups were as follows: group 1 (control) a, b, c, d—negative control, animals not exposed to laser, and group 2 (laser-treated-group) a, b, c, d—animals treated with low-level laser. All control animals were submitted to the same procedures of handling as the laser-exposed animals were.

Surgical procedure

At the beginning of the experiment, all animals underwent surgery to produce bone defects in their tibias. General anesthesia was obtained by intramuscular administration of 1% ketamine (Francotar, Virbac Ltda., São Paulo, Brazil) associated with sedative, 2% xylazine hydrochloride (Virbaxyl 2%, Virbac Ltda.) in the recommended dose of 0.1 ml/100 g. A 1 cm incision was made to expose the tibia. A cavity in the upper metaphyseal region was prepared, 5 mm deep, in the upper metaphyseal region, and the bone marrow exposed. A carbon steel round bur under copious irrigation was used, and, after each procedure, the bur was discarded and a new one was mounted for the next animal.

Laser therapy

A low-energy gallium arsenide laser, 735 nm in wavelength (DMC, Sao Carlos, Brazil), continuous wave, 3 mm laser beam diameter, at 16 J/cm^2, with irradiation time of 1 min, was used in this experiment. Laser irradiation was initiated 24 h after the surgery and was performed, punctually, every 48 h for 15 days, or until the rat was killed. Laser irradiation was performed transcutaneously, at one point, above the lesion on the injured tibias.

Histopathological analysis

The animals were killed by the administration of an overdose of the anesthetic drug, in the experimental periods established, after 2 days, 7 days, 14 days, or 21 days, for histopathological and immunohistochemical analysis. The tibias were removed, fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h and decalcified in 4%

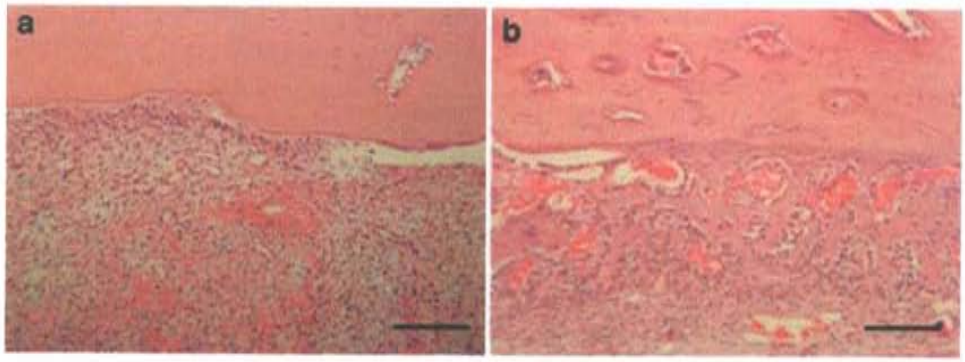

Fig. 2 Bone repair 7 days after surgery. a Control group and b laser-exposed group. H&E. The *bar* represents 48 μm

ethylene glycol tetra-acetic acid (EDTA) (Merck). Five-micrometer slices were obtained in semi-seriate fashion and stained with hematoxylin and eosin (H&E) (Merck).

Morphometry

For the morphometric analysis the regions of bone repair previously identified in the histopathological observation for each animal were measured in a blind fashion by one expert observer using an image analysis system (KS-300, Carl Zeiss, Germany) for Windows. The slices stained with H&E were observed. Three areas of the cortical region of the defect were selected, named C1 and C3, corresponding to the regions close to the wall defect, and C2, corresponding to the central region of the defect. The bone tissue presented in these regions was measured and the area registered at a magnification of ×20. After the registration, the areas were added, resulting in the total bone area of the defect. This analysis had been established in a previous study conduced by our group [18].

Immunohistochemistry

Paraffin was removed with xylene from serial sections of 4 μm and the sections were rehydrated in graded ethanol, then pretreated in a microwave with 0.01 M citric acid buffer (pH 6) for three cycles of 5 min each at 850 W for antigen retrieval. The material was pre-incubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) solution for 5 min for inactivation of endogenous peroxidase and then blocked with 5% normal goat serum in PBS solution for 10 min. The specimens were then incubated with anti-cyclo-oxygenase-2 polyclonal primary antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:50. Incubation was carried out overnight at 4°C within the refrigerator. This was followed by two washes in PBS for 10 min. The sections were then incubated with biotin-conjugated secondary antibody anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 in PBS for 1 h. The sections were washed twice with PBS followed by the application of preformed avidin biotin complex conjugated to peroxidase (Vector Laboratories) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3-3′-diaminobenzidine solution and counterstained with Harris hematoxylin. For control studies of the antibodies, the serial sections were treated with rabbit IgG (Vector Laboratories) at a concentration of 1:200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining batch.

Data analysis

Sections stained for immunohistochemistry were examined under an optical microscope for the percentages of immuno-positive bone cells. A total of 8–10 fields per slice for each animal at ×400 magnification were evaluated

by systematic sampling. These values were used as labeling indices (%).

Statistical methods

The values obtained from morphometric analysis were submitted to the Kruskal–Wallis statistical test and Dunn's post-hoc analysis if a significant effect was detected. Sigma Stat program (Jandel Scientific, Chicago, USA), version 2.0 for Windows was used.

Results

General findings

Neither postoperative complications nor behavioral changes were observed. The rats returned rapidly to their normal diet and showed no loss of weight during the experimentation (data not shown). None of the animals died during the experiment.

Histopathological analysis

Regarding the control group, all the defects were filled by blood clot after 48 h (Fig. 1). On the seventh day, the central region of the defect was filled by granulation tissue (Fig. 2a). In one of the specimens, a small focus of calcifying hyaline cartilage was noticed. After 14 days, irregular and highly cellularized woven bone was seen (Fig. 3a). Most of the specimens showed osteogenic activity also in the medular region, due to the presence of bone fragments resulting from bone perforation. Within 21 days, regular bone trabeculas were seen, covered by osteoblastic cells (Fig. 4). The repaired bone was thinner than the wall defects.

In the group treated with low-level laser, blood clot filled the bone defects after 48 h. On day 7, granulation tissue and woven bone could be seen. In the periphery, however, new bone formation could be seen coming from the bony walls (Fig. 2b). In the same way, bone tissue becoming mature was noticed after 14 days (Fig. 3b). On the 21st day, well-defined trabeculas underwent remodeling, resulting in the formation of a bone bridge thinner than the original bony walls.

Morphometry

The area of newly formed bone was examined using the slices stained with H&E. The results showed statistically significant differences ($P<0.05$) between groups after 14 days. Such findings are shown in Table 1.

Immunohistochemistry

Cyclo-oxygenase-2 expression was detected predominantly in the cytoplasm. Sections stained for immunohistochemistry were examined under an optical microscope for the percentages of immunopositive bone cells.

After 2 days of surgery, cyclo-oxygenase-2 immunoreactivity could be seen in the central region of the lesion in rats of the control group. A similar pattern occurred in the group treated with low-level laser (Fig. 5). After 7 days, cyclo-oxygenase-2 immunoexpression could be seen in the granulation tissue in the control group, whereas cyclo-oxygenase-2 was positively detected in the surrounding bone tissue in the rats exposed to laser (Fig. 6b). This was more evident on the 14th day, either in the control group

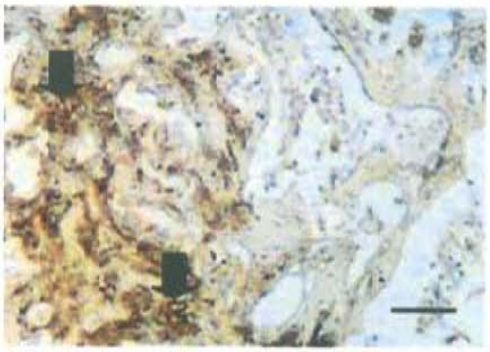

Fig. 7 After 21 days of the surgery, cyclo-oxygenase-2 (COX-2) immunoreactivity is seen (*brown stain*) in some cells of the bone marrow. Immunohistochemistry stain, *bar* represents 40 μm

(Fig. 6a) or in the laser-exposed group (Fig. 6b). Twenty-one days after the surgery, the control group and the group treated with laser showed cyclo-oxygenase-2 expression in some cells of the bone marrow (Fig. 7). All morphometric findings are also summarized in Fig. 8.

Discussion

It has been postulated that low-level laser has therapeutic efficacy on various clinical conditions [9]. Taking into consideration that some inflammatory mediators, such as prostaglandin and arachidonic acid products, play important roles during bone repair, the aim of this study was to evaluate the role of cyclo-oxygenase-2 during low-energy laser on bone repair. To the best of our knowledge, the approach has not been addressed so far. For this, we chose an experimental test system, since animal models have commonly been the basis for studying the relationship between mechanical trauma and therapies [12]. Histopathological, morphometric and immunohistochemical analyses were used in this setting to measure the quantity of formed bone, since we aimed to evaluate the putative up-regulation of osteogenesis induced by laser after mechanical trauma simulating surgical procedures in clinical practice.

After 48 h, our results demonstrated that low-level laser therapy failed to exert any activity, as depicted by similar histopathological patterns between laser-exposed and non-exposed tibias. Although more bone formation was detected in the laser-exposed group under subjective histopathological analysis after 7 days, this finding was not proven statistically, because no statistically significant differences were noticed in the morphometric analysis. Taken as a whole, therefore, we assumed that laser therapy was not able to accelerate bone repair immediately after mechanical trauma in this trial.

Conversely, low-level laser exposure resulted in significant induction of bone formation 14 days after the beginning of the therapy. This was seen in the histopathological and morphometric analyses. Therefore, the laser energy level used appears to have stimulated an increase in the healing of the bone when compared with the healing seen in the tibia controls. Our data are fully in line with others [19, 20]. It is important to stress that this positive effect is transient, since no positive results were maintained 21 days after surgery. Overall, several authors have postulated that low-power laser can stimulate bone cell proliferation and alkaline phosphatase (ALP) activity, which reflects osteoblastic activity [21]. Nevertheless, it seems that multiple doses, rather than the intensity of laser irradiation, are more effective for bone formation [22]. This emphasizes our positive results, since laser was applied every 48 h after surgery. In a study by Ozawa et al. [23], however, laser irradiation beyond 14 days failed to cause stimulation. The authors concluded that the stimulatory action of laser irradiation occurs during the proliferative and earlier stages of differentiation of immature precursors but does not occur during later stages. It is important to keep in mind that it is sometimes difficult to compare studies about the action of low-level laser on bone because the dosimetric parameters, experimental models and duration of treatments are very distinct. Further studies are necessary to elucidate this issue.

Cyclo-oxygenase is a key enzyme in the conversion of arachidonic acid to prostanoids. The expression of isoform cyclo-oxygenase-2 is relevant to many pathological processes, including inflammation, tissue repair and, ultimately, to carcinogenesis [24]. When the expressivity of cyclo-oxygenase-2 was investigated, our results showed that laser therapy promotes an up-regulation for this inflammatory mediator. In the period of 48 h, the immunohistochemistry of cyclo-oxygenase-2 confirmed the main activity of the enzyme in the central region of the defect in both experimental groups and also in the control group. This situation was not maintained after 7 days, when immunoreactivity was more intense in the granulation tissue and newly formed bone cells of the laser-exposed group than in the control group. In the following

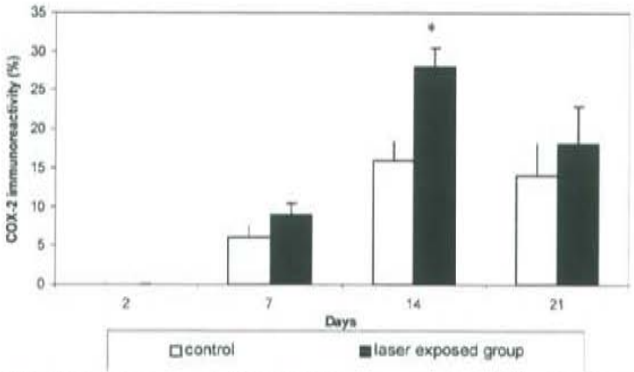

Fig. 8 Cyclo-oxygenase-2 (COX-2) immunoreactivity following laser therapy. Results are expressed in percentage as mean±SD. *$P<0.05$ when compared to control

periods, i.e., 14 days and 21 days, immuno-expression of cyclo-oxygenase-2 was observed in the bone marrow, with a more pronounced effect in the laser-exposed group. Since low-level laser therapy is able to increase the formation of new capillaries through the release of growth factors such as vascular endothelial growth factor (VEGF) [25], stimulate DNA and RNA synthesis in the cell nucleus and, consequently, increase cell proliferation and differentiation [26], we believe that the immuno-expression of cyclo-oxygenase-2 found in bone tissue could also be helpful to bone repair. In 1997, Sato et al. [27] suggested that cyclo-oxygenase-2 could be involved in the early stage of osteogenesis, probably associated with the maturation of osteoblasts. Furthermore, Zhang et al. [28] affirmed that cyclo-oxygenase-2 enzyme acts on osteoblastogenesis, regulating osteoblastic differentiation genes such as Cbfa-1 and osterix. More recently, it has been postulated that bone cells are able to produce cyclo-oxygenase-2 after mechanical trauma [29], being, therefore, important to bone formation [30]. Our results are in agreement with those of these previous studies.

In summary, our results support the notion that low-level laser therapy is able to improve bone repair in the tibia of rats after 14 days of surgery as a result of an up-regulation for cyclo-oxygenase-2 expression. However, this suggestion should be verified by further investigation.

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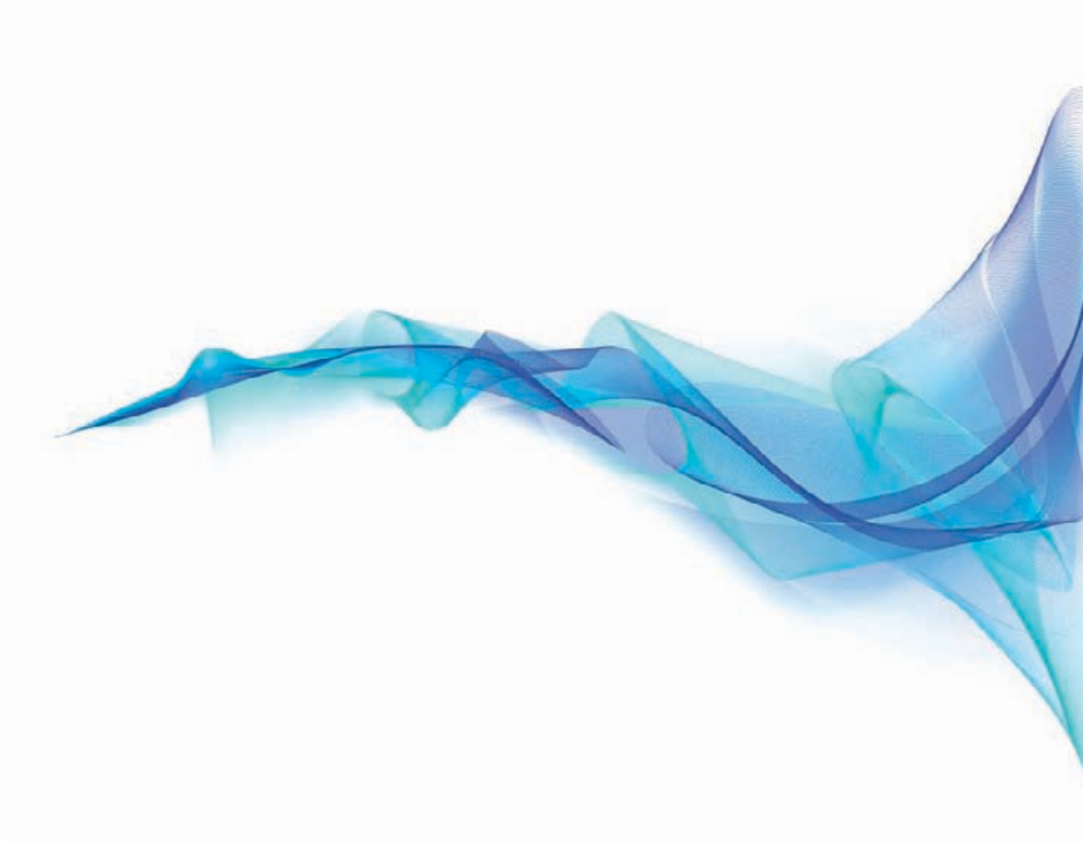

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