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ELECTROCEUTICAL TREATMENT AND RESOLUTION OF:

DIABETIC NEUROPATHY, ARTERIAL ISCHEMIA, STENOSIS, AND PROGRESSIVE GANGRENE

284 DIABETIC PATIENT CLINICAL STUDY AND CUMULATIVE RESEARCH DATA REPORT

F. Savery, M.D., R. Sorgnard, Ph.D., J. Woessner, M.D., Ph.D.

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ABSTRACT:

A group (A) of 284 diabetic patients with severe Diabetic Neuropathy experienced analgesic relief of nocturnal pain and regained normal vascularization, normal sensation, and normal EMG following a series of specific-parameter electroceutical treatments. Included in this cumulative report of 284 diabetic patients, 194 (B) presented with moderate to severe arterial ischemia and/or stenosis, and 27 (C) patients with total occlusion (progressive gangrene). Overall, (A) 247 patients (87%) reported total recovery treatment success or a *definite* positive influence with their condition (i.e., pain resolution, vascular change, new tissue growth, etc.). Although 28 patients in group (B) and 9 patients in group (C) reported no substantial improvement with their overall condition, all described some favorable influence and thought the treatment was worthwhile.

Keywords: Electroceutical treatment; Vascularization, Diabetic Neuropathy; Arterial Ischemia; Stenosis; Progressive Gangrene

INTRODUCTION:

The electroceutical treatment approach is based on the application of controlled, specific-parameter electrical impulses. Electrical current is altered via special step-down transformers into electric impulses that mimic the human bioelectric system.

Despite the complexity of the nervous system as a whole, the structure and function of individual nerve cells is understood in great detail - more than any other type of cell. It is well known that electric impulses are conducted along the length of every nerve cell and that their function is to code and transmit all biophysiological information by varying the frequency of their occurrence.

Well documented research efforts in the field of clinical electromedicine by Doctors - Sorgnard, May, Hansjurgens, Schwartz, and the Clinical Electromedical Research Academy (CERA) have helped to bring about a less confusing view of electromedical science. Electromedicine is now categorized into two (2) different, distinct classifications based upon comparing and merging the physiological effects produced via

specific bioelectric pulses with the desired biophysiological effects necessary for medical treatment success. The two (2) electromedical classifications are:

The Stimulatory Class: Physiological effects induced by repeated action impulse propagation in excitable cells - cell membrane depolarization and repolarization activity.

Multi-facilitory Class: Physiological effects induced without repeated action impulses. Some mechanisms known are; hormone/ligand imitation, cellular oscillo/torsional response, ionic transport (D.C.), sustained membrane depolarization, second messenger formation, et cetera.

With continued research comes great insight into the most accurate electroceutical parameters or the proper combination of the two (2) known electromedical classes necessary to facilitate optimum patient treatment success.

DISCUSSION:

Clinical treatment application of specific bioelectric impulses in peripheral vascular disease with an endocrine etiology has been evaluated in 284 participating diabetic patients. Pursuant to the electroceutical treatment parameters and guidelines requested by the attending research physicians, all patients were administered specific electroceutical treatments.

Initial treatments consisted primarily of the *Multi-facilitory Class (Mf)* of electroceuticals because of the direct association with second messenger formation (cyclic AMP), which activates the regeneration (repair) processes; the cellular oscillo/torsional response with assists in balancing metabolic concentration differences (pH normalization); enhanced filtration and diffusion processes (mitigation of tissue acidosis) and potent analgesia.

While Multi-facilitory (Mf) treatments are applied, neuron blockade occurs - resulting in potent analgesia. Longer-lasting analgesia is accomplished through a balance of metabolic concentration differences and increased enzyme synthesis. As a result of increased activation energy and of the cellular oscillo-torsional response, metabolic end products and pain and inflammation mediators are redistributed and more efficiently eliminated by the body.

In diabetes, accumulation of sorbitol in Schwann cells causes osmotic damage with segmental demyelination. Peripheral nerves are probably affected by small vessel disease. Ischemental changes in the nerve presumably result from proliferation of the endothelium in blood vessels and abnormalities of the capillaries.

As treatment progresses, protocols of alternating *Stimulatory Class (Si)* electroceuticals *and Multi-facilitory Class (Mf)* electroceuticals are initiated to ensure a maximum range of bio-physiological effects. Such effects are reflected by the changes in the activity of acetyleholinesterase. The effect of catecholamines, which is evidenced by an anti-curare effect and also by he ability to augment the repetitive firing and twitch potentiation produced by neostigmine and other drugs, is presumably due to the ability of bioelectric energy stimulus to increase the release of acetylcholine at nerve endings. Vasodilation produced by stimulus to increase the release of acetylcholine at nerve endings. Vasodilation produced electroceutical treatment has distinct analgesic and revascularization effects, which lead to rapid improvement of blood supply and elimination of pain in lesions of peripheral myelinated nerves.

SAMPLE CASE HISTORY:

A 47-year old female patient with diabetes developed severe neuropathy manifested by complete bilateral loss of sensation and of vibration and position senses in the lower extremities, accompanied by deep pain characterized by throbbing. Three weeks after the appearance of these symptoms she developed arterial stenosis of the left foot with evidence of total occlusion. A blister on one (1) toe developed into progressive gangrene, for which an orthopedist advised amputation of the toe. Antibiotics were administered as part of routine treatment and the patient was started on multi-facilitory (Mf) electroceutical treatments BID 20 minutes each.

After 8 treatments revascularization started characterized by complete return of sensation, cessation of pain, reduced inflammation, and arrest of the progressive gangrene. The protocol treatment was changed to an alternation between *Stimulatory (St) Class* and *Multi-facilitory (Mf) Class* electroceutical treatment. After 10 treatments, complete arrest of gangrene with new growth of tissue was noted, all pain was relieved and pharmaceutical analgesics were withdrawn. EMG prior to electroceutical treatment indicated a reduced number of motor unit waveforms with increased duration and amplitude -- fibrillation potential and positive sharp waves were also apparent. Repeat EMG appeared normal, with no fibrillation or denervation potentials. The patient continued to receive treatment intermittently (2 times weekly). Fasting blood sugar level remained the same and the patient continued regular pharmacological therapy and diet. The single most common finding was the return of normal deep tendon reflexes with normal knee and ankle jerk in all patients. At a one-year follow-up, the patient is still clinically free from pain and shows no significant vascular pathology.

SUMMARY AND CONCLUSION:

Our clinical experience and patient trial data has shown that the application of electroceutical treatment with specific electroceutical parameters favorably influences the peripheral vasculature - promoting nerve and cell nutrition while, stirnulation of motor nerve fibers results in excitation of the muscle fibers. This has two effects on the blood flow: energy is used up, the metabolic rate is increased, and blood flow in enhanced in the region of stimulating muscles. In addition, through the contraction activity of the muscle group, an active stimulation of the venous backflow occurs. Also, electroceutical applications directly influences blood flow and lymph transport via sympathetic function imitation. Exogenous stimuli at specific pulse rates induce synchronous synaptic release of the neurotransmitter, Norepinephrine without depletion. Norepinephrine reacts with Alpha-receptors causing contraction of vessel smooth muscle. Vasoconstriction is necessary to treat the inflammation and edema normally present in diabetic neuropathy patients. Vasoconstriction achieves these effects by pushing intravascular fluid in a central direction to the heart, reducing the influx from arteries and enabling the extra-capillary fluid to penetrate the intravascular space to improve the drainage function of the capillary system.

Asymmetric neuropathy or mononeuropathy is due to metabolic abnormalities of the neurons of Schwann cells, whereas symmetric or focal neuropathy is due to vascular occlusion and ischemia. Ulcers on the plantar aspect of the foot in Charcot joint neuropathy are due to weakness of the intrinsic muscles of the foot and consequent abnormal pressure distribution. Therefore, timely application of electroceutical treatment is not only effective with respect to peripheral vascular dilation, it appears that it improves or strengthens the intrinsic muscles of the foot, relieving abnormal pressure distributions. We also believe the results achieved with electroceutical treatment are as effective as aldose reductase inhibitors and glycosylation.

There appears to be enough evidence to encourage the use of electroceutical treatment in all diabetic neuropathy, arterial ischemia, arterial stenosis and progressive gangrene. These specific treatments, especially the alternation of Stimulatory Class (St) electroceuticals and Multi-facilitory (Mf) electroceuticals, have placed us at the threshold of discovery and its time to apply this knowledge in other clinical settings.

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Pulsed magnetic field therapy in refractory neuropathic pain secondary to peripheral neuropathy: electrodiagnostic parameters--pilot study.

Weintraub MI[1], Cole SP.

Author information

Abstract

CONTEXT: Neuropathic pain (NP) from peripheral neuropathy (PN) arises from ectopic firing of unmyelinated C-fibers with accumulation of sodium and calcium channels. Because pulsed electromagnetic fields (PEMF) safely induce extremely low frequency (ELF) quasirectangular currents that can depolarize, repolarize, and hyperpolarize neurons, it was hypothesized that directing this energy into the sole of one foot could potentially modulate neuropathic pain.

OBJECTIVE: To determine if 9 consecutive 1-h treatments in physician's office (excluding weekends) of a pulsed signal therapy can reduce NP scores in refractory feet with PN.

DESIGN/SETTING/PATIENTS: 24 consecutive patients with refractory and symptomatic PN from diabetes, chronic inflammatory demyelinating polyneuropathy (CIDP), pernicious anemia, mercury poisoning, paraneoplastic syndrome, tarsal tunnel, and idiopathic sensory neuropathy were enrolled in this nonplacebo pilot study. The most symptomatic foot received therapy. Primary endpoints were comparison of VAS scores at the end of 9 days and the end of 30 days follow-up compared to baseline pain scores. Additionally, Patients' Global Impression of Change (PGIC) questionnaire was tabulated describing response to treatment. Subgroup analysis of nerve conduction scores, quantified sensory testing (QST), and serial examination changes were also tabulated. Subgroup classification of pain (Serlin) was utilized to determine if there were disproportionate responses.

INTERVENTION: Noninvasive pulsed signal therapy generates a unidirectional quasirectangular waveform with strength about 20 gauss and a frequency about 30 Hz into the soles of the feet for 9 consecutive 1-h treatments (excluding weekends). The most symptomatic foot of each patient was treated.

RESULTS: All 24 feet completed 9 days of treatment. 15/24 completed follow-up (62%) with mean pain scores decreasing 21% from baseline to end of treatment ($P=0.19$) but with 49% reduction of pain scores from baseline to end of follow-up ($P<0.01$). Of this group, self-reported PGIC was improved 67% (n=10) and no change was 33% (n=5). An intent-to-treat analysis based on all 24 feet demonstrated a 19% reduction in pain scores from baseline to end of treatment ($P=0.10$) and a 37% decrease from baseline to end of follow-up ($P<0.01$). Subgroup analysis revealed 5 patients with mild pain with nonsignificant reduction at end of follow-up. Of the 19 feet with moderate to severe pain,

there was a 28% reduction from baseline to end of treatment (P<0.05) and a 39% decrease from baseline to end of follow-up (P<0.01). Benefit was better in those patients with axonal changes and advanced CPT baseline scores. The clinical examination did not change. There were no adverse events or safety issues.

CONCLUSIONS: These pilot data demonstrate that directing PEMF to refractory feet can provide unexpected shortterm analgesic effects in more than 50% of individuals. The role of placebo is not known and was not tested. The precise mechanism is unclear yet suggests that severe and advanced cases are more magnetically sensitive. Future studies are needed with randomized placebo-controlled design and longer treatment periods.

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The use of pulsed electromagnetic fields with complex modulation in the treatment of patients with diabetic polyneuropathy.

Musaev AV[1], Guseinova SG, Imamverdieva SS.

Author information

Abstract

Clinical and electroneuromyographic studies were performed in 121 patients with diabetic polyneuropathy (DPN) before and after courses of treatment with pulsed electromagnetic fields with complex modulation (PEMF-CM) at different frequencies (100 and 10 Hz). Testing of patients using the TSS and NIS LL scales demonstrated a correlation between the severity and frequency of the main subjective and objective effects of disease and the stage of DPN. The severity of changes in the segmental-peripheral neuromotor apparatus--decreases in muscle bioelectrical activity, the impulse conduction rate along efferent fibers of peripheral nerves, and the amplitude of the maximum M response--depended on the stage of DPN and the duration of diabetes mellitus. The earliest and most significant electroneuromyographic signs of DPN were found to be decreases in the amplitude of the H reflex and the Hmax/Mmax ratio in the muscles of the lower leg. Application of PEMF-CM facilitated regression of the main clinical symptoms of DPN, improved the conductive function of peripheral nerves, improved the state of Ia afferents, and improved the reflex excitability of functionally diverse motoneurons in the spinal cord. PEMF-CM at 10 Hz was found to have therapeutic efficacy, espccially in the initial stages of DPN and in patients with diabetes mellitus for up to 10 years.

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ARTICLE

E. Bosi · M. Conti · C. Vermigli · G. Cazzetta · E. Peretti · M. C. Cordoni · G. Galimberti · L. Scionti

Effectiveness of frequency-modulated electromagnetic neural stimulation in the treatment of painful diabetic neuropathy

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Abstract *Aims/hypothesis:* The largely unsatisfactory results reported for the pharmacological treatment of diabetic neuropathy has spurred the search for alternative therapies. The aim of this study was to evaluate the efficacy of frequency-modulated electromagnetic neural stimulation (FREMS) as a novel treatment for painful diabetic neuropathy. *Methods:* Patients (n=31) with painful neuropathy associated with decreased nerve conduction velocity (<40 m/s) and increased vibration perception threshold (>25 V) were enrolled in a randomised, double-blind, crossover study designed to compare the effects of FREMS with those of placebo. Each patient received two series of ten treatments of either FREMS or placebo in random sequence, with each series lasting no more than 3 weeks. The primary efficacy end point was the change in pain measured by a visual analogue scale (VAS). *Results:* FREMS induced a significant reduction in daytime and night-time VAS pain score (all p<0.02). Furthermore, FREMS induced a significant increase in sensory tactile perception, as assessed by monofilament; a decrease in foot vibration perception threshold, as measured by a biothesiometer; and an increase in motor nerve conduction velocity (all p<0.01). No significant changes were observed after placebo. Comparison of measurements at the 4-month follow-up with those at baseline revealed that a significant benefit persisted for all measures that showed an improvement at the end of treatment, with an additional improvement in quality of life evaluated by the Short Form-36 questionnaire (all p< 0.05). No significant side effects were recorded during the study. *Conclusions/interpretation:* FREMS is a safe and effective therapy for neuropathic pain in patients with diabetes and is able to modify some parameters of peripheral nerve function.

Keywords Clinical trial · Diabetes · Electromagnetic stimulation · Painful neuropathy

Abbreviations FREMS: frequency-modulated electromagnetic neural stimulation · MNCV: motor nerve conduction velocity · SF36: Short Form-36 questionnaire · SNCV: sensory nerve conduction velocity · TENS: transcutaneous electrical nerve stimulation · VAS: visual analogue scale · VEGF: vascular endothelial growth factor

Introduction

Peripheral neuropathy is a frequent and disabling microvascular complication of both type 1 and type 2 diabetes [1]. This condition may be prevented by good blood glucose control [2]; however, it is at best halted, once established, even after long-term blood glucose normalisation, such as that observed following successful pancreas transplantation [3, 4]. The pathological hallmarks of diabetic neuropathy are microangiopathy of the vasa nervorum, loss of axons and axonal atrophy, all of which are the result of a combination of different mechanisms of tissue damage that are common to all long-term complications of diabetes [5].

The pharmacological treatment of diabetic neuropathy is largely unsatisfactory, mainly due to a lack of drugs that act on the underlying pathogenetic mechanisms. Aldose reductase inhibitors are among the few compounds with this mode of action; however, the results of clinical trials performed to date have been disappointing [6]. Consequently, current therapy is purely symptomatic, aiming to relieve the pain associated with neuropathy through the administration of various analgesics, tricyclic antidepressants, anti-arrhythmics [7] and, more recently, the new anti-epileptic agents gabapentin [8] and lamotrigine [9], and opioids [10].

E. Bosi (✉) · M. Conti · E. Peretti · G. Galimberti
Diabetes and Endocrinology Unit,
Department of General Medicine,
Vita-Salute San Raffaele University Hospital,
Via Olgettina, 60,
20132 Milan, Italy
e-mail: bosi.emanuele@hsr.it
Tel.: +39-02-26432818
Fax: +39-02-26432827

C. Vermigli · G. Cazzetta · M. C. Cordoni · L. Scionti
Department of Internal Medicine, University of Perugia,
Perugia, Italy

Non-pharmacological symptomatic treatments have also been proposed, including acupuncture [11], near-infrared phototherapy [12], low-intensity laser therapy [13], static and pulsed magnetic field therapies [14, 15], and various electrotherapies, including transcutaneous electrical nerve stimulation (TENS) [16, 17], percutaneous electrical nerve stimulation [18] and spinal cord electrostimulation [19]. The rationale for the use of electrical nerve stimulation in diabetic neuropathy is based on its historical, though controversial, use in various painful clinical conditions [20] and on some beneficial effects reported in the treatment of other diabetic complications, such as foot ulcers [21].

Frequency-modulated electromagnetic neural stimulation (FREMS) has recently been developed as a novel electrotherapy. This method is different from TENS and other known electrotherapy systems, as it uses sequences of modulated electrical stimuli that vary automatically in terms of pulse frequency, duration and voltage amplitude. The FREMS method was designed on the basis of the hypothesis that the summation of sub-threshold electrical stimuli, conveyed through the skin proximal to a motor nerve in a non-invasive system, would induce composite motor action potentials in excitable tissues. A single impulse of low intensity and short duration, such as that used by conventional electrotherapies, is unable to overcome the dielectric skin barrier to excite the underlying nervous or muscular tissue. However, FREMS achieves this effect through specific sequences of weak impulses, characterised by a rapid increase and decrease in pulse frequency and duration, which result in the gradual recruitment of membrane potentials in the stimulated tissues [22].

These characteristics prompted us to evaluate the therapeutic potential of FREMS in human diabetic neuropathy. In this paper we report the results of a two-centre, randomised, double-blind, placebo-controlled, crossover clinical trial on FREMS treatment of patients with painful diabetic neuropathy.

Subjects and methods

Study design and end points The study had a randomised, double-blind, placebo-controlled, crossover design. The primary end point was the change in grading of daytime and night-time pain, as assessed using a visual analogue scale (VAS). Secondary end points were changes in: sensitivity to monofilament; vibration perception threshold, as measured by a biothesiometer; quality of life, as assessed by questionnaire; motor nerve conduction velocity (MNCV); and sensory nerve conduction velocity (SNCV). The treatment consisted of ten sessions of placebo followed by ten sessions of FREMS (sequence 1) or vice versa (sequence 2) at random, separated by a wash-out period of 1 week. Each treatment session was administered at intervals of at least 24 h, and each ten-session series lasted no more than 3 weeks. Randomisation to sequence 1 or sequence 2 was performed centrally at the time of enrolment. Principal investigators, physicians, nurses, technicians and statisticians were unaware of treatment assignment.

Characteristics of FREMS Treatment with FREMS was performed using sequences of monophase-compensated negative potential electrical pulses that are characterised by a sharp spike and an asymmetrical shape (peak amplitude variable from 0–255 V, pulse frequency variable within the range 1–50 Hz, pulse duration variable within the range 10–40 μs).

Administration of FREMS and placebo Electrotherapy and placebo were administered using the Physioflog ETS 501 (Lorenz Therapy System; Lorenz Biotech, Medolla, Italy) via four electrodes applied to the lower extremities; the original device was modified by the addition of a switch to apply treatment A (later revealed to be placebo) or treatment B (later revealed to be FREMS). Each session of either placebo or FREMS lasted for 30 min. Placebo consisted of no electric current transmission. This placebo was chosen after a preliminary study had shown that patients with a vibration perception threshold higher than 25 V effectively had no perception of the electrical stimuli administered by the FREMS device (data not shown). These findings were in accordance with those reported by two other studies showing a direct correlation between vibration perception threshold measured by a biothesiometer and current perception threshold measured by a Neurometer (Neurotron, Baltimore, MD, USA) across the same range of frequencies used by FREMS [23, 24]. During sessions of either placebo or FREMS, patients were invited to modulate the delivery of neurostimulation themselves, by progressively increasing the voltage of electrical stimulation along a scale of 0–255 V through a manually gradable remote control device that increased the voltage by 1 V per step up to the maximal allowed, which corresponded to the possible perception of burning at the site of the electrode.

Subjects Patients who met the following criteria were invited to participate in the study: (1) type 1 or type 2 diabetes according to American Diabetes Association criteria [25]; (2) age between 18 and 70 years; (3) painful diabetic neuropathy with reduced sensory and/or MNCV (<40 m/s in at least one nerve trunk of lower limbs); and (4) vibration perception at big toe >25 V. Exclusion criteria were: (1) the presence of any other severe disease; (2) pregnancy; (3) renal disease with serum creatinine levels >1.77 μmol/l; (4) a history or actual presence of foot ulcers; and (5) lower limb vasculopathy as indicated by an ankle-brachial index <0.9 or a transcutaneous partial pressure of oxygen <50 mmHg. Any analgesic or other drug administered for the chronic treatment of painful neuropathy was discontinued at least 3 weeks before randomisation. Patients were enrolled at two centres: Milan and Perugia. The study protocol was approved by the ethics committees of San Raffaele University Hospital and Perugia University Hospital, and written informed consent was obtained from all patients prior to enrolment.

Clinical assessments Patients were evaluated four times: at baseline, at the end of each series, and 4 months after the completion of the study. Each patient saw the same phy-

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Pulsed electromagnetic fields to reduce diabetic neuropathic pain and stimulate neuronal repair: a randomized controlled trial.

Weintraub MI[1], Herrmann DN, Smith AG, Backonja MM, Cole SP.

Author information

Abstract

OBJECTIVE: To determine whether repetitive and cumulative exposure to low-frequency pulsed electromagnetic fields (PEMF) targeting painful feet can reduce neuropathic pain (NP), influence sleep in symptomatic diabetic peripheral neuropathy (DPN), and influence nerve regeneration.

DESIGN: Randomized, double-blind, placebo-controlled parallel study.

SETTING: Sixteen academic and clinical sites in 13 states.

PARTICIPANTS: Subjects (N=225) with DPN stage II or III were randomly assigned to use identical devices generating PEMF or sham (placebo) 2 h/d to feet for 3 months.

INTERVENTIONS: Nerve conduction testing was performed serially.

MAIN OUTCOME MEASURES: Pain reduction scores using a visual analog scale (VAS), the Neuropathy Pain Scale (NPS), and the Patient's Global Impression of Change (PGIC). A subset of subjects underwent serial 3-mm punch skin biopsies from 3 standard lower limb sites for epidermal nerve fiber density (ENFD) quantification.

RESULTS: Subjects (N=225) were randomized with a dropout rate of 13.8%. There was a trend toward reductions in DPN symptoms on the PGIC, favoring the PEMF group (44% vs 31%; P=.04). There were no significant differences between PEMF and sham groups in the NP intensity on NPS or VAS. Twenty-seven subjects completed serial biopsies. Twenty-nine percent of PEMF subjects had an increase in distal leg ENFD of at least 0.5 SDs, while none did in the sham group (P=.04). Increases in distal thigh ENFD were significantly correlated with decreases in pain scores.

CONCLUSIONS: PEMF at this dosimetry was noneffective in reducing NP. However neurobiological effects on ENFD, PGIC and reduced itching scores suggest future studies are indicated with higher dosimetry (3000-5000 G), longer duration of exposure, and larger biopsy cohort.

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Review

Photobiomodulation Therapy (PBMT) in Peripheral Nerve Regeneration: A Systematic Review

Marcelie Priscila de Oliveira Rosso [1], Daniela Vieira Buchaim [2,3], Natália Kawano [2], Gabriela Furlanette [2], Karina Torres Pomini [1] and Rogério Leone Buchaim [1,2,*]

1 Department of Biological Sciences (Anatomy), Bauru School of Dentistry, University of São Paulo (USP), Alameda Dr. Octávio Pinheiro Brisola 9-75, Vila Nova Cidade Universitária, Bauru, São Paulo CEP 17012-901, Brazil; marcelierosso@usp.br (M.P.d.O.R.); karinatorrespomini@gmail.com (K.T.P.)

2 Medical School, Discipline of Human Morphophysiology, University of Marilia (UNIMAR), Av. Higino Muzi Filho, 1001 Campus Universitário, Jardim Araxa, Marília, São Paulo CEP 17525-902, Brazil; danibuchaim@usp.br (D.V.B.); natalia.kawano@hotmail.com (N.K.); gafurla@hotmail.com (G.F.)

3 Medical School, Discipline of Neuroanatomy, University Center of Adamantina (UNIFAI), Rua Nove de Julho, 730, Centro, Adamantina, São Paulo CEP 17800-000, Brazil

* Correspondence: rogerio@fob.usp.br; Tel.: +55-14-3235-8226

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Abstract: Photobiomodulation therapy (PBMT) has been investigated because of its intimate relationship with tissue recovery processes, such as on peripheral nerve damage. Based on the wide range of benefits that the PBMT has shown and its clinical relevance, the aim of this research was to carry out a systematic review of the last 10 years, ascertaining the influence of the PBMT in the regeneration of injured peripheral nerves. The search was performed in the PubMed/MEDLINE database with the combination of the keywords: low-level laser therapy AND nerve regeneration. Initially, 54 articles were obtained, 26 articles of which were chosen for the study according to the inclusion criteria. In the qualitative aspect, it was observed that PBMT was able to accelerate the process of nerve regeneration, presenting an increase in the number of myelinated fibers and a better lamellar organization of myelin sheath, besides improvement of electrophysiological function, immunoreactivity, high functionality rate, decrease of inflammation, pain, and the facilitation of neural regeneration, release of growth factors, increase of vascular network and collagen. It was concluded that PBMT has beneficial effects on the recovery of nerve lesions, especially when related to a faster regeneration and functional improvement, despite the variety of parameters.

Keywords: low-level laser therapy; nerve regeneration; peripheral nerve repair; photobiomodulation therapy; tissue regeneration

1. Introduction

Low-level laser therapy (LLLT), now commonly referred to as photobiomodulation therapy (PBMT), using low-level infrared light spectrum lasers is considered a therapeutic advance. Its effects are related to tissue biostimulation, presenting therapeutic responses from photoelectric, photoenergetic, and photochemical reactions [1]. Scientific research has shown the application of PBMT in bone tissue and peripheral nerves with good results whether or not it is associated with other supporting methods in tissue repair [2–7].

Laser photobiomodulation presents itself as an electromagnetic technology that is being inserted into clinical practice due to its characteristics that differ from other conventional thermal sources [8,9]. It was observed that there are several features of PBMT that are related to the reduction of tissue repair time and its capacity to increase cell proliferation [10].

In rehabilitative health, PBMT was inserted to promote the repair and recovery of tissues. For example, in physical therapy, the use of PBMT is applied in postoperative phases as an aid in the muscular, nervous, joint, and other functional recovery processes, and in dentistry it is applied in the processes of dental extraction, grafting, osteonecrosis, and periodontal lesions [11–13].

The wavelength of infrared irradiation is easily absorbed by tissues and the loss of intensity is minimal, affecting metabolic modifications, DNA activity, adenosine triphosphate (ATP) formation, and the mitochondrial chain. The effect of photobiomodulation is due to the absorption of the photons by cytochrome C oxidase in the mitochondrial respiratory chain, consequently increasing the cytochrome C oxidase activity and therefore ATP formation. ATP from injured or regions of impaired blood perfusion can reactivate injured cells and metabolic disorders [10]. PBMT is also related to pain and inflammation relief and prevention of tissue death to avoid neurological degeneration [14,15].

The wavelength is the key point that regulates the depth and penetration of the laser irradiance in the tissue, noting that the absorption and dispersion coefficients are larger at the lower wavelengths. Regarding the type of wave, whether continuous or pulsed, there are still divergences in which is the best and for which factors are the pulse parameters to be chosen [16]. PBMT presents difficulties in selecting the most suitable parameters for its application due to the lack of standardization, since wavelength, power density, irradiation time, and light polarization have repercussions on the biological effects [9].

Due to the photochemical and photobiological effects of PBMT at the cellular level, there is a relationship between the improvement of trophic conditions and the reduction of inflammatory processes, closely related to a more efficient nervous regeneration and, also, promoting the secretion of neural factors [16,17]. Thus, photobiomodulation therapy in the neurological area acts as an adjuvant in the treatment of traumatic brain degeneration/injury, spinal cord trauma, and in the process of peripheral nerve regeneration.

Peripheral nerve lesions are a reality today, but there is a deficit in relating effective treatments for recovery of the nerves, resulting in considerable functional changes in the daily life of the individual. When injured, the nerve can lose its function, causing motor or sensitive deficits. There is retrograde axonal degeneration to the area of the lesion, so regeneration occurs slowly and sometimes incompletely [18,19].

At the end of the 80's, the scientific interest in the therapeutic approach of rehabilitation for neural lesions was initiated [20], due to the good results with the use of PBMT in the recovery of injured peripheral nerves but, until the present day, there are still difficulties related to the application parameters [19,20]. Its beneficial effects are independent of the repair technique, neurorrhaphy techniques, and the use of fibrin sealants [3,6,7,21].

PBMT leads to changes in important vascular levels such as elevation of the secretion of antiapoptotic factors in ischemic organs, providing a better wound healing [22,23]; the presence of angiogenesis when ischemic organs were injured [24,25]; a decrease in the site of infarction in rats; as well as elevation in neurological scores following embolic stroke in rats [26,27].

Due to the high range of benefits that PBMT has shown and its clinical relevance of application, the aim of this research was to carry out a systematic review of the scientific papers published in the last 10 years verifying the relation of PBMT with the regeneration of injured peripheral nerves.

2. Materials and Methods

A search was performed in the PubMed/MEDLINE database, combining low-level laser therapy AND nerve regeneration keywords, over the last 10 years and restricted to the English language. The next step was to restrict the verification and consultation of articles that used animals as a study object (non-human species).

We verified those articles that presented titles and summaries that approached the subject of this research, as well as methodology, results, and relevance for its practical application.

The articles included should necessarily be presented with full access to the text. The acquired texts were analyzed and synthesized in a reflexive way in order to obtain consistent information on the subject.

3. Results

Initially, 54 articles were obtained from the PubMed/MEDLINE database, of which 28 were excluded because they were not included in the search criteria (in English, study in animals, and full access to content). At the end, 26 articles related to the subject were included. Figure 1 schematizes the search system, according to PRISMA Flow Diagram [28].

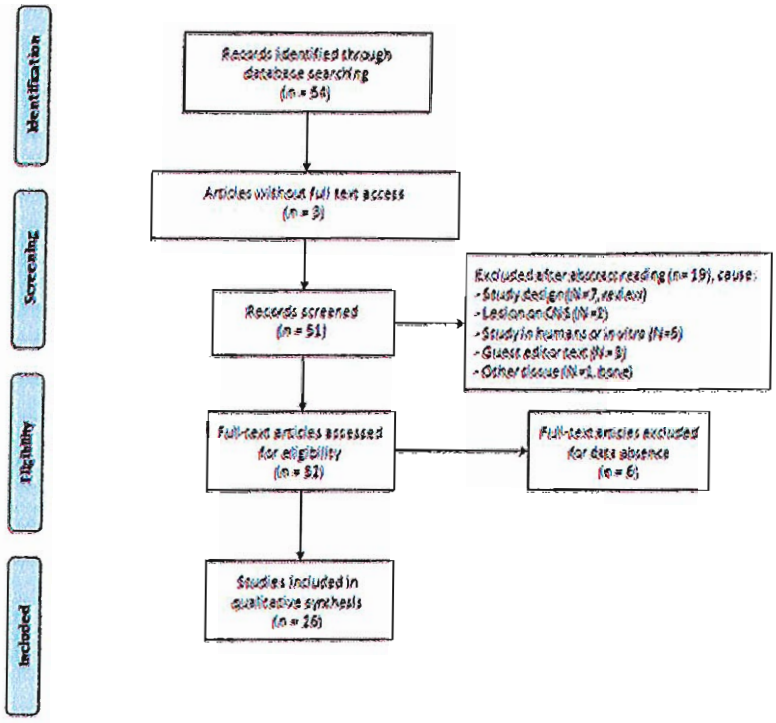

Figure 1. Design used to select the articles.

Table 1 summarizes the data presented in the 26 articles selected for this research.

Table 1. Data of selected articles.

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam	Energy (mW)	Energy Density (J/cm²)	Radiation Amount	Variables	Irradiation Site	Evaluation Time	Main Results
Buchaim et al. [5]	GaAlAs (Laserpulse IBRAMED, Brazil)	660/0.116	30	4	16 s per point; 3 points	Sural nerve graft was coapted to the vagus nerve using the fibrin glue.	Right side of the neck.	Application on the 1st day post-operatory, 5 weeks, 3 times a week. Evaluation 30 days after irradiation.	LLLT improved the nerve regeneration.
Buchaim et al. [3]	GaAlAs (Laserpulse IBRAMED, Brazil)	830/0.116	30	6	24 s per point; 3 points	Neurotmeses of buccal branch of facial nerve, followed by end-to-end suture or coaptation with heterologous fibrin sealant derived from snake venom	On the surgical site, on both sides of the face	Application 1st day post-operatory, 3 times/week for 5 weeks. Evaluation 5 and 10 weeks after the surgery.	LLLT showed satisfactory results on facial nerve regeneration.
Buchaim et al. [6]	GaAlAs (Laserpulse IBRAMED®, Brazil)	830/0.116	30	6.2	24 s per point; 3 points	Neurotmeses of buccal branch of facial nerve, end-to-end anastomosis. Use of epineural suture or coaptation with heterologous fibrin sealant derived from snake venom.	On the surgical site, on both sides of the face	Application on the 1st day post-operatory, 3 times a week, for 5 weeks.	Laser stimulated axonal regeneration accelerated the process of functional recovery of whisker, and the two techniques used allowed the growth of axons.
Rosso et al. [7]	GaAlAs (Laserpulse IBRAMED®, Brazil)	830/0.116	30	6.2	24 s per point; 3 points	Neurotmeses in buccal branch of facial nerve, end-lateral anastomosis in the zygomatic branch of the facial nerve with epineural suture or heterologous sealant of fibrin derived from snake venom.	Or. the surgical site, on both sides of the face	Application on the 1st day post- operatory, 3 times a week, for 10 weeks.	Laser groups presented faster functional recovery, similar results to the control group. It was observed that PBMT provided accelerated morphological and functional repair in the two techniques used.
Ziago et al. [19]	GaAlAs (Twin Laser, MMO, São Carlos, SP, Brazil)	780/0.04	40	4 10 50	4, 10 e 50 s per point; 3 points	Crushing of the left sciatic nerve.	On the surgical site	Application during 6 sessions on alternate days.	Best morphological quantitative and morphometric results on L10 group after 15 days of nerve lesion.
Alessi Pissulin et al. [29]	GaAs (Endophoton, KLD Biosystems, Amparo, Brazil)	904/0.035	50	69	48 s per point	0.5% bupivacaine injection to the right and 0.9% sodium chloride injection to the left on sternocleidomastoid muscle and accessory nerve exposed in surgery.	Ventral side of the neck	Application 1st day post-operatory, during 5 successive days.	LLLT reduced the aggressive effects of bupivacaine on the nerve and the muscle, of muscular degeneration, of myonecrosis and fibrosis, kept the morphology of the axon and the myelin sheath.
Takhtfooladi; Sharifi [30]	GaAlAs (pulsed) LED (red and blue) (—)	680/0.04 650/1.5 red 450/1.5 blue	10	10	200 s per point; 3 points	Neurotmeses of right sciatic nerve followed by epineural neurorrhaphy.	On the surgical site, sciatic nerve	Application 1st day post-operatory, during 14 successive days	LLLT increased Schwann cells on the great myelinic axons and on neurons, sped up and potentialized nerve regeneration.

Table 1. *Cont.*

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam	Energy (mW)	Energy Density (J/cm²)	Radiation Amount	Variables	Irradiation Site	Evaluation Time	Main Results
Takhtfooladi et al. [31]	InGaAlP (Teralaser; DMC®São Carlos, SP, Brazil)	685/0.028	15	3	10 s per point	Crushing of the left sciatic nerve.	On the surgery site on sciatic nerve.	Application on the 1st day post-operatory, during 21 successive days.	LLLT accelerated and improved the nerve function after crushing lesion.
Wang et al. [32]	GaAlAs (Transverse IND. CO., LTD., Taipei, Taiwan)	808/3.8	170	3 8 15	67.2 s 179 s 335.6 s	Crushing of the right sciatic nerve.	On lesion on sciatic nerve.	Application during 20 successive days.	LLLT (3 and 8 J/cm²) accelerated functional and morphologic recovery of the nerve, increased the expression of the marker GAP43.
Shen; Yang; Liu [33]	AlGaInP (Megalas1-AM-800, Konftec Co., Taipei, Taiwan, ROC)	660/—	0.0032	3.84	5 min per day	Neurotmeses of the left sciatic nerve, 10 mm gap and use of biodegradable tube containing genipin-cross-linked gelatin annexed with β-tricalcium phosphate ceramic particles (genipin-gelatin-tricalcium phosphate, GGT)	Applied to the surgical site.	Application 1st day post-operatory, during 20 successive days. Euthanasia after 8 weeks.	LLLT obtained better functional, electrophysiological and histomorphometric results and assisted on neural repair.
Shen; Yang; Liu [34]	AlGaInP (MegalasVR -AM-800; Konftec, Taipei, Taiwan)	660/—	50	Immediate post-surgery (5.76) 9 following days (0.96)	Immediate post-surgery (30 min) 9 successive consecutive (5 min)	Neurotmeses of the left sciatic nerve, 15 mm gap and the use of 1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide (EDC) cross-linked gelatin, annexed with β-tricalcium phosphate (TCP) ceramic particles (EDC-Gelatin-TCP, EGT).	On the surgery site.	Application immediately after the lesion, during 9 successive days. Euthanasia after 12 weeks.	LLLT showed better results on the functional index, on development, on electrophysiology, on nerve regeneration, larger neural tissue area, larger axon, and myelin sheath diameter.
Medalha et al. [35]	GaAlAs (Teralaser, DMC São Carlos, São Paulo, Brazil)	660/0.028 808/0.028	30 30	10 e 50 10 e 50	9 s and 47 s; 3 points 9 s and 47 s; 3 points	Neurotmeses of the sciatic nerve, approximately 3 mm distal to the tendon of the internal obturator. Anastomosis with 3 sutures using nylon monofilament 10-0.	Applied to the surgical site.	Application 1st day post-operatory during 5 successive days and 2 days interval until completing 15 days.	LLLT 808 nm on 50 J/cm² obtained higher fiber density. LLLT 660 nm on 50 J/cm² presented larger diameters of axons and of fibers of gait functional recovery.
Shen et al. [36]	GaAlAsP (Aculas-AM-100A, Konftec Co., Taipei, Taiwan)	660/0.1	50	2	2 min per day; 2 points at the same time	A biodegradable nerve conduit containing genipin-cross-linked gelatin was annexed using beta-tricalcium phosphate (TCP) ceramic particles (genipin-gelatin-TCP, GGT) with a 15 mm sciatic nerve transection gap.	On the sciatic nerve.	Application 1st day post-operatory during 10 successive days.	LLLT accelerated the nerve regeneration due to the larger neural tissue, larger diameter and thicker myelin sheath, motor function, electrophysiology and muscular innervation.

Table 1. *Cont.*

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam	Energy (mW)	Energy Density (J/cm²)	Radiation Amount	Variables	Irradiation Site	Evaluation Time	Main Results
Chen et al. [37]	GaAlAs (Transverse IND. CO., LTD., Taipei, Taiwan)	808 ± 5/≤0.5	190	8	207 s	Chronic compression on dorsal root ganglion. A thin L shaped needle (0,6 mm of diameter) was inserted 4 mm in the L4 and L5 intervertebral foramen.	On the dorsal root of L4 and L5.	Application 1st day post-operatory, per 8 successive days. Euthanasia 4 e 8 days.	LLLT decreased the levels of inflammatory cytokines and of pain, facilitating the nerve regeneration, demonstrated by levels of TNF-a, IL-1b e GAP-43.
Belchior et al. [38]	GaAlAs (KLD® Endophoton model)	660/0.63	26.3	4	96.7 s; 3 points	Crushing of the right sciatic nerve.	On the surgical site.	Application 1st day post-operatory, during 20 successive days.	LLLT was positive on the functional index after the 21st day.
Barbosa et al. [39]	GaAlAs (Ibramed® Equipamentos Médicos)	660/0.06 830/0.116	30	10 10	20 s 38.66 s	Crushing of the right sciatic nerve.	On the surgical site.	Application 1st day post-operatory, during 20 successive days.	LLLT 660 nm promoted functional recovery in a faster manner.
Marcolino et al. [40]	AlGaAs (Laser Diode, Ibramed)	830/0.116	30	10 40 80	38.66 s 154.66 s 309.33 s	Crushing of the right fibular nerve.	On the right sciatic nerve.	Application immediately after surgery and during the 21 successive days.	40 J/cm² and 80 J/cm² LLLT influenced the functional recovery of the nerve.
Akgul; Gulsoy; Gulcur [41]	Laser diode (model: DH650-24-3(5), Huanic, China)	650/≈0.14	25	10	57 s on 3 points	Crushing of the sciatic nerve.	On the sciatic nerve.	Early group: Application after surgery, up to the 14th day. Delayed group: Application on the 7th day post-operatory and up to the 21st day.	LLLT accelerated nervous recovery. The group with delayed application showed better functional results.
Gigo-Benato et al. [42]	GaAlAs (TWIN LASER; MM Optics, São Carlos, SP, Brazil)	660/0.04 780/0.04	40 40	10, 60 and 120 10, 60 and 120	0.3 s, 1 min and 2 min 0.3 s, 1 min and 2 min; 2 points	Crushing of the left sciatic nerve.	Applied to the surgical site.	Application 1st day post-operatory, during 10 successive days.	LLLT (660 nm, 10 J/cm² or 60 J/cm²) accelerated the neuromuscular recuperation.
dos Reis et al. [43]	AlGaAs (KLD®; Endophoton model)	660/0.63	26.3	4	96.7 s per point, 3 points	Neurotmeses and epineural anastomosis on the right sciatic nerve.	On the surgical site.	Application 1st day post-operatory, 20 successive days.	LLLT significantly changed the morphometry (myelin sheath), but did not interfere on functionality.
Yang et al. [44]	GaAlAs (Aculas-Am series, Multi-channel LLLT System, Konftec Corp., Taipei, Taiwan)	660/≈0.2	30	9	60 s per point; 4 points	Use of Mesenchymal stem cells (MSC) on the lesion by crushing of sciatic nerve.	On the sciatic nerve	7 successive days.	LLLT+MSC improved the electrophysiologic function, S100 immunoreactivity, less inflammatory cells and less vacuole formation.
de Oliveira Martins et al. [45]	GaAs (Laserpulse-Laser, Ibramed Brazil) pulsado	904/0.1	70 Wpk	6	18 s on 5 points	Pulsed LLLT. Lesion on alveolar nerve, by a hemostatic Crile clamp.	On the sciatic nerve.	10 sessions every 10 days.	LLLT obtained better nociception, higher expression of neural growth factor (NGF) 53% and of expression of neurotrophic factor (BDNF) 40%.

Table 1. *Cont.*

Authors	Type of Laser (Manufacturer)	Wavelength (nm)/Spot Beam	Energy (mW)	Energy Density (J/cm^2)	Radiation Amount	Variables	Irradiation Site	Evaluation Time	Main Results
Gomes; Dalmarco; André [46]	HeNe (—)	632.8/0.1	5	10	20 s on 10 points	Crushing of the right sciatic nerve.	On the sciatic nerve.	1st Application 24 h after surgery; 7, 14 and 21 successive days.	LLLT increased the expression of mRNA and the factors BDNF and NGF after 14 days and maximum expression was observed on the 21st day.
Hsieh et al. [47]	GaAlAs (Aculas-Am series, Multi-channel laser system; Konftec, Taipei, Taiwan)	660/≈0.2	30	9	60 s per point; 4 points	Lesion on the sciatic nerve with 4 ligatures, using chromic suture 4-0.	On the surgery site.	Application 7th post-operatory, during 7 successive days.	LLLT improved functional index, decreased HIF-1a, TNF-a, and IL-1b, increased VEGF, NGF, and S100, reduced tissue ischemia and inflammation, helped the nerve recovery.
Sene et al. [48]	GaAsAl (Physiolux Dual, BIOSET, Rio Claro, Brazil)	830/0.02	30	5 10 20	Maximum time of application was 40 s	Crushing of the right fibular nerve.	Application fibular nerve region.	Application immediately after the lesion, during 21 successive days.	LLLT simulation group obtained a larger nerve transverse area; group 10 J/cm^2 obtained higher density of the fiber. LLLT did not speed up nerve recovery.
Dias et al. [49]	GaAlAs (Mm Twin Laser Optics, São Carlos, Brazil)	780/0.4	30	25	20 s per point; 3 points	Latex protein (F1) on lesion per crushing of sciatic nerve.	On the surgery site, sciatic nerve.	Application per 6 sessions on alternate days.	LLLT associated to the F1 protein did not present positive results and did not potentialize the effects of this protein.

4. Discussion

With the evolution of the technology in the health field and the evolution of the adjunct methods for rehabilitation and functional restoration of injured nerves [3,6–9], the PBMT has shown a wide range of benefits with clinical relevance. Thus, the aim of this research was to carry out a review of the scientific papers published in the last 10 years in order to verify the relation of PBMT in the regeneration of injured peripheral nerves. Regarding the varied benefits of PBMT, the highlight is the reduction of regeneration time and the aid in nerve function.

Among the effects of PBMT on nerve injury, it was verified that the laser minimized the side effects of bupivacaine on the nerve and on the muscle [29], potentiated the process of nerve regeneration observed by morpho-quantitative analysis of the axons and of the nerve fibers [2,3,19,30–35], in addition to assisting muscular reinnervation [36].

Photobiomodulation in the nerve injury was also related to a decrease in inflammatory cytokine levels, in pain, and to the facilitation of neural regeneration, demonstrated by the levels of TNF-a, IL-1b, and GAP-43 [32,37].

The functional analysis evidenced the evolution of functional recovery associated with PBMT [6,7,34,38,39]. Marcolino et al. [40] found a functional recovery with both 40 J/cm^2 and 80 J/cm^2 (830 nm), Akgul; Gulsoy; Gulcur [41] also scored improvement in functionality with late application PBMT (650 nm) (7 days after injury), as well as Medalha et al. [35] at 660 nm at 50 J/cm^2. PBMT 660 nm, 10 J/cm^2, or 60 J/cm^2 accelerated neuromuscular recovery when compared to 780 nm and 830 nm PBMT [42]. Differently, dos Reis et al. [43] observed that PBMT significantly altered morphometry (myelin sheath thickness values) but did not interfere with the functionality.

Yang et al. [44], when associating PBMT with MSC, demonstrated a better electrophysiological function, immunoreactivity of S100, and fewer inflammatory cells. de Oliveira Martins et al. [45] demonstrated that PBMT (904 nm) had better nociception, greater expression of neural growth factor (NGF) 53% and neurotrophic factor expression (BDNF) 40%. As seen, Gomes; Dalmarco; André [46] evidenced that PBMT (632.8 nm) increased mRNA expression, BDNF and NGF factors after 14 days and maximum expression was observed on day 21. PBMT (660 nm) improved functional index, reduced HIF-1a, TNF-a, and IL-1b, elevated VEGF, NGF, and S100, and decreased tissue ischemia and inflammation [47]. Sene et al. [48] (830 nm) observed that PBMT did not accelerate nerve recovery and the study by Dias et al. [49] when associating PBMT (780 nm) with latex protein also did not find positive results.

The effects of PBMT on nerve damage were verified in the sciatic nerve in 17 articles [19,30–36,38,39,41–44,46,47,49], facial nerve in 3 [3,6,7], fibular nerve in 2 articles [40,48], and vagus nerve [2], accessory nerve [29], alveolar nerve [45], and dorsal root [37] in one article each. Of the 26 articles inserted in this review, it was observed that 14 [19,31,32,37–42,44–46,48,49] presented compression as nerve damage (crushing) and 11 [2,3,6,7,30,33–36,43,47] articles evaluated the effects of PBMT on neurotmeses, which is the worst type of nerve injury.

It has been observed that the diversity of PBMT application protocols in nerve lesions is large, with the wavelength varying from 632.8 to 904 nm, a varied range of energy and energy density, in addition to the time of application, despite the similarity in the type of lesion targeted in each experiment. As shown, the infrared spectrum has good experimental results. The red spectrum (600 to 700 nm) [50] was seen in 15 studies with satisfactory morphological and electrophysiological results, immunological factors, and tissue markers [2,30,31,33–36,38,39,41–44,46,47]. It was also possible to verify the lack of standardization in relation to the application protocols, noting that 6 studies were discarded due to lack of data information regarding energy density and time of application of PBMT.

In a general critical analysis of the articles for the detailed study, a consensus was observed on the effectiveness of PBMT, with the use of low-level laser therapy on the improvement of the morphological and morphometric aspects of the regenerated peripheral nerve, as well as on the reduction of events inflammatory and painful sensitivity, providing faster and higher quality functional recovery [51,52].

In the perspective of new fronts of study, in the last decade, optogenetic and chemogenetic techniques have been used more frequently in the investigation of neuronal circuits, as well as in the study of non-neuronal cells in the brain and peripheral nerves. Optogenetics is effective in generating patterns that mimic neuron responses using a pulse generator that produces lights with different frequencies and pulse durations. Photostimulation can be performed in different subcellular regions, being useful for the study of neuronal circuits in the brain. Chemogenetics are less invasive in animal experiments and do not require the installation of a fiber optic cable into the brain or the connection of the cable to a light source, such as a laser or a light emitting diode (LED).

5. Conclusions

At the end of the present study, it can be seen that the data presented in the current articles helped us to understand the beneficial and helpful effects of photobiomodulation on regeneration and functionality after nerve injury. In spite of the great variety of parameters presented, great results were observed, mainly when related to the faster nervous regeneration process.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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RESEARCH REPORT

830 nm laser irradiation induces varicosity formation, reduces mitochondrial membrane potential and blocks fast axonal flow in small and medium diameter rat dorsal root ganglion neurons: implications for the analgesic effects of 830 nm laser

Roberta T. Chow[1,2], Monique A. David[2], and Patricia J. Armati[3]

[1]Castle Hill Medical Centre, Private Medical Practice; [2]Discipline of Medicine and; [3]Nerve Research Foundation, The University of Sydney, Sydney, Australia

Abstract We report the formation of 830 nm (cw) laser-induced, reversible axonal varicosities, using immunostaining with β-tubulin, in small and medium diameter, TRPV-1 positive, cultured rat DRG neurons. Laser also induced a progressive and statistically significant decrease ($p < 0.005$) in MMP in mitochondria in and between static axonal varicosities. In cell bodies of the neuron, the decrease in MMP was also statistically significant ($p < 0.05$), but the decrease occurred more slowly. Importantly we also report for the first time that 830 nm (cw) laser blocked fast axonal flow, imaged in real time using confocal laser microscopy and JC-1 as mitotracker.

Control neurons in parallel cultures remained unaffected with no varicosity formation and no change in MMP. Mitochondrial movement was continuous and measured along the axons at a rate of 0.8 μm/s (range 0.5–2 μm/s), consistent with fast axonal flow. Photoacceptors in the mitochondrial membrane absorb laser and mediate the transduction of laser energy into electrochemical changes, initiating a secondary cascade of intracellular events. In neurons, this results in a decrease in MMP with a concurrent decrease in available ATP required for nerve function, including maintenance of microtubules and molecular motors, dyneins and kinesins, responsible for fast axonal flow. Laser-induced neural blockade is a consequence of such changes and provide a mechanism for a neural basis of laser-induced pain relief. The repeated application of laser in a clinical setting modulates nociception and reduces pain. The application of laser therapy for chronic pain may provide a non-drug alternative for the management of chronic pain.

Key words: 830 nm, axonal varicosities, fast axonal flow, mitochondrial membrane potential

Introduction

Low-level laser therapy (LLLT) is the clinical use of laser for the treatment of medical conditions at power densities not associated with macroscopic thermal effects, in contrast to thermally mediated surgical applications. Efficacy of LLLT in painful clinical conditions has been established by several recent systematic reviews and meta-analyses [level 1 evidence, according to the Australian Government, NHMRC *(1999)*]. This level of evidence relates to chronic neck pain *(Chow and Barnsley, 2005)*, tendonitis *(Bjordal et al., 2001)*, chronic joint disorders *(Bjordal et al., 2003)*, and chronic pain *(Enwemeka et al., 2004)*.

Address correspondence to: Dr. Roberta T. Chow, MB BS (Hons), FRACGP, FAMAC, MApplSci (Med Acu), Nerve Research Foundation, Room 475, Blackburn Building, D06, The University of Sydney, NSW 2006, Australia. E-mail: rtchow@bigpond.net.au

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Randomised controlled trials (RCTs) provide level II evidence for the efficacy of laser therapy in chronic low back pain *(Umegaki et al., 1989; Soriano and Rios, 1998; Basford et al., 1999)*. In other reviews of laser therapy for painful conditions such as rheumatoid arthritis *(Brosseau et al., 2005)* and musculoskeletal pain *(Gam et al., 1993; de Bie et al., 1998)*, the evidence is equivocal. Such variability in outcomes may be due to the multiplicity of parameters used, including wavelengths, energy, and power densities, with differing frequencies of application *(Chow and Barnsley, 2005)*.

For clinical use, wavelength is generally recognised as one of the most important parameters *(Tunér and Hode, 2002)*. In the treatment of painful conditions, both visible (e.g., λ = 632.8, 670 nm) and infrared (e.g., λ = 780, 810–830, 904 nm) wavelengths have been used *(Beckerman et al., 1992)*. In some conditions, infrared wavelengths have been confirmed to be more effective than visible wavelengths, such as in the modulation of neck pain *(Chow and Barnsley, 2005)*. Furthermore, there is mounting evidence that the narrow wavelength spectrum from 810 to 830 nm is effective in a range of painful clinical conditions *(Palmgren et al., 1989; Umegaki et al., 1989; Toya et al., 1994; Fukuuchi et al., 1998; Ozdemir et al., 2001)*. In this wavelength range, we showed statistically significant, clinically relevant improvement in chronic neck pain using 830 nm laser *(Chow et al., 2004; 2006)*.

A number of reports suggest that neural mechanisms are the basis for laser-induced pain relief. These include increased production of serotonin *(Walker, 1983)*, increase in β-endorphin synthesis *(Laakso et al., 1994)*, increased synaptic activity of acetylcholine esterase *(Navratil and Dylevsky, 1997)*, and, at high energy densities (EDs), inhibition of Na^+-K^+-ATPase, responsible for maintaining the resting potential of nerves *(Kudoh et al., 1990)*. Following on from our clinical studies *(Chow et al., 2004; 2006)*, we therefore sought to test the hypothesis that 830 nm laser acts to modulate pain via direct neural inhibition.

In support of inhibitory mechanisms are a number of studies that demonstrate that 830 nm, continuous wave (cw), laser slows nerve conduction velocity and increases latencies in median *(Baxter et al., 1994)* and sural nerves *(Cambier et al., 2000)*. This has led to the proposal that conduction block underpins 830 nm laser-mediated analgesic effects *(Baxter et al., 1994; Kasai et al., 1996; Navratil and Dylevsky, 1997; Cambier et al., 2000)*. Consistent with this is the finding that 830 nm (cw) laser irradiation specifically suppresses nerve conduction in small diameter, thinly myelinated Aδ and unmyelinated C fibres following electrical stimulation of nerves *in vivo (Tsuchiya et al., 1993; 1994; Wakabayashi et al., 1993)*. It is these nociceptors that respond to noxious heat, mechanical, and chemical stimuli and can be identified by the presence of transient receptor potential vanilloid type-1 (TRPV-1) receptors on cell bodies *(Tominaga et al., 1998)*.

To further explore laser-induced inhibitory neural mechanisms at a neuronal level, we investigated the effects of 830 nm (cw) laser irradiation on those domains of nerve function which are critically dependent on mitochondrial function and ATP production because mitochondria are known to be the primary site of laser energy absorption and transduction. We therefore chose to observe effects on microtubule arrays, fast axonal flow (FAF), and mitochondrial membrane potential (MMP), which are functionally interdependent, relying on normal mitochondrial activity and production of ATP, essential to normal nerve function and action potential propagation. We therefore tested the hypothesis that 830 nm (cw) laser irradiation induces such changes by a direct effect on nociceptors in the peripheral nervous system (PNS) and propose that neural inhibition is the basis for the pain modulating effects of laser irradiation.

The laboratory study concentrates on the effect of 830 nm (cw) laser at EDs shown to be effective in laser-induced modulation of neck pain in our clinical trials. Based on this, we focussed on the effect of a single 30 s, 830 nm (cw) laser exposure, while examining EDs and irradiation times around the clinically relevant exposure time of 30 s, in a rat dorsal root ganglion (DRG) culture model. We also defined the subclasses of neurons that were affected and used real-time confocal microscopy and video imaging of living neurons in real time to define 830 nm (cw) laser effects on (1) the morphology of axons; (2) distribution of mitochondria; and (3) changes in MMP and FAF.

Materials and Methods

Preparation of DRG cultures

The study was approved and carried out in accordance with the guidelines of the Animal Ethics Committee, Sydney University # L04/12-2002/1/3669. One- to three-day-old Sprague-Dawley rat DRG were removed aseptically and placed in Hanks calcium- and magnesium-free saline plus 0.05% collagenase and 0.25% trypsin, incubated for 25 min at 37°C, centrifuged at 700 *g* for 4 min and the pellet resuspended in culture medium composed of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.6% additional glucose. Centrifugation was repeated and the pellet resuspended in medium, plated onto collagen-coated glass coverslips in 24-well culture plates, in alternate

wells and maintained at 37°C in 5% CO_2-humidified environment for 4 days. Well plates were designated as experimental or control.

Laser irradiation

The laser device used for irradiation of the cells was a Spectramedics 830 nm, 1 W, cw, diode laser, (parameters of irradiation are outlined in Table 1). To ensure that the EDs delivered to the cultures were equivalent to those used in our clinical studies *(Chow et al., 2003; 2004; 2006)*, the laser output was measured at the Department of Laser Physics, Macquarie University, Australia, using a Trimedyn Optical Power Meter, Model: 210/100, with a 22 mm aperture of detection. These measurements determined that a distance of 4.5 cm between the laser aperture of the handpiece and the surface of the coverslip on which cultures were growing would deliver the appropriate EDs.

The laser handpiece was mounted vertically in a retort stand in a class II laminar flow cabinet. All artificial light sources in the laboratory were switched off. To ensure that laser irradiation did not cause a confounding rise in the temperature of the culture medium, a thermocouple was inserted into replicate wells of culture medium, which was irradiated for 120 s and the temperature of the culture medium measured before and after irradiation. No temperature change occurred after 30 s, with a rise of 0.1°C only at 120 s. This is within the range defined for LLLT *(Kert and Rose, 1989)*. For each experiment, laser irradiation was delivered under sterile conditions to replicate cultures ($n = 2$) for 5 s [total energy (TE) of 1.5 J at ED: 1.4 J/cm^2], 30 s (TE: 9 J at ED: 8.3 J/cm^2), 60 s (TE: 18 J at ED; 16.7 J/cm^2) or 120 s (TE; 36 J at ED: 33.3 J/cm^2). Each experiment was repeated twice. Control cultures were handled in the same way as experimental cultures but without laser irradiation.

Immunohistochemistry

Experimental cultures were fixed at 1, 4, and 24 h post-laser irradiation in chilled acetone at room temperature for 20 min. Control cultures were fixed at corresponding times.

Replicate experimental and control cultures were immunostained with (1) monoclonal anti-β-tubulin isotype 111 (Sigma), specific for neurons; or (2) the nociceptor receptor VR-1 antibody, now known as TRPV-1 *(Tominaga et al., 1998)*. Replicate cultures incubated for 1 h at room temperature with anti-β-tubulin were washed three times in phosphate-buffered saline (PBS), incubated in anti-mouse fluoro-isothiocyanate (FITC; Amersham), diluted 1 : 100 in trizyme buffered saline (TBS) for 1 h at room temperature and washed as above. Replicate cultures incubated overnight with goat anti-VR-1 polyclonal antibody (1 : 100; Santa Cruz) primary antibody were washed three times in PBS buffer, incubated in rabbit anti-goat FITC, diluted in TBS for 1 h at room temperature, then washed as before. Replicate cultures were treated in the same manner with the omission of primary antibody to ensure there was no non-specific staining. Anti-fade solution (Vectashield, Vector Laboratories) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) diluted to 2 μg/ml were added to the cultures and the coverslips sealed onto microscope slides.

Table 1. Parameters of laser device for 830 nm irradiation of rat dorsal root ganglion neurons in culture.

Diode	Gallium aluminum arsenide (GaAlAs)
Power	1 W delivering 400 mW at 4.5 cm above coverslips in well plate
Mode	Continuous wave
Spot size	1.4 cm^2
Power density	300 mW/cm^2

Identification and measurement of neuronal subsets

β-tubulin-positive cells were identified as neurons. These cells also had characteristic neuronal morphology with distinct cell bodies and extended axons forming a neuronal network. TRPV-1 positive cells exhibited the same neuronal morphology. Non-neuronal cells (fibroblasts and Schwann cells) were identified by their morphology and were neither β-tubulin nor TRPV-1 positive with fibroblasts characterized by their irregular, flattened shape and Schwann cells by their primarily filiform shape.

Images were collected by a blinded observer from three to five fields of view from each replicate of the experimental and control coverslips. Images from each source (FITC, DAPI, and Differential Interface Contrast) were collected by CCD (SensiCam) using a Nikon E800 microscope and merged using Adobe Photoshop 5.0. A minimum of 100 cell body diameters from each of the replicate experimental and control cultures was measured from the collected images.

Measurement of MMP

To measure MMP in living cultures, each coverslip in a parallel set of experimental and control cultures was incubated just prior to observation in 3 μM of the mitotracker JC-1, (Md. Probes Inc.) in PBS for 15 min at 37°C, washed with PBS, then mounted in a chamber filled with physiologic solution *(Dedov and Roufogalis, 1998)*.

Each experimental culture was at room temperature in a darkened room, irradiated for 30 s, and imaged by inverted confocal scanning microscopy using Leica TCS SP2 (Leica Microsystem Heidelberg

GMBH) with neutral density filters of 10%, a 488 nm argon laser, and a pinhole of 1 mm. JC-1 was imaged as red fluorescent emission (570 nm) indicating high MMP, while green fluorescent emission (530 nm) indicated low MMP. Background fluorescence was subtracted from the ratios of fluorescent intensity of the red and green channels. 3D reconstructions of J-aggregates, representing mitochondria, were performed using "VoxBlast" NT Version 1.3.2 software (Image Analysis Facility, University of Iowa).

The ratio of red to green fluorescence was measured every 5 min for 30 min, from each of 10 randomly selected fields of view from each culture, and recorded independently in (1) axons and (2) cell bodies, in replicate laser-irradiated ($n = 3$) and control cultures ($n = 2$).

Statistical analysis

Data were analysed by a one-way ANOVA with a level of significance set at $p < 0.05$.

Measurement of FAF by video imaging

In a further set of experiments, living experimental cultures irradiated with laser as above ($n = 3$) and control, non-laser irradiated cultures ($n = 2$) were video imaged in real time with images collected at 1-min intervals for 10 min. The rate of FAF was calculated by measurement of the distance travelled by randomly selected mitochondrial clusters over a 10-min observation period.

Results

Identification of neuronal subsets

Morphology of β-tubulin-positive neurons showed that 86.9% of cell bodies were 10–30 μm in diameter, before and following laser irradiation. This is consistent with their being Aδ and C fibre classes of small and medium diameter nociceptors *(Light and Perl, 1984)* (Fig. 1). These neurons were also TRPV-1 positive.

Laser irradiation induced axonal varicosities in DRG neurons

Single laser exposures of 5, 30, 60, or 120 s of DRG neurons induced intensely β-tubulin III-positive axonal varicosities at all exposure times (Fig. 2). At 120 s, some axonal processes partially detached from the substratum showing disruption of adhesion molecules. There was no cell body detachment. Non-laser irradiated control cultures showed no detectable morphological changes with axonal processes remaining firmly attached to the substratum, which also confirmed that the argon laser of the microscope was not a factor in the experiments. JC-1 staining, discussed

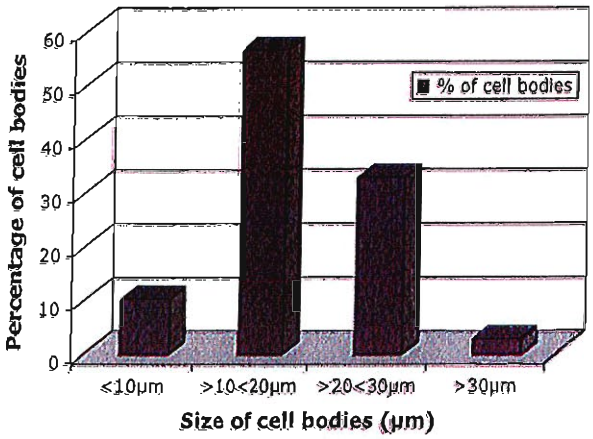

Figure 1. Histogram of cell body measurements of subsets of rat dorsal root ganglion neurons showing 86.9% of the cells are within the cell size range of Aδ and C nociceptors.

in detail later, also showed that the axonal varicosities contained clusters of mitochondria (Fig. 3). Importantly, following laser irradiation, varicosities had resolved by 24 h (Fig. 4). Axonal processes that had shown some partial detachment had reattached.

Real-time confocal imaging of living DRG neurons

Confocal imaging of living laser-irradiated cultures showed that mitochondria within the varicosities were static over the 10-min observation time (Fig. 5A). This was of great interest as, in contrast, control neurons showed no varicosities, with mitochondria continuing to move along the axons over the 10-min period of observation at a rate of 0.8 μm/s. This rate is consistent with FAF because FAF has a range of 0.5–2 μm/s *(Hirokawa et al., 1991)* (Fig. 5B).

MMP decreased in laser-irradiated small and medium diameter neurons resulting in block of FAF

Five minutes after laser irradiation, there was a statistically significant decrease in MMP ($p < 0.002$) within the axons, including mitochondrial clusters within the axonal varicosities (Fig. 6A). This decrease continued over the 30-min period of observation ($p < 0.005$). MMP of mitochondria in the cell bodies did not decline significantly for 20 min after which there was a significant decrease ($p < 0.05$) (Fig. 6B).

Discussion

We report for the first time that 830 nm (cw) laser irradiation of rat DRG cultures at all EDs used induced the formation of static axonal varicosities in small and

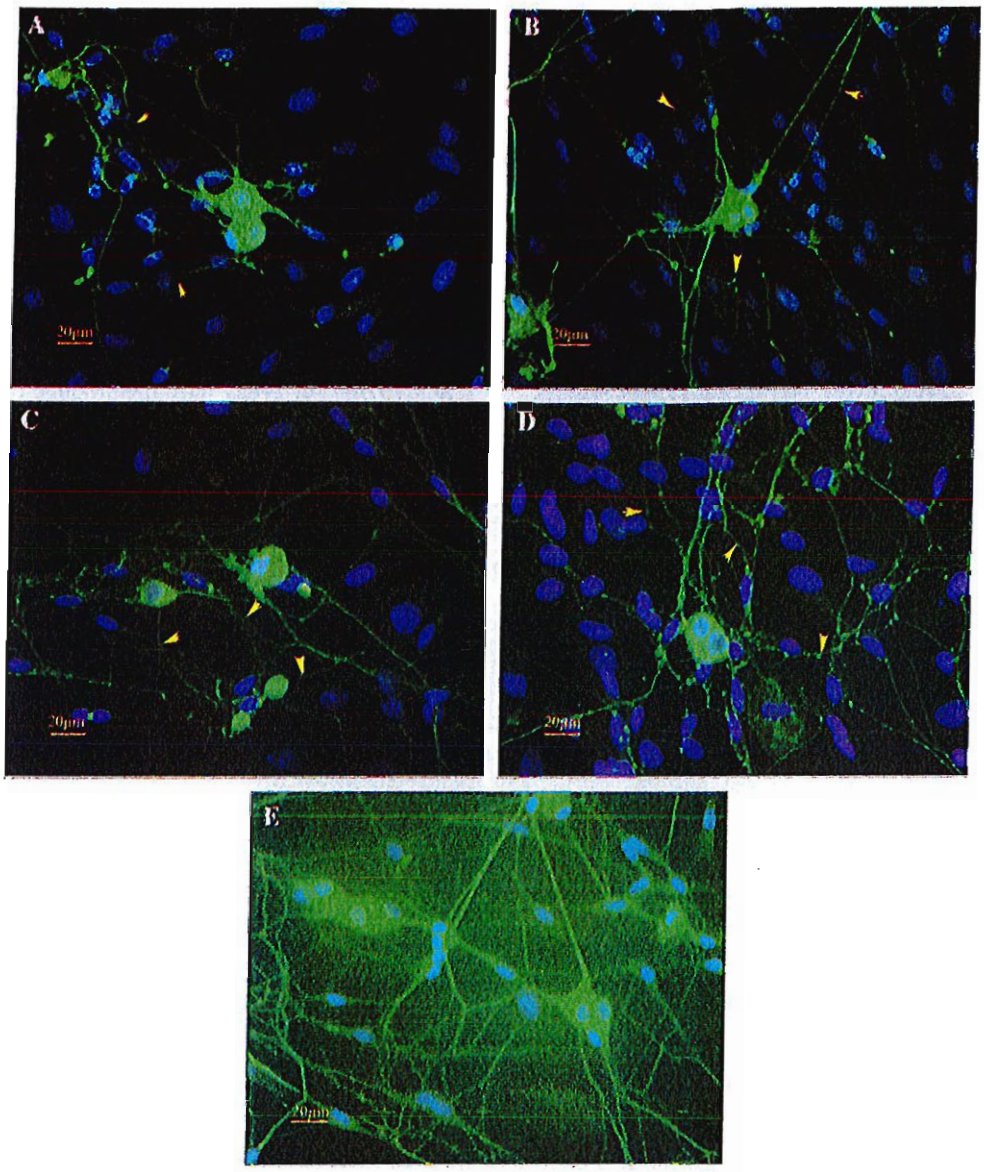

Figure 2. Representative photomicrographs of β-tubulin-positive neurons 4 h after 830 nm (continuous wave) laser irradiation of (A) 5 s, (B) 30 s, (C) 60 s, and (D) 120 s. Note axonal varicosities (➢) and (E) control non-laser irradiated neurons.

medium diameter, TRPV-1 positive neurons, which resolved after 24 h. Laser irradiation also induced a progressive and statistically significant decrease in MMP in and between the axonal varicosities where the mitochondria were seen as clusters. The MMP decrease in the cell bodies was also statistically significant but occurred more slowly. Most interestingly, 830 nm laser irradiation blocked FAF.

The changes occurred in the TRPV-1 positive subset of neurons, consistent with their being nociceptors, i.e., the Aδ and C fibre neurons *(Rang et al., 1991; Gold et al., 1996; Kress and Reeh, 1996; Julius and Basbaum, 2001; Mandadi, 2001)*. The importance of TRPV-1, which responds to noxious stimuli such as capsaicin, high temperatures (>43°C) and acidity (pH < 6.5), is highlighted by the elegant studies of Caterina *et al. (2000)* in TRPV-1 knockout mice, where the mice exhibited little or no response to noxious thermal stimuli. Moreover, the mice failed to exhibit pain behaviour when the skin of their hind paws was injected with capsaicin, sensitivity to which is a hallmark of unmyelinated, small-diameter nociceptors.

In other studies, when 830 nm laser irradiation (40 mW, cw) was applied proximal to the site of electrical stimulation of rat saphenous nerve, there was selective inhibition of the slowest component of the action potential, again indicative of selective Aδ and C fibre inhibition *(Tsuchiya et al., 1993; 1994)*. In

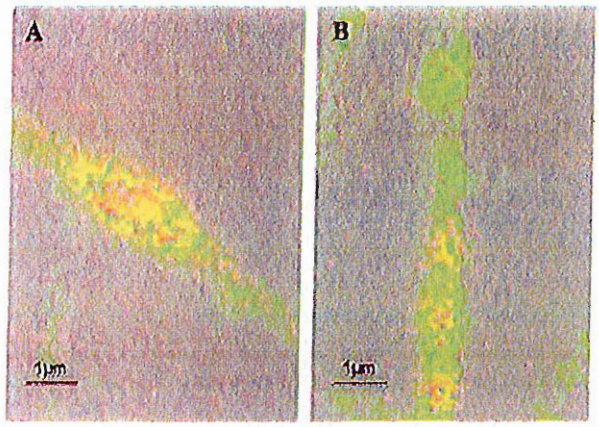

Figure 3. Representative pixelated images of JC-1 stained mitochondria seen as red/green fluorescence (a) clustered within an axonal varicosity (►) after 30 s, 830 nm (continuous wave) laser irradiation and (b) in a control non-lasered axon.

a parallel study, rats treated with capsaicin at birth and devoid of Aδ and C fibres subjected to the same protocol had action potentials with no slow component but no change in the conduction of large, myelinated fibres *(Tsuchiya et al., 1993)*. In yet another study, 830 nm laser irradiation (120 s, 350 mW, cw), applied to the rat incisor and the tooth pulp electrically stimulated, suppressed only the slowest component, i.e., C fibres *(Wakabayashi et al., 1993)*.

Such studies are directly relevant to our hypothesis that 830 nm laser irradiation acts on nociceptor-specific neurons with varicosity formation as a major morphological feature. Varicosity formation has previously been reported in only one other study of 830 nm laser irradiation, where mouse DRG neurons had varicosities restricted to neurons that were substance P-positive, another characteristic of nociceptors *(Chen et al., 1993)*. In these studies, the neurites ceased outgrowth following irradiation but recommenced after 5 h. This is relevant to our finding that varicosities had resolved by 24 h, showing that the neurons were undamaged. Consistent with these findings are those of Park *et al. (1996)* who applied N-methyl-D-aspartic acid (NMDA) to mouse neocortex neurons, stimulating NMDA receptors and inducing varicosities, which resolved 2 h after stimulation ceased. The authors propose that reversible varicosity formation is a physiologically adaptive response to non-toxic stimuli.

In other non-laser studies, varicosities were observed when substance P was applied to rat DRG neurons *(Tanelian and Markin, 1997)* and following application of high concentrations of substance P to central nervous system (CNS) neurons, varicosities were found in dendrites *(Mantyh et al., 1995a)*. Mantyh *et al.* considered that the varicosities were a response to internalization of substance P bound to its receptor and depressed the neuronal response to nociceptive stimuli. They also suggest that small-diameter fibres are selectively more sensitive to varicosity formation.

Of direct relevance to our findings are ultrastructural studies of the local anaesthetics lidocaine and procaine, which demonstrated by transmission electron microscopy that these agents caused cytoskeletal changes including disruption of the microtubules and associated structures *(Poste et al., 1975; Nicolson et al., 1976)*. As well, the microtubule destabilizing agent, colchicine, used for the treatment of gout *(Ahern et al., 1987)*, affects some of its therapeutic benefit by reversible disruption of microtubule structure. These studies are consistent with pain relief by neuronal-specific changes such as those induced in our study by 830 nm laser irradiation.

The varicosities that we report here are intensely β-tubulin positive, indicative of microtubule disruption. This varicosity formation has important functional implications as high-energy mitochondria are carried by FAF along microtubules to provide ATP for maintenance, generation, and restoration of the axon potential. FAF is also essential for retrograde transport of low-energy, ATP-depleted mitochondria back to the cell body. Microtubule disruption would therefore block ATP supply also essential for the delivery of components of the synaptic vesicles, in particular synaptophysin, which is required for neurotransmission *(Nakata et al., 1998)*.

The disorganization of the microtubule infrastructure for FAF has additional implications for disruption to transport of ATP. Our finding of a significant decrease in MMP, a surrogate measure for ATP, after laser irradiation of live neurons, reflects decreased ATP availability which, in turn, will decrease ATPase activity *(Goldstein and Yang, 2000)*. ATPases include kinesins and dyneins, the molecular motors for organelle transport and Na^+-K^+-ATPases. As 830 nm irradiation of rat saphenous nerve at high EDs inhibits Na^+-K^+-ATPases *(Kudoh et al., 1990)*, this would be consistent with our findings of decreased MMP and ATP with a resultant conduction block. It would also be expected that decreased MMP could affect Ca^{2+}ATPase activity, which regulates intracellular calcium *(Budd and Nicholls, 1996; Liu and Barth, 2004)*, essential for neurotransmission, further compromising neurotransmission.

With regard to conduction block, varicosity formation reported in the studies discussed above led Tanelian and Markin to formulate a biomathematical model to quantify changes in electrophysiological parameters associated with such morphological alterations *(Tanelian*

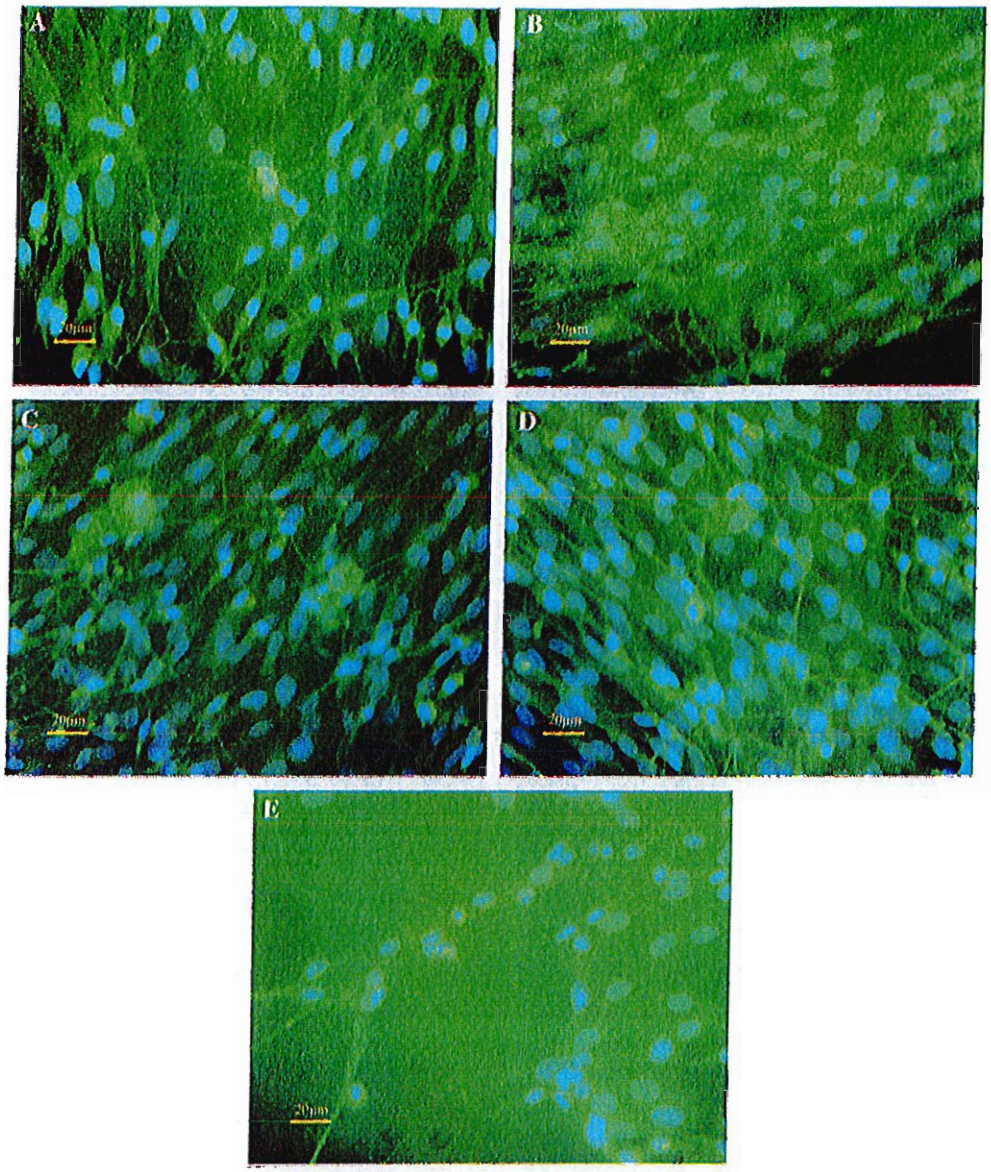

Figure 4. Representative photomicrographs of β-tubulin-positive neurons 24 h after 830 nm (continuous wave) laser irradiation of (A) 5 s, (B) 30 s, (C) 60 s, and (D) 120 s, and (E) control non-laser irradiated neurons.

and Markin, 1997). Their model proposes that the biophysical change seen as varicosities would result in slowing of nerve conduction in the affected neurons, thereby directly affecting information transfer within the nervous system.

The resolution of varicosities, found in our study after 24 h, indicates that any such laser-induced microtubule disruption, blockade of fast axonal transport, conduction, and neurotransmission block would be reversible and therefore temporary.

While this is the first report of an 830 nm (cw) laser irradiation effect on mitochondria and MMP of DRG neurons, one other study does report the effect on MMP of 780 nm (200 mW, ED: 2 J/cm^2) in the keratinocyte HaCaT cell line *(Gavish et al., 2004).* This showed a significant increase in MMP during the first hour of observation, in contrast to our findings, and a decrease after 3 h. Studies using visible wavelengths on MMP of non-neuronal cells consistently show an increase in MMP, similar to the initial period of observation by Gavish *et al. (Passarella et al., 1994; Vacca et al., 1994; 1997; Alexandratou et al., 2002).* Such differences may be related to the higher EDs (8.3 J/cm^2) used in our study, four times greater than that used by Gavish *et al.* This is an important point for the model we propose because the ED delivered to the neurons used in our laboratory studies were carefully measured to be equivalent to that we used

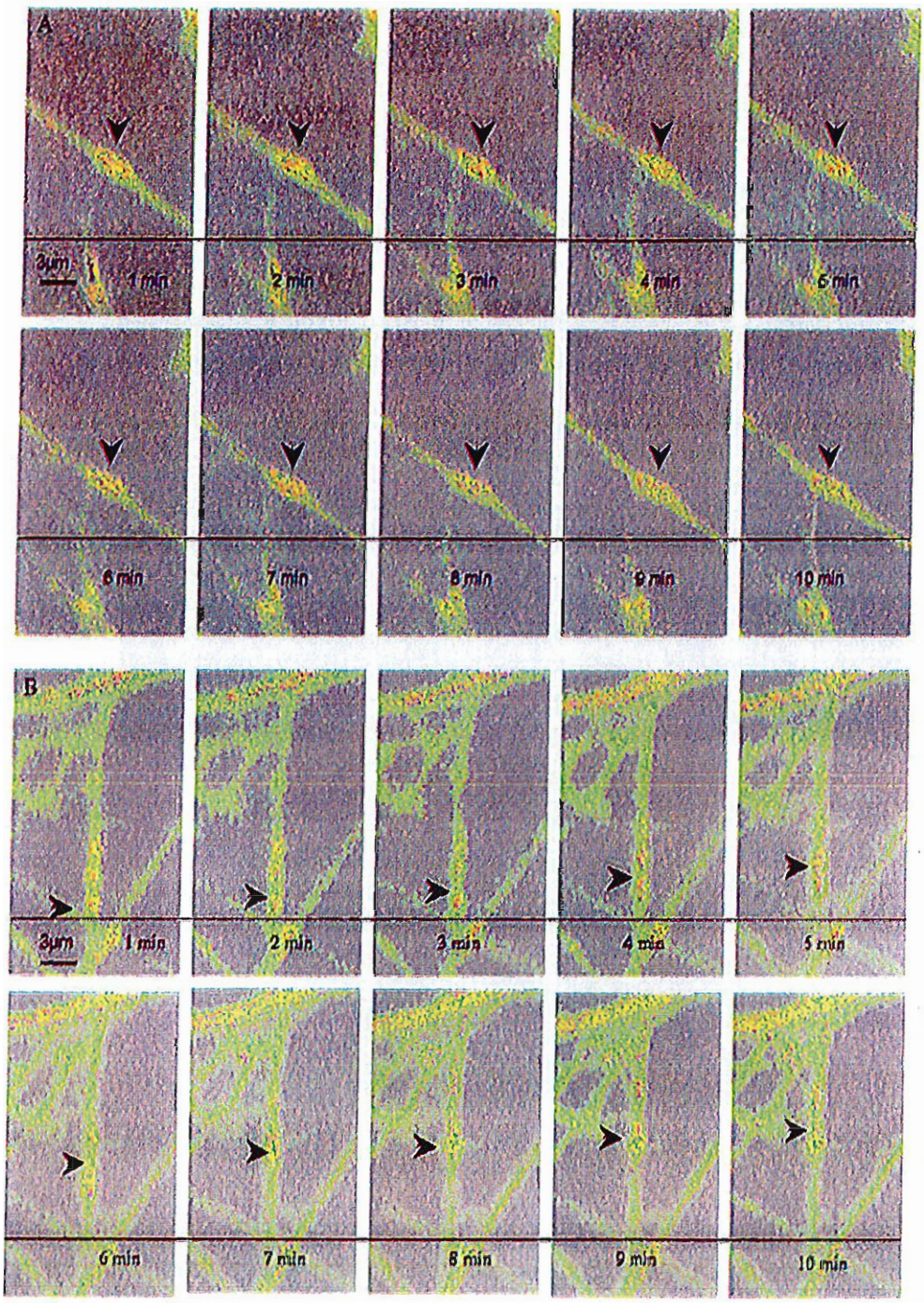

Figure 5. (A) Real-time confocal images of a JC-1 stained axon after 30 s of 830 nm (continuous wave) laser irradiation showing a static varicosity (▼) indicating blockade of fast axonal flow (FAF) and a decrease in MMP over 10 min of observation. (B) Real-time confocal images of JC-1 stained, non-laser irradiated, control axons showing movement of mitochondria (➤) at 0.8 μm/s, consistent with FAF and no decrease in MMP over 10 min of observation.

in the clinic for long-term reduction of chronic neck pain *(Chow et al., 2004; 2006)*.

Our data, showing varicosity formation and decreased MMP at all the EDs used, potentially reflect only the inhibitory phase of the Arndt-Schultz Law *(Ohshiro, 1990)*, a biphasic phenomenon where stimulation of biological activity occurs at low EDs and inhibition at high EDs. While this response is often observed in studies of visible laser irradiation on non-neuronal cells in culture *(Lam et al., 1986; Bolton*

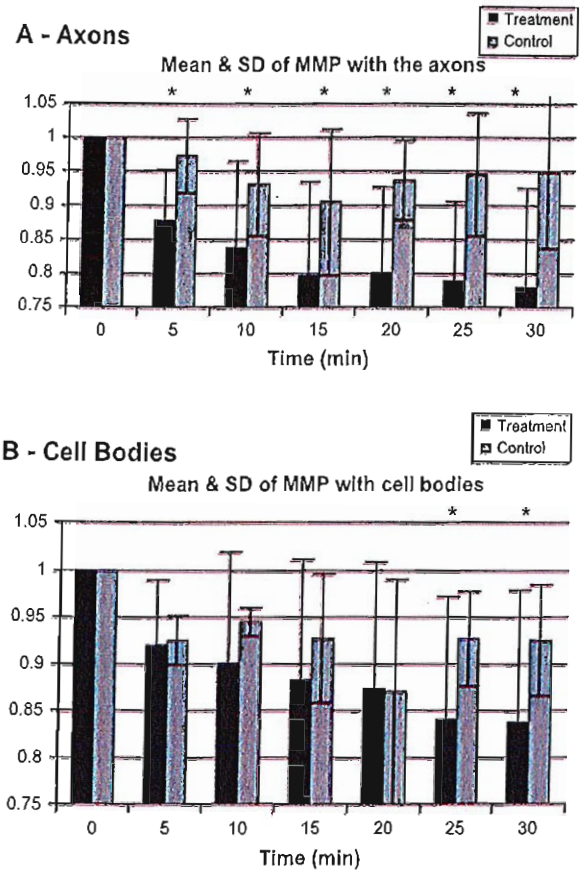

Figure 6. Histogram showing changes in mitochondrial membrane potential levels in (A) axons and (B) cell bodies after 30 s, 830 nm (continuous wave) laser irradiation (black), and control, non-laser irradiated neurons (gray)

et al., 1990; Sommer et al., 2001; Pereira et al., 2002), it does not occur universally *(Ozawa et al., 1997; Pogrel et al., 1997; Luger et al., 1998).* Moreover, it may well be that ED is not the only relevant factor but that the morphology of sensory neurons with their single axons up to 1 m in length may be important. It is only in neurons that there is a distinct cell body where protein synthesis occurs and high energy, ATP-rich mitochondria are generated. We have supportive evidence for this from other studies related to cell morphology. An unpublished study on NTera 2 cells showed that H_2O_2-induced oxidative stress resulted in varicosity formation in those cells with a neuritic morphology, whereas NTera 2 cells with the more epithelial-like morphology did not *(David, 2002).* This is supported by studies of primary human brain tissue in culture where neurites also became varicose under the same experimental protocol *(Roediger and Armati, 2003).*

Thus, the 830 nm (cw) reversible, laser-induced blockade of FAF and decreased MMP we consider to be related to unique neuronal morphology, particularly as sensory neurons have a single axon and no dendrites, as well as the incident EDs.

We provide for the first time, a direct mechanism by which 830 nm laser irradiation exerts pain relieving effects, namely via PNS, nociceptor-specific inhibition. We propose that this is directly relevant to our LLLT clinical trial data *(Chow et al., 2006)* leading to the pertinent question: How does 830 nm laser irradiation initiate such analgesia? The First Law of Photochemistry states that laser energy must be absorbed to exert its effect *(Smith, 1999).* In mitochondria, photoacceptors within the membranes absorb light, including 830 nm laser *(Karu, 1999),* inducing conformational change in enzymes *(Friedmann and Lubart, 1995)* and increasing reactive oxygen species, such as singlet oxygen *(Lubart et al., 2000),* which can regulate signal transduction *(Lubart et al., 2006).* Cytochrome c oxidase has been identified by its absorption spectrum as a primary photoacceptor for 830 nm laser irradiation *(Karu et al., 2005).* Thus, cytochrome c oxidase and possibly other photoacceptors within neuronal mitochondrial membranes provide a primary site for 830 nm laser energy absorption and transduction.

We further propose that absorption of laser energy and its transduction into electrochemical or electrophysical events triggers a secondary cascade of cell-specific events as suggested by Karu *(Karu, 1999)* and that this is represented by changes in neuronal architecture seen as varicosity formation, mitochondrial clustering, and microtubule disarray. These morphological changes reflect functional events such as the statistically significant decrease in MMP and blockade of FAF. We further propose that this leads to conduction block and failure of neurotransmission from PNS nociceptors to CNS neurons. Tanelian and Markin, in developing their mathematical model of the functional, biophysical changes of varicosity formation causing slowing of action potential propagation, propose that the morphological changes may be the basis for several forms of counter-irritation–induced analgesia such as transcutaneous electrical nerve stimulation, peripheral efferent nerve stimulation, and spinal cord and deep brain stimulation *(Tanelian and Markin, 1997).* We would add LLLT to this list. More importantly for long-term pain modulation, Mantyh *et al. (1995a; 1995b)* suggest that morphological reorganisation might alter the integrative properties of neurons and many constitute "an important mechanism of neural plasticity," which is the basis for adaptive change in the CNS, including memory, in response to altered nociceptive input, again consistent with our proposed mechanism of effect.

As laser irradiation was applied to skin surface in our clinical trials, it is important to consider how this relates to the laboratory studies. Criticism could be

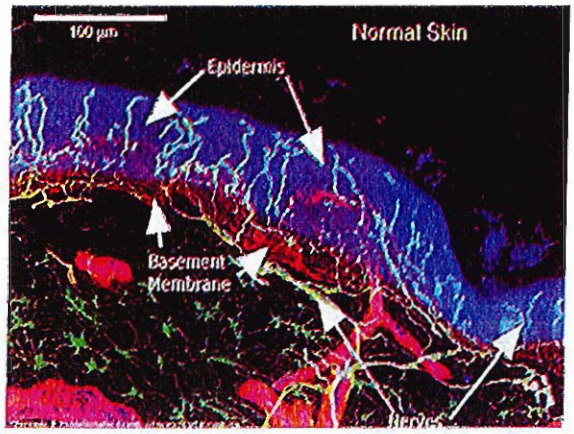

Figure 7. Cross-section of skin and subcutaneous tissues showing peripheral afferent terminals in the epidermis. Reprinted from Kennedy et al. (2005), with permission from Elsevier.

made that our neuron-specific model is based on irradiation of the whole neuron and that EDs were excessive for DRG neurons in culture. However, our laboratory studies show that the axon is more affected than the cell body, the neurons are not irrevocably damaged, and the varicosities resolve. Also, comparison of our study with other studies of 830 nm laser irradiation of cultured human foreskin keratinocytes and lung fibroblasts *(Pogrel et al., 1997)*, human periodontal cells *(Ozawa et al., 1997)*, and a number of cell lines such as the clonal osteoblastic cell line (RCJ) *(Luger et al., 1998)* show that the EDs used in these studies were of the same order of magnitude. These cells exhibited a variety of effects with no biphasic response, and there was no cell death or damage even at the higher EDs.

In our clinical studies, laser irradiation was delivered to skin surface overlying tender points. Aδ and C fibre terminals course through the epidermis, including the superficial layers of keratinocytes *(Kennedy et al., 2005)* (Fig. 7). Thus, they would be directly affected by the incident laser beam, with minimal light flux attenuation because there is also backscatter associated with such irradiation. Moreover, as 830 nm laser penetrates up to 5 cm *(Gursoy and Bradley, 1996)*, there would also be an effect on the deeper dermis and underlying muscle *(Nicolau et al., 2004)*, tendons *(Bjordal et al., 2001)*, and lymphatic tissues *(Carati et al., 2003)*, also involved in clinical pain modulation.

Although we delivered only a single laser exposure in contrast to the clinical course of 10–14 treatments over 7 weeks *(Chow et al., 2004; 2006)*, there is, however, a single RCT showing that a single treatment with 830 nm laser relieved chronic neck pain for up to 24 h, suggesting an immediate clinical effect *(Toya et al., 1994)*. How does our model relate to the clinical trials showing long-term benefit of repeated laser treatments? We propose that laser induces acute and chronic pain relief via a reversible blockade of FAF and mitochondrial transport, with a resultant decreased MMP, reduction in ATP availability, conduction block and neurotransmission failure of Aδ and C fibre nociceptors, temporary in a single treatment but long term when delivered repetitively as in our clinical trials. We further propose that this would have a flow-on effect to the CNS by inhibition of second order neurons and modulation of ascending and descending pain-associated pathways and depression of long-term potentiation as proposed by Klein *et al. (2004)*.

As 830 nm laser therapy offers a non-invasive, non-pharmacological therapy for the treatment of pain with an absence of adverse events, further defining of the mechanism is essential, however, we present a plausible platform on which to base further study. The cost benefit of 830 nm (cw) laser therapy has substantial implications for health budgets in the reduction of ongoing, costly medication with serious adverse side effects, for the current "epidemic" of chronic pain *(Cousins, 1997)*. This alone should encourage exploration of optimal protocols for other painful conditions.

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Low-Level Laser Therapy in Acute Pain: A Systematic Review of Possible Mechanisms of Action and Clinical Effects in Randomized Placebo-Controlled Trials

JAN MAGNUS BJORDAL, P.T., Ph.D.,[1] MARK I. JOHNSON, Ph.D.,[2]
VEGARD IVERSEN, Ph.D.,[3] FLAVIO AIMBIRE, M.SC.,[4] and
RODRIGO ALVARO BRANDAO LOPES-MARTINS, M.Pharmacol., Ph.D.[5]

ABSTRACT

Objective: The aim of this study was to review the biological and clinical short-term effects of low-level laser therapy (LLLT) in acute pain from soft-tissue injury. *Background Data:* It is unclear if and how LLLT can reduce acute pain. *Methods:* Literature search of (i) controlled laboratory trials investigating potential biological mechanisms for pain relief and (ii) randomized placebo-controlled clinical trials which measure outcomes within the first 7 days after acute soft-tissue injury. *Results:* There is strong evidence from 19 out of 22 controlled laboratory studies that LLLT can modulate inflammatory pain by reducing levels of biochemical markers (PGE_2, mRNA Cox 2, IL-1 , TNF), neutrophil cell influx, oxidative stress, and formation of edema and hemorrhage in a dose-dependent manner (median dose 7.5 J/cm², range 0.3–19 J/cm²). Four comparisons with non-steroidal anti-inflammatory drugs (NSAIDs) in animal studies found optimal doses of LLLT and NSAIDs to be equally effective. Seven randomized placebo-controlled trials found no significant results after irradiating only a single point on the skin overlying the site of injury, or after using a total energy dose below 5 Joules. Nine randomized placebo-controlled trials ($n = 609$) were of acceptable methodological quality, and irradiated three or more points and/or more than 2.5 cm² at site of injury or surgical incision, with a total energy of 5.0–19.5 Joules. Results in these nine trials were significantly in favor of LLLT groups over placebo groups in 15 out of 18 outcome comparisons. Poor and heterogeneous data presentation hampered statistical pooling of continuous data. Categorical data of subjective improvement were homogeneous (Q-value = 7.1) and could be calculated from four trials ($n = 379$) giving a significant relative risk for improvement of 2.7 (95% confidence interval [CI], 1.8–3.9) in a fixed effects model. *Conclusion:* LLLT can modulate inflammatory processes in a dose-dependent manner and can be titrated to significantly reduce acute inflammatory pain in clinical settings. Further clinical trials with adequate LLLT doses are needed to precisely estimate the effect size for LLLT in acute pain.

INTRODUCTION

TREATMENT OF PAINFUL DISORDERS with LLLT is still considered to be experimental by mainstream medicine. Proponents of LLLT have put forward multiple hypotheses about its biological actions, but these have been met with scepticism. Recently, there has been renewed interest in the clinical use of LLLT by mainstream medicine following the publication of articles in prestigious medical journals. For example, a scholarly paper in the *Journal of Rheumatology*[1] suggests that LLLT could be a viable alternative to drug medication in arthritis management. Ten years ago, a review of basic and clinical re-

[1]Section of Physiotherapy Science, University of Bergen, and Institute of Physiotherapy, Bergen University College, Bergen, Norway.
[2]Faculty of Health, Leeds Metropolitan University, Leeds, United Kingdom.
[3]Institute of Biomedical Sciences, Department of Physiology, University of Bergen, Bergen, Norway.
[4]Laboratory of Animal Experiments, IP&D Universidade Vale do Paraiba (UNIVAP), São José dos Campos, SP, Brazil.
[5]Institute of Biomedical Sciences, Pharmacology Department, Universidade Sao Paulo, São Paulo, SP, Brazil.

search concluded that, despite positive laboratory findings, LLLT had not established itself as a therapeutic tool.[2] Since then there have been an additional 79 controlled studies in cell cultures, 77 controlled studies in animals, and 58 randomized controlled clinical trials published in peer-reviewed journals. The bulk of new evidence needs to be systematically reviewed in order to determine the factors that influence LLLT outcome and to determine the optimal characteristics for treatment success.

LLLT is no longer believed to be a mythical alternative therapy with diffuse and hypothetical mechanisms of biological action, as it has distinct biophysical properties[3,4] and a dose-dependent mechanism of action.[5] Nevertheless, well-designed randomized controlled trials continue to use LLLT doses that are well below those expected to achieve biological responses.[6,7] This is likely to bias studies towards showing no effect from LLLT, and this may have contributed to the contradictory findings. This "shoot in the dark" approach to LLLT needs to be replaced by selecting LLLT parameters and titrating LLLT dose according to evidence gathered in a systematic manner.

We have shown in a previous systematic review that LLLT is effective for chronic joint disorders such as osteoarthritis, if LLLT is administered at the anatomic location of the pathology and the dose is titrated to achieve the desired biological action. For instance, in osteoarthritis of the knee when a minimum of 3 cm^2 of the joint capsule is exposed, the optimal parameters for infrared GaAs 904-nm pulse lasers are an intensity of 12–60 mW/cm^2 and a dose of 1–4 Joule per point. Optimal parameters for infrared GaAlAs 820–30-nm lasers are an intensity of 30–210 mW/cm^2 and a dose 6–24 Joule per session.[8] Similarly, this approach to developing optimal parameters and dosage has been adopted by the World Association of Laser Therapy (WALT) in their recommendations for treating musculoskeletal disorders with LLLT (www.walt.nu).

LLLT has been used in pain management for over two decades. Pain is a subjective experience, and acute pain is a warning signal which expresses that body tissue is about to be injured. If injury actually occurs, then a cascade of pathophysiological events will take place in a well-mapped simultaneous and chronological order.[9] Pain intensity is usually most prevalent in the inflammatory phase during the first hours and days after injury, and in most cases, pain decreases as the tissue repair processes get under way. In chronic pain, the experience of pain may be different, and pain may be present in the absence of known pathology or tissue damage. This may be due to a state of persistent central sensitization within the central nervous system despite the healing of the original injury. In peripheral nerve injury, pain may occur from persisting mechanical pressure, neurogenic inflammation, or damage to the nerve structure. Inflammation may also be present in some chronic musculoskeletal pain disorders. Particularly in episodes with flares of symptom aggravation in degenerative and systemic arthritis, increased synovial inflammatory activity may be similar to what is seen in acute injuries.[10,11] For tendon disorders, short-lived flares in disease activity seem to be associated physical overload, although a definite link between pain aggravation and inflammatory activity is still uncertain.[12] On the other hand, NSAIDs have been shown to reduce pain in both acute and subacute tendinopathies.[13] Reducing oxidative stress with anti-oxidants has also been shown to preserve tendon structure *in vitro*,[14] and LLLT has been found to reduce oxidative stress[15] and improve healing[16] in acute tendon injuries. For chronic muscle pain, both the capacity of the muscle cells to withstand fatigue and subsequently cell damage, and the vasoactive response to muscle contractions, seems impaired.[17,18] In this plethora of pathophysiological processes, LLLT has been suggested to modulate several of the processes involved. One hypothesis has been that LLLT can modulate inflammatory processes,[19] and a second hypothesis is that LLLT acts by altering excitation and nerve conduction in peripheral nerves.[20] A third hypothesis has been that LLLT stimulates the release of endogenous endorphins.[21]

In order to test the evidence behind the most common hypotheses for acute pain modulation by LLLT, first, we decided to search and critically appraise the evidence from laboratory trials which assess possible pain-relieving effects within the first 72 h of the inflammatory phase. Secondly, we wanted to assess the effect of LLLT in randomized controlled clinical trials within 1 week after an acute musculoskeletal injury. And thirdly, we wanted to subgroup the clinical trials by the adequacy of the doses used and the recommended doses that can be extrapolated from controlled dose-finding laboratory trials.

METHODS

A review protocol was specified prior to conducting the review.

Review protocol specification for laboratory studies

1. To search published literature for controlled LLLT trials performed in cell cultures, or acute injuries in animals and healthy humans with outcomes measured within 7 days after induction of injury.
2. To extract power density and dose of LLLT used in positive outcome studies in order to reveal putative mechanisms of pain relief and potential dose-response patterns.

Review protocol specification for randomized controlled clinical trials

1. To search published literature for randomized controlled trials that applied LLLT to acute injuries or post-surgery, and outcomes were recorded during the first 7 days.
2. To evaluate the methodological quality of each study using the Jadad scale.[22]
3. To estimate the size of effect at 4, 6, 8, 12, 24, 48, 72, or 168 h after injury.
4. To conduct a subgroup analysis to compare the effect size of adequate versus inadequate LLLT dose and treatment procedure, as determined by the findings from the review of laboratory studies.

Literature search

A search of published literature was performed using Medline, Embase, The Cochrane Library, CINAHL, and the Physiotherapy Evidence database (PEDro). The search string used

for laboratory trials was as follows: acute OR injury OR soft-tissue OR pain OR inflammation OR edema OR neutrophil influx AND low laser therapy AND controlled. The search string used for clinical trials was as follows: acute OR injury OR soft-tissue OR surgery AND pain AND low laser therapy AND randomized OR randomized. In addition, hand searches of national Scandinavian physiotherapy journals, conference abstracts, and reference lists of systematic reviews were performed, and experts in the field were consulted. No language restrictions were applied.

Procedure

Inclusion criteria. Laboratory studies were included for review if they used (1) a no-treatment or sham treatment control group; and (2) a quantitive measure of acute injury such as neutrophil cell influx, presence of inflammatory markers, cytokine presence, edema, withdrawal latency, physical function, nerve latency time, nerve conduction velocity, hemorrhagia, microcirculation, or pain. Clinical trials were included for review if they used (1) a method of randomisation to allocate patients to groups; (2) a placebo laser control group; (3) outcome measures for either pain, and/or edema and/or function; and (4) assessors who were blinded to treatment group.

Exclusion criteria. Clinical trials were excluded if there was concomitant use of steroid therapy during the trial period or steroid therapy had ended within 4 weeks preceding the start of the trial.

Statistical analysis

For continuous data, mean differences of change for intervention groups and placebo groups, and their respective standard deviations (SD), were included in a statistical pooling. If variance data were not reported as SDs, they were calculated from the trial data of sample size and other variance data such as *p*-value, *t*-value, standard error of the mean, or 95% confidence interval (CI). Results were presented as weighted mean difference (WMD) between test drug and placebo with 95% CI in mm on VAS (i.e., as a pooled estimate of the mean difference in change between the treatment and the placebo groups, weighted by the inverse of the variance for each study).[23] For heterogeneous trial samples, a random effects model was used for calculation, and for confirmed absence of heterogeneity ($p < 0.05$), a fixed effects model was applied.

For categorical data, improvement was calculated by the relative risk ratio and the number-needed-to-treat (NNT) values.[24] NNT can be expressed as the reciprocal of the absolute risk reduction. The 95% CI for the NNT is constructed by inverting and exchanging the limits of a 95% CI for the absolute risk reduction.

RESULTS

The literature search revealed 131 laboratory trials and 102 randomized controlled clinical trials with LLLT. Of these trials, 33 laboratory trials and 15 randomized placebo-controlled satisfied our inclusion criteria for treating acute injury or postoperative pain, and provided outcomes measured within 7 days after trauma (Table 1).

Laboratory studies

A variety of biological mechanisms were identified as potential contributors of pain-relieving responses associated with LLLT (Fig. 1).

Neurophysiological effects. Seven studies found none, or only minor, changes in neurophysiological processes or nerve conduction velocities in intact peripheral nerves after LLLT.[20,25–30] One study in healthy subjects found LLLT reduced nerve conduction velocity and increased negative peak latency with energy dose of 1 Joule per stimulation point, but there were no effects from energy doses at 0.5 or 1.5 Joules when applied over the sural nerve.[31] There was no convincing evidence that LLLT could act by substantial rapid modulation of neurophysiological processes in intact peripheral nerves in the absence of inflammation. Although a possible narrow therapeutic window cannot be ruled out, available evidence suggests that the effect of LLLT on neurophysiological processing was of limited practical use.

Release of endogenous opioids. One study found increased levels of endorphins,[21] although local injection of the opioid antagonist naloxone produced only minor reductions of LLLT-induced pain relief in two studies.[32,33] There was limited evidence that the pain-relieving effects of LLLT are due to an increase in the levels of endorphins.

Local effects on delayed onset muscle soreness. Two studies by the same investigators found that LLLT did not affect delayed onset muscle soreness (DOMS) in healthy humans undergoing eccentric exercises. These investigators used a cluster probe combining a single 820-nm laser with five different wavelengths (range 660–950 nm) of superluminous LED therapy and high doses.[34,35]

Local microcirculatory and angiogenetic effects. There was strong evidence that LLLT improves angiogenesis, through increased growth factor secretion and formation of collateral vessels in the injured region in cell and animal studies during the first 7 days after injury.[36–39] This effect is dose-dependent, with therapeutic windows ranging from 0.5 to 6 J/cm², and it has been demonstrated for laser with wavelengths 632, 820, and 904 nm.

Local anti-inflammatory effects. There was strong evidence that LLLT modulates biochemical inflammatory markers and produces local anti-inflammatory effects in cells and soft tissue (Fig. 1).

Effects on biochemical markers. Five studies found that LLLT inhibited the release of PGE_2 when compared to a placebo control.[40–44] One study found that LLLT did not affect levels of tumor necrose factor (TNF), blood monocytes, and vein endothelial cells.[45] However, these findings were contradicted by two other studies.[46,47] This may indicate a narrow therapeutic range for LLLT inhibition of TNF release. Three studies found that LLLT increased plasma fibrinogen levels,[46,48,49] and three studies found that LLLT reduced levels of interleukin-1 .[40,50,51] One study on periodontal inflammation in humans found that LLLT did not alter interleukin-1 but did

TABLE 1. TRIAL CHARACTERISTICS AND DOSAGE IN LABORATORY TRIALS WITH SIGNIFICANT LLLT MODULATION OF INFLAMMATION

First author, year, model	*Inflammatory agent*	*Laser type, mean output power (mW)*	*Power density (mW/cm²)*	*Dose (Joules/cm²)*
Honmura, 1992, rat paw edema	Carrageenan	830 nm, 60 mW	32	9.6
Campana, 1993, arthritis animal	Urate crystals	633 nm, 5 mW	6	0.72
Honmura, 1993, rat paw edema	Carrageenan	830 nm, 60 mW	32	9.6
Shimizu, 1995, ligament cells	Mechanically stretched	830 nm, 30 mW	12	2.3–7.4
Ozawa, 1997, ligament cells	Mechanically stretched	830 nm, 700 mW	6–13	3.9
Sattayut, 1999, myofibroblast cells	Carrageenan	820 nm, 200 mW	22	4–19
Campana, 1999, arthritis animal	Urate crystals	633 nm, 30 mW	30	8
Nomura, 2001, fibroblast cells	Lipopoly-saccharide	830 nm, 50 mW	6–13	4–7.9
Sakurai, 2001, fibroblast cells	Lipopoly-saccharide	830 nm, 700 mW	21	1.9–6.3
Shefer, 2001, skeletal muscle cells	Cell starvation	633 nm, 4.5 mW	112	0.34
Campana, 2003, arthritis animal	Pyrophosphate crystals	633 nm, 6.5 mW	200	8.0
Dourado, 2004, mice	Snake venom	904 nm, 50 mW	90	2.8
Albertini, 2004, rat paw edema	Carragenan	660 nm, 2.5 mW	31	7.5
Ferreira, 2004, rat paw edema	Carrageenan PGE_2	633 nm, 12 mW	171	7.5
Pessoa, 2004, rat skin wound	Excised skin flap 0.5 cm²	904 nm, 2.8 mW	5	0.66
Avni, 2005, rat muscle ischemia	Hypoxia	810 nm, 400 mW	42	5.0
Lopes-Martins, 2005, mice pleurisy	Carrageenan	660 nm, 25 mW	31	7.5
Aimbire, 2005, airway hyperreactivity	Lipopoly-saccharide	660 nm, 2..5 mW	31	7.5
Aimbire, 2005, rat lung injury	Bovine serum albumin	660 nm, 2.5 mW	31	7.5
Median results		830 nm (633–904)	31 mW/cm² (5–171)	7.5 J/cm² (0.3–19)

The first column gives the name of first author, year of publication, and the experimental model used. Other columns give inflammatory agent used, laser type, and mean optical output, power density, and dose.

affect other inflammatory outcomes.[52] Two studies found reductions of cyclooxygenase 2 (Cox2) mRNA after LLLT exposure.[44,53] One study found that LLLT reduced levels of plasminogen activator in stretched periodontal ligament cells.[48]

Effects on cells and soft tissue. Laboratory investigations using animal models found that LLLT reduced inflammatory cell infiltration in four studies[47,54–56] and edema volume in four studies.[5,19,57,58] Four studies using cell cultures, rats, and mice found that LLLT reduced the formation of hemorrhagic le-

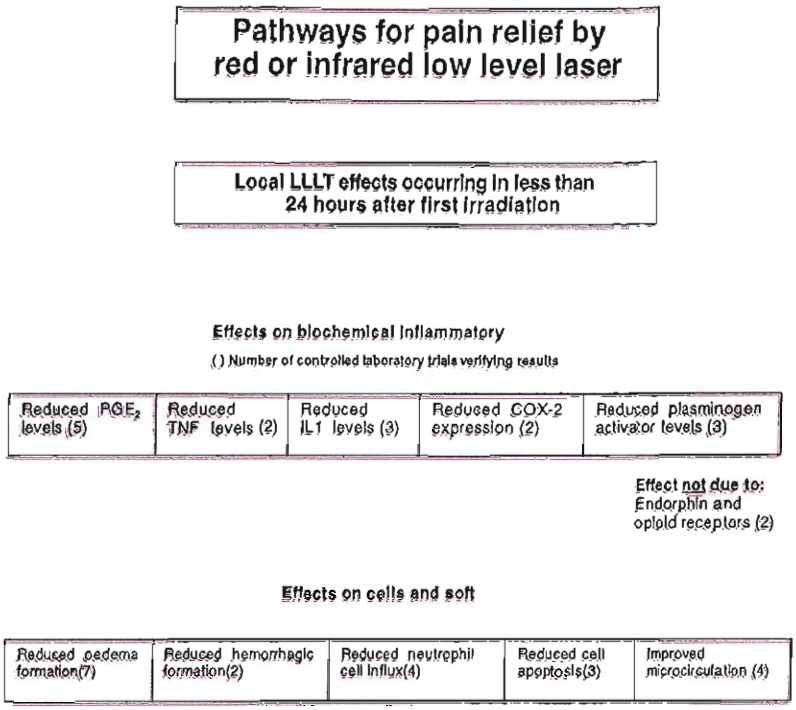

FIG. 1. Flow chart of the evidence behind biological effects of LLLT laboratory trials of acute pain mechanisms. Each identified outcome is listed, as well as the number of laboratory trials supporting or refuting that the specific outcome can be affected by LLLT.

sions,[58] reduced apoptosis,[59] reduced necrosis of muscle cells after ischemia,[60] and increased myotube proliferation[61] when compared to sham-irradiated controls.

Anti-inflammatory effects of LLLT versus non-steroidal anti-inflammatory drugs. Head-to-head comparisons between LLLT and pharmacological substances in four animal studies found that there were no differences in anti-inflammatory effects between LLLT and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin,[32] meloxicam,[62] celecoxib,[55] and diclofenac[5] when they were administered at doses equivalent to those given in clinical practice (Fig. 2).

Interpretation of evidence on mechanisms for acute pain relief by LLLT

There was strong evidence from 18 out of 19 studies that red and infrared wavelengths of LLLT can act locally and rapidly to modulate the inflammatory processes in injured tissue. These anti-inflammatory effects include changes in biochemical markers, altered distribution of inflammatory cells, and reduced formation of edema, hemorrhage, and necrosis. These anti-inflammatory effects are dose-dependent. LLLT wavelength does not appear to influence outcome by a significant degree providing it lies within the red and infrared range. However, this result does not exclude the possibility that certain wavelengths may be more effective than others in some diseases where specific cell types or specific parts of pathophysiological processes are targeted. There was no convincing evidence that LLLT produces pain relief through any other mechanism during the first hours and days after acute injury.

Transition of laboratory findings into clinical dose recommendations

The median dose at the target location of studies reporting anti-inflammatory effects was 7.5 J/cm² (range 0.7–19 J/cm²) and a power density of 5–171 mW/cm² for continuous red lasers with wavelengths of 632–660 nm or infrared lasers with wavelengths of 810–830 nm. For infrared 904-nm lasers, having strong pulses peaking above 1 Watt, efficacy was demonstrated with lower doses at 0.7 and 2.8 Joules. This difference in dose levels coincides with similar findings in meta-analyses of clinical trials.[8,63] In animal studies, the entire inflamed area can be treated by LLLT stimulation at one point by single diode laser. In contrast, the volume of inflamed tissue and edema containing inflammatory cells is larger in the clinical situation and cannot be effectively irradiated with a single diode laser. In clinical practice, LLLT dose is titrated according to the volume of inflamed tissue and edema. If the skin surface is intact, the depth to the target tissue and subsequent energy must also be considered. Lasers without strong pulses and an output of less than 50 mW can effectively irradiate tissue that lies within 10–15 mm of the laser source. Lasers with an output of 100–500 mW can effectively irradiate tissue that lie no more than 30–40 mm from the laser source. However, it should be remembered that excessively high power densities may inhibit cell activity if too near to the laser source.

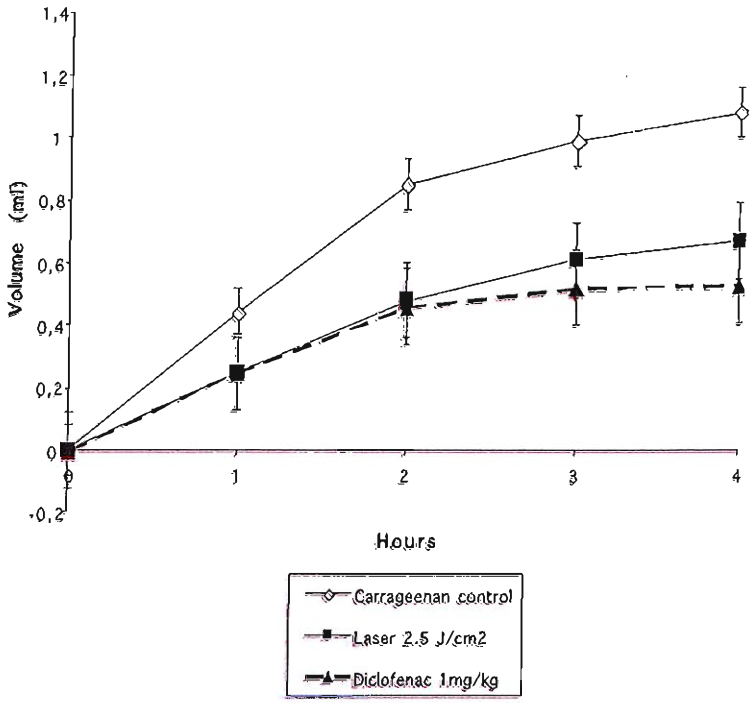

FIG. 2. Development of carrageenan-induced rat paw edema and treatment by LLLT at 2.5 J/cm² and a dose of diclofenac potassium at 1 mg/kg, which is 41% higher than the recommended diclofenac dose for humans. For both active treatments, edema development was significantly reduced compared to the control group ($p < 0.05$). (Modified from an experiment from our research group; for full details, see Albertini et al., 2004.)

Clinical trials

Fifteen placebo-controlled trials were included in the review (Table 2). Six of these trials used daily energy doses 5 Joules or less and found no significant effects from LLLT for ankle sprains[64,65] or oral surgery.[41,66] Nine trials ($n = 609$) administered LLLT with daily doses higher than 5 Joules for acute ankle sprains,[67] acute Achilles tendonitis,[68–70] medial tibial shin splint,[71] oral surgery,[56] and cholecystectomy.[72] Eight of these nine trials found that LLLT was significantly better than placebo in at least one of the outcomes measured (Table 3).

The number of cases with subjective improvement on the first day could be calculated from four trials that had administered an adequate dose of LLLT (i.e., 5J/day, $n = 379$). There were 83 patients in the active LLLT group and 27 in the placebo-control group reporting improvement, thus giving a significant Relative Risk for improvement at 2.7 (95% CI, 1.8–3.9) in a fixed effects model ($Q = 7.1$, not significant for heterogeneity) (Fig. 3). The corresponding value for numbers-needed-to-treat is 2.1 (95% CI, 1.4–2.9).

DISCUSSION

The results of this review demonstrate that an adequate dosage of LLLT produces anti-inflammatory effects and pain relief over that seen with placebo. The effect size in laboratory studies during the first hours after injury equals that of NSAIDs when optimal doses are administered. Inhibition of inflammatory processes after injuries may hinder beneficial processes later in the proliferative and remodelling phases of tissue repair. For example, steroids are very potent therapeutic agents which inhibit inflammatory processes and relieve pain, but they also impair proliferation and delay tissue repair.[16,73,74] Placebo-controlled clinical trials of NSAIDs for ankle injuries also show significant pain relief during the first few days, but this is also associated with impaired edema absorbtion for several weeks.[75] LLLT can be advantageous because its therapeutic window for anti-inflammatory actions overlaps with its ability to improve tissue repair.[3] The ability of LLLT to promote tissue repair in a dose-dependent manner, with optimal doses being 2 J/cm² at target tissue, has been extensively studied and was outside the scope of the present review.[76] However, when taken together, the available evidence strongly suggests that, for acute pain, optimal LLLT effects will be achieved if it is administered at high doses, typically 7.5 J/cm² at the target tissue, in the first 72 h (to reduce inflammation), followed by lower dosages, typically 2 J/cm² at target tissue, in subsequent days (to promote tissue repair).

The speculation about putative biological mechanisms and the difficulty of translating laboratory findings to the clinical situation are likely to have hindered the acceptance of LLLT as an effective therapeutic agent for acute pain.[77] Claims that LLLT irradiation of intact nerves produces meaningful changes in nerve activity and/or endorphin release was not supported by the findings of this review. Evidence for LLLT irradiation of injured

TABLE 2. LLLT THERAPY IN ACUTE PAIN: CHARACTERISTICS FOR TRIALS MEASURING EFFECTS WITHIN 7 DAYS

First author, year, surgical procedure or type of injury	*Laser type, mean output power (mW)*	*Spot size in cm²*	*Number of irradiated points or area (cm²)*	*Total Joules delivered in 24 h*	*Dose above minimum dose limit*	*Method score max 5 (Jadad scale)*
Carillo, 1990, third molar	633 nm 5 mW	0.02	6 point	0.72[a]	No	3
Taube, 1990, third molar	633 nm 4 mW	0.02	1 point	0.48[a]	No	3
Fernando, 1993, third molar	830 nm 30 mW	0.02	1 point	4.0[a]	No	3
Masse, 1993, third molar	633 and 904 nm 5 mW	0.02	1 point	0.37[a]	No	3
Axelsen, 1993, ankle sprain	830 nm 30 mW	0.02	1 point	0.9[a]	No	4
de Bie, 1998, ankle sprain	904 nm 2.5 and 25 mW	0.64	1 point	0.5 and 5[a]	No	5
Røynesdal, 1993, third molar	830 nm 40 mW	0.1	1 point	6	Yes	4
Nekcel, 2001, third molar	809 nm 50 mW	1.0	2.5 cm²	7.5	Yes	3
Kreisler, 2004, endodontic	809 nm 50 mW	1.0	2.5 cm²	7.5	Yes	3
Moore, 1992, cholecystectomy	830 nm 60 mW	0.02	20 points	9.6	Yes	3
Tabau, 1985, ankle sprain	904 nm 6.5 mW	5	5 cm²	19.5	Yes	3
Stergioulas, 2004, ankle sprain	820 nm 40 mW	0.16	10 points	24	Yes	4
Darre, 1994, achilles	830 nm 30 mW	0.2	4 points	16	Yes	4
Bjordal, 2005, achilles	904 nm 10 mW	0.5	3 points	5.4	Yes	5
Nissen, 1994, shin splint	830 nm 40 mW	0.2	2.4 J/cm²	2.4 to ?[a]	?	3

[a]Small dose.

Trials in italics have used doses outside the optimal dose range for LLLT determined from laboratory studies or have failed to cover over one-third of the inflamed tissue volume.

nerves is considerably more mature, with a growing number of laboratory and clinical trials finding positive effects.[78,79]

New hypotheses about LLLT mechanisms, such as systemic effects through nitric oxide synthesis (NOS), cannot be ruled out. But at the moment, targeting modulation of systemic NOS and local TNF levels by LLLT are only experimental possibilities that need to be explored further. Our understanding of how LLLT can be used therapeutically to relieve pain by these two mechanisms is novel, and far from what is required for safe and effective clinical use.

This review demonstrated that a prerequisite for treatment success is that laser energy be distributed across the inflamed tissue using a sufficiently high anti-inflammatory dose (i.e., Joules per day). Clinical trials that fail to do this will bias trial outcome towards negative outcome for LLLT (i.e., no effect). Several trials in this review used doses just above the lower limit of the therapeutic range, and the exact effect size under optimal conditions remains to be estimated. Further weaknesses in published trial data observed in this review were considerable inter-trial variability in baseline pain scores, and inter-trial variability in the selection and reporting of clinical outcomes.

Pharmaceutical companies seeking approval by the U.S. Food and Drug Administration (FDA) for NSAIDs in acute pain tend to use evidence from randomized placebo-controlled trials with impacted third molar surgery.[80] Surprisingly few trials have been performed on more common soft-tissue injuries.

In this review NNT calculations were only possible for measurements taken during the first 24 h after injury or sur-

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Address reprint requests to:
Dr. Jan Magnus Bjordal
Section of Physiotherapy Science
University of Bergen
5018 Bergen, Norway Kalfarveien 31

E-mail: jmb@hib.no

The Mechanistic Basis for Photobiomodulation Therapy of Neuropathic Pain by Near Infrared Laser Light

Vanessa Milanesi Holanda, MD, PhD,[1,2*] Maria Cristina Chavantes, MD, PhD,[1,3] Xingjia Wu, BS, and Juanita J. Anders, PhD[4]

[1]*Post Graduate Program in Biophotonics Applied to the Health Sciences, University Nove de Julho—UNINOVE, São Paulo, SP 01504-001, Brazil*

[2]*Center of Neurology and Neurosurgery Associates (CENNA), Beneficência Portuguesa of São Paulo Hospital, São Paulo, SP 01323-030, Brazil*

[3]*Post Graduate Program in Medicine, University Nove de Julho—UNINOVE, São Paulo, SP 01504-001, Brazil*

[4]*Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814*

Background and Objective: Various irradiances have been reported to be beneficial for the treatment of neuropathic pain with near infrared light. However, the mechanistic basis for the beneficial outcomes may vary based on the level of irradiance or fluence rate used. Using *in vivo* and *in vitro* experimental models, this study determined the mechanistic basis of photobiomodulation therapy (PBMT) for the treatment of neuropathic pain using a high irradiance.

Study Design/Materials and Methods: In vitro experiments: Cultured, rat DRG were randomly assigned to control or laser treatment (LT) groups with different irradiation times (2, 5, 30, 60, or 120 seconds). The laser parameters were: output power = 960 mW, irradiance = 300 mW/cm^2, 808 nm wavelength, and spot size = 3 cm diameter/area = 7.07cm^2, with different fluences according to irradiation times. Mitochondrial metabolic activity was measured with the MTS assay. The DRG neurons were immunostained using a primary antibody to β-Tubulin III. In vivo experiments: spared nerve injury surgery (SNI), an animal model of persistent peripheral neuropathic pain, was used. The injured rats were randomly divided into three groups ($n = 5$). (i) Control: SNI without LT; (ii) Short term: SNI with LT on day 7 and euthanized on day 7; (iii) Long term: SNI with LT on day 7 and euthanized on day 22. An 808 nm wavelength laser was used for all treatment groups. Treatment was performed once on day 7 post-surgery. The transcutaneous treatment parameters were: output power: 10 W, fluence rate: 270 mW/cm^2, treatment time: 120 seconds. The laser probe was moved along the course of the sciatic/sural nerve during the treatment. Within 1 hour of irradiation, behavior tests were performed to assess its immediate effect on sensory allodynia and hyperalgesia caused by SNI.

Results: In vitro experiments: Mitochondrial metabolism was significantly lower compared to controls for all LT groups. Varicosities and undulations formed in neurites of DRG neurons with a cell body diameter 30 μm or less. In neurites of DRG neurons with a cell body diameter of greater than 30 μm, varicosities formed only in the 120 seconds group. In vivo experiments: For heat hyperalgesia, there was a statistically significant reduction in sensitivity to the heat stimulus compared to the measurements done on day 7 prior to LT. A decrease in the sensitivity to the heat stimulus was found in the LT groups compared to the control group on days 15 and 21. For cold allodynia and mechanical hyperalgesia, a significant decrease in sensitivity to cold and pin prick was found within 1 hour after LT. Sensitivity to these stimuli returned to the control levels after 5 days post-LT. No significant difference was found in mechanical allodynia between control and LT groups for all time points examined.

Conclusion: These *in vitro* and *in vivo* studies indicate that treatment with an irradiance/fluence rate at 270 mW/cm^2 or higher at the level of the nerve can rapidly block pain transmission. A combination therapy is proposed to treat neuropathic pain with initial high irradiance/fluence rates for fast pain relief, followed by low irradiance/fluence rates for prolonged pain relief by altering chronic inflammation. Lasers Surg. Med.

Key words: dorsal root ganglion; fluence rate; laser irradiation; photoneuromodulation; transient neuronal injury

INTRODUCTION

Neuropathic pain is a common, debilitating disorder with a complex etiology [1]. Although a number of pharmacologic agents have been used to treat neuropathic

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*Correspondence to: Vanessa Milanesi Holanda, Rua Martiniano de Carvalho, 807-suite 705, Bela Vista, São Paulo, SP 01321-001, Brazil. E-mail: vanessamila@gmail.com

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pain, reported outcomes have been poor with less than half of the patients reporting satisfactory relief of their symptoms [2]. Photobiomodulation therapy (PBMT), previously referred to as low level laser therapy, has been used to decrease various types of neuropathic pain in preclinical animal models and in randomized controlled clinical trials. PBMT has been reported to reduce pain and improve function in compressive neuropathies such as carpal tunnel syndrome [3]. Also, systematic reviews and meta-analysis of randomized controlled trials found that PBMT reduced acute and chronic neck pain [4,5].

Since PBMT can act in part by causing an anti-inflammatory effect in the target tissue [6–9], it has promise as an effective treatment for neuropathic pain associated with inflammation. Considering the involvement of the central nervous system in pain, it is important to note that photobiomodulation (PBM) can alter microglial phenotypes from pro- to anti-inflammatory across the M1/M2 spectrum in a dose-dependent manner [10]. A number of pre-clinical animal studies investigated the effect of PBMT on inflammatory markers in neuropathic pain models. Hsieh et al. [11] reported a decrease in pro-inflammatory markers (tumor necrosis factor [TNF], interleukin 1 beta [IL-1β], and hypoxia-inducible factor 1-alpha [HIF-1α]) at a chronic constriction injury site in the rat sciatic nerve in PBMT treated animals compared to non-treated-injured animals. Cidral-Filho et al. [12], using a mouse sciatic nerve crush model, reported that PBMT reduced mechanical hypersensitivity and decrease spinal cord and sciatic nerve levels of TNFα. Recently, our laboratory reported that PBMT effectively reduced mechanical hypersensitivity in a spared nerve injury preclinical model of neuropathic pain and modulated macrophage/microglial activation to an anti-inflammatory phenotype [13]. Both studies provide evidence that PBMT is effective for treating neuropathic pain by altering the inflammatory response. In these studies, the fluences (energy densities) were 9 J/cm^2 [11], 2.5 J/cm^2 [12], and 8 J/cm^2 [13] and the irradiances (power densities) were 150 mW/cm^2 at the skin surface [11], 80 mW/cm^2 at the skin surface [12], and 43.25 mW/cm^2 at the target tissue [13].

In contrast, the pioneering work of Dr. Chow et al. on the clinical efficacy of PBMT for neck pain [14] and the mechanistic basis of pain suppression [15] used irradiances of 670 mW/cm^2 at the skin surface for the clinical study [14] and 300 mW/cm^2 at the cell surface for the *in vitro* experiments [15] at a wavelength of 830 nm. A systematic review on the inhibitory effects of laser irradiation on peripheral mammalian nerves and analgesic effects examined 44 studies: 18 human studies and 26 animal studies [16]. Although inconsistently reported, irradiances that suppressed conduction velocity and/or reduced the amplitude of the action potentials ranged from 300 mW/cm^2 to 1.73 W/cm^2 in the human studies. One important conclusion of this systematic review was that the inhibition of nerve conduction requires comparatively high therapeutic doses [16].

Recently, we completed a pilot, clinical study for treatment of low back pain that compared three treatment modalities: lidocaine injection, radiofrequency, or PBMT with 808 nm wavelength light with high irradiance and fluence (measured at the tip of the fiber optic). The data showed that PBMT applied bilaterally to the dorsal root ganglia (DRG) of the second lumbar spinal nerves decreased low back pain within 5 minutes which was comparable to lidocaine injection [17]. These results lead to the development of this combined *in vitro* and pre-clinical animal study to better understand the mechanistic basis underlying the photoneuromodulation of the neuropathic low back pain at high irradiances. The data from this study and our previous experiments on nerve regeneration and pain suppression serve as the basis for discussion of mechanisms of action for pain suppression based on irradiance.

METHODS

In Vitro Experiments

Cell culture. Primary rat dorsal root ganglion neurons (Lonza Walkersville, Inc. Walkersville, MD) were seeded in Poly-D-Lysine/Laminin (30 μg/ml poly-D-lysine and 2 μg/ml laminin) coated 4-well chamber slides with a seeding density of 2×10^4 cell/well in primary neural growth medium (PNGM) according to manufacturer's instructions. The cells were incubated at 37°C, 5% CO_2 for 48 hours.

Laser irradiation. The laser device used for irradiation of the cells was a CW, 808 nm wavelength diode laser with adjustable output power up to 2 W. Based on our previous clinical pilot study on low back pain in which a high irradiance was delivered to the DRG [17] and a previous *in vitro* study on DRG neurons, in which 300 mW/cm^2 at different treatment times caused varicosity formation and slowing of nerve conduction in the small DRG neurons [15], an irradiance of 300 mW/cm^2 was used in this study. Measurements were made to determine the output power that was needed to deliver 300 mW/cm^2 to the cells (LabMaster Ultima power meter with LM-3 HTD sensor, Coherent, Inc., Santa Clara, CA). After incubating for 48 hours, the cultures were randomly assigned to either control or laser treatment (LT) groups (irradiation times = 2, 5, 30, 60, or 120 seconds). The laser parameters were: output power = 960 mW, irradiance = 300 mW/cm^2, 808 nm wavelength, and spot size: 3 cm diameter/area = 7.07 cm^2.

MTS assay. MTS assay (Promega, Madison, WI) was used to measure the metabolic activity in the living cells. This assay is based on the reduction of tetrazolium salts into formazan, which can be measured colorimetrically. The conversion is presumably accomplished by reductase enzymes in mitochondria. Forty minutes after laser irradiation, MTS solution was added to each well. Forty minutes were chosen based on reports in the literature and previous work in our laboratory that identified that the peak time post-irradiation for change in mitochondrial metabolism was 40 minutes [18]. After incubation for 1.5 hour at 37°C, the supernatant was read for absorbance at 485 nm using FLUOstar OPTIMA plate reader (BMG Labtech, Inc., Cary, NC). Blank controls were medium

alone with MTS solution. Each light parameter setting was measured in triplicate. This experiment has been repeated twice and the data were combined.

Immunocytochemistry of β-tubulin III. Cells were fixed with 4% paraformaldehyde and then blocked in PBS with 10% goat serum and 0.1% Triton for 30 min at room temperature (RT). Cells were then incubated with Mouse anti-β-Tubulin III antibody (1:75 in PBS with 1% goat serum, Sigma, St. Louis, MO) for 1 hour at RT, followed by incubation with secondary antibody (AlexaFluor488 Goat anti-mouse IgG, 2.5 μg/ml in PBS with 1% goat serum, Life Technologies, Grand Island, NY) for 30 min at RT. After washing with PBS, samples were coverslipped with Vectashield mounting medium with DAPI (Vectors Laboratories, Inc. Burlingame, CA) and sealed with nail polish. The cells were photographed digitally using an Olympus BX43 fluorescence microscope equipped with an Olympus DP72 microscope digital camera (Olympus Imaging America, Inc. Center Valley, PA). The diameters of cell body were measured using Olympus CellSens software (Olympus Imaging America, Inc.). A minimum of 100 cells from each treatment and control groups was measured.

In Vivo Experiments

Animals. The animal use protocol (APG-14-808) was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Use Committee. Sixteen male Sprague–Dawley (SD) rats (201–225 gram, Charles River Laboratories International, Inc., Wilmington, MA) were used in this study. One rat was used for power penetration measurements and the other 15 rats were divided into three groups ($n = 5$) as follows: (i) Control: surgery without LT; (ii) Short term: surgery with LT on day 7 and euthanized on the same day after behavior test; (iii) Long term: surgery with LT on day 7 and euthanized on day 22. Animals were housed two per cage, under a 12-hour light/dark cycle, with access to food and water *ad libitum*.

Power penetration measurement. One male SD rat was anesthetized for light penetration measurement. The fluence rate was measured by a near infrared detector which was designed and built by B&W Tek, Inc. (Newark, DE). A small photo sensor ($2.0 \times 2.5\ mm^2$) was sealed in a glass tube. The output voltage of this sensor was calibrated such that a reading of 1 mV represented $1\ mW/cm^2$. The sensor was placed below the lumbar 4 and 5 DRGs. An 808 nm wavelength laser (Model BWF5-808-20, B&W Tek, Inc.), connected with a probe that had a rolling ball with an irradiation diameter of 4 cm, was placed in direct contact with the skin surface. Light penetration was measured for output powers of 3, 5, and 10 W (which was the maximum output power of this laser). The probe was moved until the highest reading was identified and recorded.

Surgery. Spared nerve injury (SNI) surgery, an animal model for peripheral neuropathic pain, was performed on all rats [19]. Briefly, rats were anesthetized with isoflurane (5% for induction and 0.5–3% for maintenance). An incision was made on the lateral left thigh and the bicep femoris was separated to expose the sciatic nerve and its branches. The common peroneal and tibial nerves were tight-ligated and 3–4 mm of each nerve (distal to the ligation) were cut and removed. Great care was taken to avoid any contact with the intact sural nerve. The muscle and skin were then sutured in two layers.

Laser irradiation. An 808 nm wavelength laser (Model BWF5-808-20, B&W Tek, Inc.) was used for both the long and short term treatment groups. Transcutaneous laser treatment was performed once on day 7 post-surgery with parameters: output power: 10 W, treatment time: 120 seconds. The probe had a small massage ball (circular area with a diameter of 4 cm) and was scanned along the nerve track during the 2 minutes of treatment from the thoracic 13 and lumbar 1 (T13/L1) spinal cord level to the lumbar 4 and 5 (L4/L5) DRG, the sciatic nerve, the sural nerve, and the involved dermatomes on the lateral plantar surface of the hind paw (Fig. 1). The irradiance rate was $270\ mW/cm^2$ at the DRG L4/L5 region according to the power penetration measurements. The rats were lightly anesthetized with isoflurane for the LT. The rats in the control group were handled in exactly the same manner as the irradiated rats but the laser was off. Within 1 hour of treatment, behavior tests were performed to assess the immediate effects of the laser treatment on sensory allodynia and hyperalgesia caused by the SNI model [20].

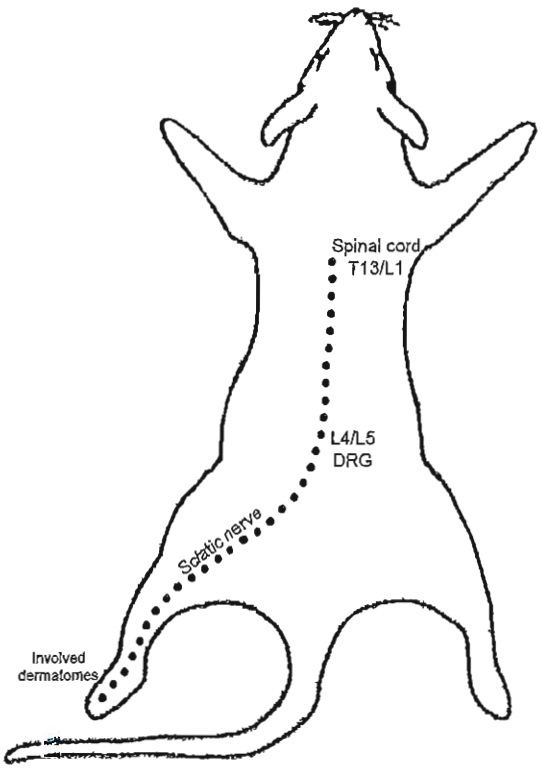

Fig. 1. Illustration of *in vivo* laser scanning pathway and primary targets.

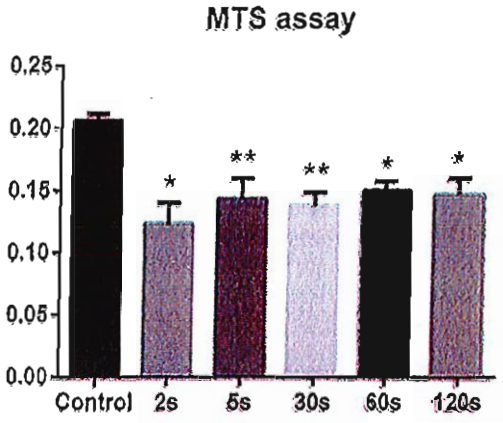

Fig. 2. Statistically significant reduction of mitochondrial metabolism as measured by MTS assay. $^{*}P < 0.005$, $^{**}P < 0.01$ compared to control group.

Behavior tests. Behavior tests included Heat Hyperalgesia, Cold Allodynia, Mechanical Hyperalgesia (Pin Prick), and Mechanical Allodynia (Electronic Von Frey). The animals were placed in an inverted plastic box on an elevated metal grid to allow for stimulation on the lateral plantar surface of the hind paw. Tests were performed before surgery (baseline), day 7 before LT and 1 hour after LT and on days 10, 12, 15, 18, and 21. The behavior tests done on day 7 post-irradiation were done at 1 hour after PBMT because the animals had been anesthetized and needed this amount of time to completely wake up. The behavior data from day 7 before and after LT represents the immediate effects from the laser irradiation. For long term effects, data from long term group were compared with the control group.

Heat hyperalgesia. The test used to measure heat hyperalgesia was modified from Hargreaves et al. [20]. The thermal stimulation was generated by a beam of radiant heat using an 808 nm wavelength laser with a 2 W output power and 3 mm diameter. Animals were placed on the elevated grid and acclimated for 5 min. The laser beam was positioned on the lateral plantar surface of the hind paw which is the region innervated by the intact sural nerve. The time when the hind paw was briskly withdrawn was recorded with a maximum cut-off time of 10 seconds. Both left (injured) and right (uninjured) sides were tested. The ratio was calculated as response time of left side divided by the response time of the right site. Rats were

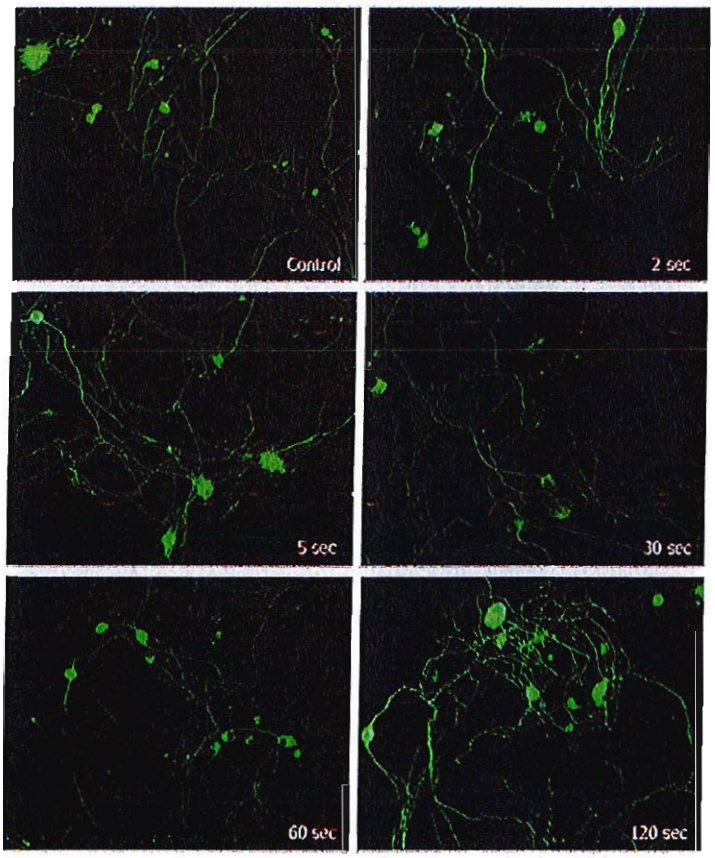

Fig. 3. Photomicrographs of control and LT DRG neurons immune-labeled with β-Tubulin III. Varicosities and undulations were present in LT groups.

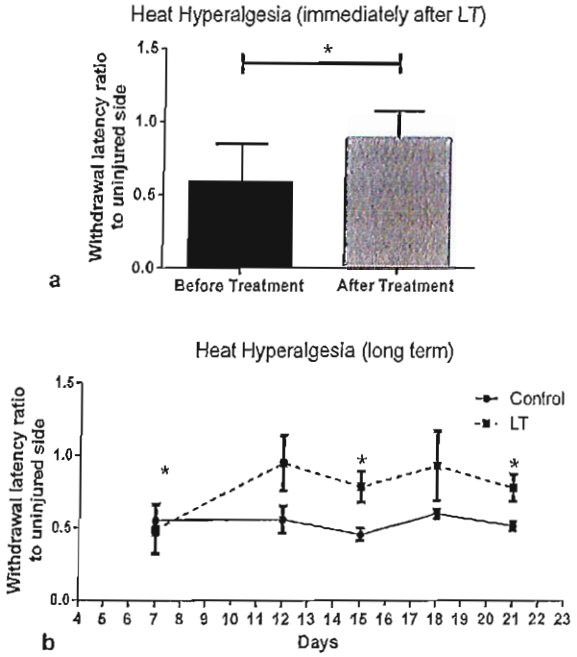

Fig. 4. Heat hyperalgesia. There was a decrease in sensitivity to the heat stimulus ($P < 0.05$) within 1 hour after irradiation (**a**). For the long term, a decrease was found in the LT group ($P < 0.05$) on days 5 and 21 (**b**).

tested three times with a 5-minute interval between each test.

Cold allodynia. After 5 min of acclimation on the elevated grid, cold acetone (20 μl, −20°C) was sprayed on the lateral plantar surface of the hind paw. The duration of the withdrawal response to the cold stimulation was recorded and graded in five levels: 0, no visible response; 1, startle response without paw withdrawal; 2, clear withdrawal of the paw; 3, prolonged withdrawal (2–30 seconds) often combined with flinching and licking of the paw; and 4, prolonged, repetitive withdrawal (30 seconds) and/or vocalization.

Mechanical hyperalgesia (pin prick). Pin prick test was performed using a safety pin after 5 min acclimation of the elevated grid [13]. A brief stimulation was applied to the lateral part of the plantar surface of the hind paw. The duration of paw withdrawal was recorded. An arbitrary minimal time of 0.5 second was used as normal response time. The maximum cut-off time was 15 seconds.

Mechanical allodynia (electronic von frey). SNI animals developed hypersensitivity to mechanical stimulation, which was measured using an electronic von Frey (Bioseb, Chaville, France) device [21,22]. The hand-held force transducer of the device can generate force from 0 to 500 g in 0.1 g intervals. After an acclimation period of 15 min, the plastic tip of the transducer was applied perpendicularly to the lateral plantar surface of the hind paw. The lowest force at which a brisk withdraw of the hind paw was recorded. The test was repeated three times with a 5-minute interval between each measurement, and the mean of the three measurements was computed for analysis.

Statistical Analysis for *In Vivo* and *In Vitro* Experiments

For the MTS assays, one way ANOVA with Tukey's multiple comparisons test were used to compare the effects between groups. For behavior data, results were presented as mean + standard error of the mean (SEM). Unpaired two-tailed t-test was used to compare the immediate effects between before and after irradiation. For long-term behavior data, Two-way ANOVA with Sidak's multiple comparisons test was used to compare control and long-term LT groups. Family-wise significance and confidence levels were set at 0.05 (95% confidence interval).

RESULTS

In Vitro Experiments

All the LT groups with treatment times of 2, 5, 30, 60, and 120 seconds had statistically significant lower mitochondrial metabolism compared to controls (Fig. 2). There was no difference in the metabolic inhibition for all irradiation times tested. This finding is due to the fact that 40 minutes post irradiation was previously identified as the peak time post-irradiation for change in mitochondrial metabolism [18].

In all LT groups, varicosities, and undulations were present in neurites of DRG neurons with a cell body diameter of 30 μm

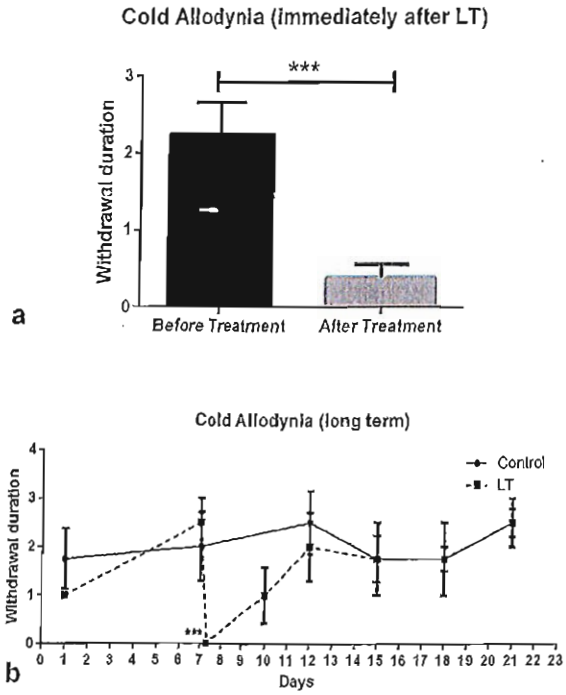

Fig. 5. Cold allodynia. A decrease in sensitivity to the cold stimulus was found within 1 hour post-LT ($P < 0.001$, **a**). The sensitivity to the cold stimulus returned to the control levels after 5 days post-LT (day 12) (**b**).

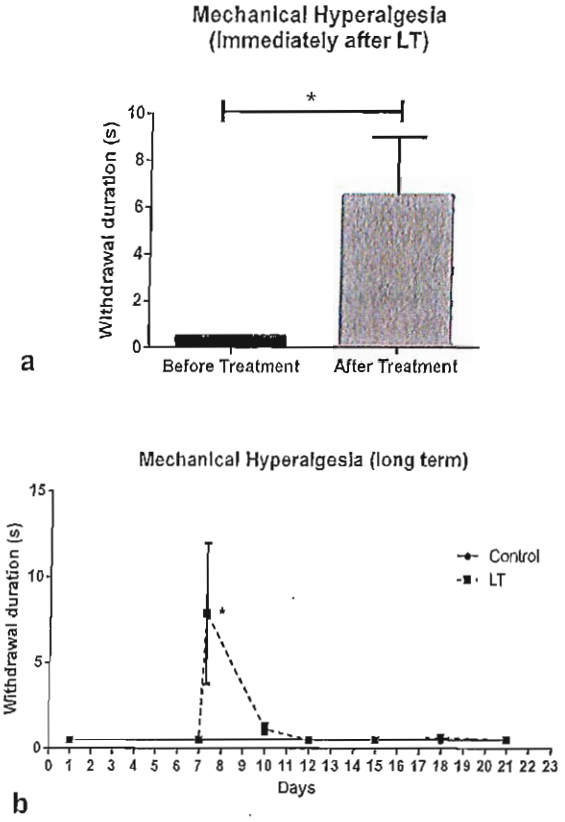

Fig. 6. Mechanical hyperalgesia. A significant decrease in sensitivity to the pin prick stimulus ($P < 0.05$) occurred within 1 hour after LT (**a**) and returned to the control level after 5 days post-LT on day 12(**b**).

or less compared to the control group (Fig. 3). These neurons are associated with C and Aδ fiber types which *in vivo* are the unmyelinated and lightly myelinated axons conveying pain and temperature sensory information. The neurites of DRG neurons with a cell body diameter $\geq 30\,\mu m$ began to form varicosities only in the 120 seconds group (Figure 3). Neurons of this size are associated with Aα and Aβ fiber types, which *in vivo* are myelinated and convey proprioceptive, two-point tactile and vibration sensory information. It is critical to remember that in our culture model there are no Schwann cells and therefore no myelination of the neurites. Therefore, the response of the neurons to the laser irradiation is not related to the degree of myelination.

In Vivo Experiments

Power measurement. For an output power of 3, 5, and 10 W, the fluence rate was measured as 80, 165, and 270 mW/cm^2, respectively. The fluence rate of 270 mW/cm^2 was the closest power density to the target 300 mW/cm^2 which was used in the *in vitro* experiments. Therefore, an output power of 10 W was chosen for *in vivo* laser irradiation experiments.

Behavior tests. For all hyperalgesia and allodynia behavior tests done, there was no significant difference found between control and experimental groups on day 7 post-surgery prior to the laser treatments. On day 7 within 1 hour after irradiation, the involved area of the lateral plantar surface of the hind paw was tested.

For heat hyperalgesia, there was a statistically significant decrease in sensitivity to the heat stimulus ($P < 0.05$) compared to the measurements done on day 7 prior to the laser treatment (Fig. 4a) within 1 hour after irradiation. For the long term measurements, a decrease in the sensitivity to the heat stimulus was found in the LT group compared to the control group ($P < 0.05$) on days 15 and 21 (Fig. 4b). The lack of a significant difference on days 12 and 18 was due to the greater variability in the standard error of the means. A greater number of animals in each group would likely result in decreased heat hyperalgesia for all time points. For cold allodynia, a significant decrease in sensitivity to the cold stimulus was found within 1 hour post-LT ($P < 0.001$, Fig. 5a). The sensitivity to the cold stimulus returned to the control levels after 5 days post-LT (day 12) (Fig. 5b). For mechanical hyperalgesia, a significant decrease in sensitivity to the pin prick stimulus ($P < 0.05$) occurred within 1 hour after LT (Fig. 6a) and also returned to the control level after 5 days post-LT on day 12 (Fig. 6b). For mechanical allodynia using electronic Von Frey test, no significant difference was found (Fig. 7a and b) between control and LT groups for all time points examined post-laser treatment.

DISCUSSION

In the present study, *in vitro* and pre-clinical animal experiments explored the mechanistic basis underlying

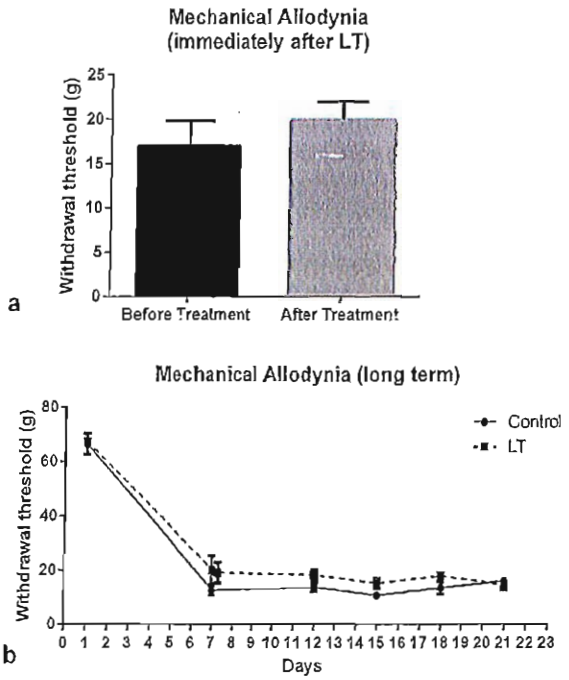

Fig. 7. Mechanical allodynia. No significant difference was found (a and b).

photoneuromodulation of neuropathic pain at an irradiance of 300 and 270 mW/cm^2. Chow et al. [15] reported that laser irradiation using a CW 830 nm wavelength laser with an irradiance of 300 mW/cm^2 induced axonal varicosities in DRG small and medium neurons which represent the type C and Aδ fibers related to pain, temperature, and light touch perception. Chow et al. [11] hypothesized that the laser irradiation blocks nociceptor-specific neurons by microtubule disruption with varicosity formation as the key morphological feature. With our treatment parameters, varicosities, and undulations formed in *in vitro* neurites of DRG neurons with a diameter of 30 μm or less at all irradiation times examined and in neurites of DRG neurons with a diameter greater than 30 μm in the 120 seconds irradiation group in contrast to Chow's study. These large neurons represent the Aα and Aβ large myelinated neurons related to the perception of proprioception, vibration, and two point tactile discrimination.

Early varicosity formation in axons have been associated with injury of the central and peripheral nervous systems [23–25] and many neurodegenerative diseases [26,27]. Interestingly, many reports suggest that varicosity formations in dendrites are neuroprotective (see the recent publication by Liebert for discussion) [28]. Besides laser irradiation, a number of chemical and physical agents including anesthetics have been reported to cause varicosity formation in neurons of various sizes [29–31]. Varicosities form when there is breakage of the microtubules [23]. This cytoskeletal disruption affects axonal flow and mitochondrial function [15,28], impairs nerve conduction [32], and signal transduction [33]. In the present study, mitochondrial metabolism measured using the MTS assay was significantly lower for all irradiated groups compared to controls. These data were based on the addition of the MTS solution at 40 minutes post-irradiation which has been previously identified as the peak time for change in mitochondrial metabolism [18]. Previously, Chow et al. (2007) examined mitochondrial membrane potential in DRG neurons treated with 830 nm wavelength light with an irradiance of 300 mW/cm^2 for 30 seconds. A statistically significant decrease in the mitochondrial membrane potential was found by 5 min post-irradiation and progressed over the total 30 minutes post-irradiation time [15]. In preliminary studies to determine a maximal irradiance that could block pain transmission and not cause permanent damage, the effect of 600 mW/cm^2 at irradiation times of 60 or 120 seconds was examined. This combination of parameters caused a statistically significant decrease in mitochondrial metabolic activities compared to the controls and the cells irradiated with 300 mW/cm^2 at 60 or 120 B indicating that an irradiance higher that 300 mW/cm^2 may be more effective (data not shown).

Different sizes and types of neurons have differential susceptibility to stimuli that cause axonal varicosity formation. Magdesian et al. [34] designed experiments to apply gradual nano-scale forces to compress axons of rat hippocampal or DRG neurons in a microfluidic chamber. They found that the two types of neurons undergo similar morphological changes including varicosity formation but their response differed in intensity and time. The hippocampal axons completely recovered axonal transport when compressed to pressures up to 65 ± 30 Pascal (Pa) for 10 min while the DRG axons resisted pressures up to 540 ± 220 Pa. The authors related the differences in the neuronal response to the composition of the cytoskeletal elements and thus the viscoelastic properties of the axons. They determined that the DRG axons had seven times more neurofilaments than the hippocampal axons [34]. Also, neurofilament/microtubule ratios are three times higher in the peripheral nervous system than in the central nervous system [35]. Furthermore, there are higher numbers of microtubules in unmyelinated axons compared to myelinated axons [36]. It is important to remember that in our culture model there were no Schwann cells and therefore no myelination of the neurites. Therefore, the response of the neurons to the laser irradiation was not related to the degree of myelination but may be related to differences in the neurofilament/microtubule ratios.

The primary target of the near-infrared light within neurons that results in the cytoskeletal disruption and varicosity formation has not been identified. It has been suggested that the light may be directly absorbed by proteins involved in microtubule stability/instability inducing a conformational change [28]. Another possibility is that a calcium influx occurs which is followed by the activation of calcium-dependent proteases, such as calpain, which cleave and degrade cytoplasmic proteins [37].

The present study demonstrates that transcutaneous irradiation with an output power of 10 W delivered 270 mW/cm^2 to the DRG and sciatic nerve and blocked pain and thermal transmission, but did not affect mechanical allodynia which relayed by large myelinated fibers. Laser irradiation of the rat sciatic nerve decreased small and medium sizes fiber somatosensory evoked potentials but did affect fast conducting large fibers [32].

Of clinical relevance is the duration of the effect. As demonstrated, even after 21 days, the laser treated group showed less sensitivity to the heat stimulus compared to control group. For cold allodynia and mechanical hyperalgesia, the duration of the effect was 5 days. In contrast to our results, a study on the assessment of neuropathic pain relief in a chronic constriction injury rat sciatic nerve model reported that laser treatment (980 nm wavelength, irradiance on the surface of the skin = 248 mW/cm^2, irradiation times 16 s at three sites) caused an increase in mechanical allodynia and thermal threshold at 7 and 14 days post-surgery. Unlike the present study in which irradiation was done only once, irradiation was done daily for 14 days [38].

Based on our studies on PBMT and nerve regeneration as well as neuropathic pain and a survey of the literature, we propose two methods that can be used to modulate neuropathic pain based on irradiance levels of near infrared light at the level of the target tissue. The first of these methods uses low irradiances in the range of 10–100 mW/cm^2 and causes a decrease in pain response by altering chronic inflammation [13] and decreasing mechanical

allodynia [13,39]. The mechanisms involved at these irradiance levels are the currently know and accepted mechanisms of PBM [40–42]. The second method utilizes irradiances in a range from 250 mW/cm^2 to 1.73 W/cm^2 which suppress conduction velocity and/or reduce the amplitude of the action potentials [16] and rapidly block pain transmission as demonstrated in the current pre-clinical animal data and human data [17]. The mechanism involved at these irradiance levels is the alteration of the neuronal microtubules as discussed above. It is important to note that to transcutaneously deliver these irradiance levels at the target tissue much high levels need to be used at the surface of the skin. We further propose that a combination therapy approach may result in the improved clinical outcomes for treating neuropathic pain. This approach would involve initial use of a high irradiance treatment to block the pain transmission followed by a series of low irradiance treatments along the course of the involved nerve to alter the chronic pathology and inflammation.

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