Clinical Studies

ORTHO LAZER

Orthopedic Laser Center

The OrthoLazer Mission | Scott A. Sigman, MD

As Chief Medical Officer and Founder of OrthoLazer, I routinely get asked, "How did this Opioid Crisis happen? How did my child get addicted to drugs after a surgery? How did my child or loved one die of a heroin overdose six months after surgery? How is it possible that four out of five new heroin users started on prescription opioids?"

Honestly, I wish I saw it coming. As orthopedic surgeons, we were told our patients needed to have pain control. We were told opioids were inexpensive and non addictive. The truth is, opioids are highly addictive and extremely costly to society.

As a proud Opioid Sparing Surgeon, I have been on a personal mission since 2013 to change the culture of postoperative pain management for doctor and patients. There is good news – across the country, doctors like yourselves are embracing our postoperative Opioid Sparing techniques.

My passion for Opioid Sparing Surgery is the reason I founded OrthoLazer. I have been using the MLS M8 Robotic Laser as an opioid alternative treatment for acute and chronic orthopedic pain. With the use of the MLS M8 Laser I have had great success in treating patients with chronic orthopedic conditions without surgery or injections. In addition, the M8 Laser, when used postoperatively, has reduced patients' pain and hastened recovery, reducing the need for opioids. Acute orthopedic injuries have also responded well to MLS Laser treatment, returning our athletes to their sport faster. My patients are embracing the concept of laser treatment.

Scott A. Sigman, MD, board-certified orthopaedic surgeon, has provided comprehensive care to patients at Orthopedic Surgical Associates of Lowell, Massachusetts since 1996. Specializing in Sports Medicine, Dr. Sigman possesses the skills and experience to diagnose and treat sports injuries and conditions affecting the knee and shoulder. In addition, he has served as the Team Physician for the US Ski Jump Team, served for the last 20 years as the Team Physician at UMASS Lowell, and is the past Chief of Orthopaedics at Lowell General Hospital. Dr. Sigman has also contributed to numerous publications and research studies advancing the field of orthopaedic surgery. Taking great pride in utilizing latest state-of-the-art arthroscopic techniques for both knee and shoulder surgery, Dr. Sigman lectures and instructs fellow surgeons worldwide.

ORTHO LAZER
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CLINICAL STUDIES

Fundamental Action: Basic Science

Musculoskeletal Analysis and Effects

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CLINICAL STUDIES

Musculoskeletal Analysis and Effects (continued)

Molecular BioSystems

RSC Publishing

PAPER

Effect of IR laser on myoblasts: a proteomic study

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Laser therapy is used in physical medicine and rehabilitation to accelerate muscle recovery and in sports medicine to prevent damages produced by metabolic disturbances and inflammatory reactions after heavy exercise. The aim of this research was to get insight into possible benefits deriving from the application of an advanced IR laser system to counteract deficits of muscle energy metabolism and stimulate the recovery of hypotrophic tissue. We studied the effect of IR laser treatment on proliferation, differentiation, cytoskeleton organization and global protein expression in C2C12 myoblasts. We found that laser treatment induced a decrease in the cell proliferation rate without affecting cell viability, while leading to cytoskeletal rearrangement and expression of the early differentiation marker MyoD. The differential proteome analysis revealed the up-regulation and/or modulation of many proteins known to be involved in cell cycle regulation, cytoskeleton organization and differentiation.

Introduction

Since the seventies laser therapy has been widely used in sports medicine, physiatry and rehabilitation to treat muscle diseases of different origins: myalgias, contusions, sprains, lacerations and damage due to heavy exercise.[1–3] These diseases have in common the painful symptomatology, the inflammatory component and, in the case of injuries, the need to repair the tissue portion in which the muscle fibers have suffered damage. The application of laser therapy, either alone or combined with other treatments, both pharmacological and instrumental, has its rationale in the therapeutic effects that are attributed to laser radiation: analgesic, anti-inflammatory, anti-oedema and ability to promote wound healing and tissue repair. A large amount of literature shows that laser radiation can affect the cell energy metabolism and ATP production,[4,5] the response of immune cells to injury,[6] the production of inflammation mediators,[7–9] the behavior of fibroblasts[10–12] and endothelial cells.[13,14] Moreover, laser radiation can improve microcirculation[15] and relieve pain both indirectly through the effects mentioned above, and directly, by acting on receptors and nerve endings.[16–19]

Although many clinical studies give evidence for the effectiveness of IR laser therapy in the treatment of muscular disorders, thus justifying the wide application of laser treatments in clinical rehabilitation and sports medicine, in the literature there are conflicting results[1,20–27] most likely caused by differences in the laser sources and treatment parameters that have been used.

In the last few years significant progress has been made in understanding the mechanisms by which the IR laser therapy promotes the healing process and recovery of muscle tissue. Recent studies, carried out both in animal models and human subjects, demonstrated that pre-exercise treatment with IR laser can significantly delay muscle fatigue and accelerate post-exercise recovery.[3,28] In rats, it has been found that laser treatment reduces the inflammatory response caused by experimentally induced muscle trauma and is able to block the effects of reactive oxygen species (ROS) release and the activation of NF-kB.[29] Laser-induced changes in inflammatory biomarkers and significant muscle recovery have been observed also in a rat model of myopathy.[30] Other authors found that, in traumatized muscle tissues, laser therapy induces an increase in activity of the complexes I, II, III and VI of the respiratory chain that may lead to an increase in ATP synthesis and faster muscle recovery.[5] A study aimed at evaluating the effectiveness of IR laser radiation in promoting the recovery of atrophied skeletal muscles demonstrated that the laser treatment favours tissue repair through activation of satellite cells and induction of neoangiogenesis.[31]

However, despite the fact that over the past decade numerous studies have significantly improved our knowledge, in many respects the effects of radiation emitted by different laser sources on muscle tissue and its repair mechanisms are far to be completely understood.

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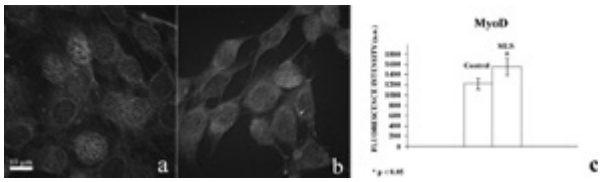

Fig. 1 MyoD expression in C2C12 cells after MLS laser treatment. a: control C2C12 cells. b: MLS treated C2C12 cells. c: quantitative expression of MyoD. Images obtained by immunofluorescence microscopy show that, in cells treated with MLS laser, MyoD expression increased and the transcription factor was mainly distributed in the nuclear and perinuclear area. Image analysis revealed that in treated cells MyoD increased by about 26%, in comparison with controls.

This paper reports the results of a study aimed to investigate the behavior of C2C12 cells exposed to the emission of a dual wavelength IR laser. The C2C12 skeletal muscle cell line has been derived from murine satellite cells and is widely accepted as a model to study the behavior of satellite cells,[32] which play a crucial role in skeletal muscle regeneration and repair[33] and are capable of repopulating damaged and atrophied muscle.[34] The source chosen to perform the treatments was a synchronized IR dual emission laser system, with wavelengths of 808 and 905 nm, respectively. Most of the recent studies reported the effectiveness of laser emissions in the range 800–830 nm as well as emissions around 905 nm in triggering a biological response in muscle tissue. Sources with multiple emissions are widely used in clinics but little is studied from the point of view of the effects that induce in the cellular component of muscle tissue. We considered interesting to study the effects of a source that simultaneously emits two wavelengths which, on the basis of the literature, both can favour the recovery of homeostasis in diseased muscles.

The behavior of C2C12 cells was assessed before and after exposure to laser treatments in terms of cell viability, morphology, proliferation, differentiation and proteomic profile. To the best of our knowledge this is the first time that the effect of IR laser radiation on the proteomic profile of myoblasts is being studied.

Results and discussion

Effect of MLS laser treatment on viability, proliferation and differentiation of C2C12 cells

Murine myoblasts C2C12 were treated with a Multiwave Locked System laser (MLS laser) as described in the experimental section. Before proceeding to the appropriate morphological and proteomic studies, we analyzed the effect of the laser treatment on cell viability and proliferation. The trypan blue assay, performed both after a single exposure to the laser radiation and after 3 exposures carried out on consecutive days, showed that in treated samples there were no significant changes in cell viability (over 98%) compared to the control. The cell count did not show significant differences after a single exposure, whereas after 3 treatments with MLS laser, the cell number decreased moderately (about 23.4%) with respect to the control. Since MLS laser treatment does not affect cell viability, we hypothesized that the reduced proliferation rate of MLS laser treated cells was caused by the triggering of a differentiative process. To verify our hypothesis we analyzed expression and distribution of MyoD, which is widely recognized as an early marker of myoblast differentiation. In fact it is known that in skeletal myogenesis, gene expression is initiated by MyoD and includes the expression of specific Mef2 isoforms and activation of the p38 mitogen-activated protein kinase (MAPK) pathway.[35] In treated cells MyoD increased by about 26%, in comparison with controls (Fig. 1c), and was mainly distributed in the nuclear and perinuclear area (Fig. 1a and b). The increase in MyoD expression and the morphological changes in the cytoskeleton structure observed in treated cells strongly suggest that MLS laser radiation triggered a differentiation process in C2C12 cells.

Effect of MLS laser treatment on cytoskeleton organization

The organization of the cytoskeleton network is a crucial factor in determining cell shape, regulating cell adhesion/migration, transducing signals and triggering intra and extracellular pathways. Therefore, the three major cytoskeleton components, *i.e.* actin microfilaments, microtubules and intermediate filaments were studied by immunofluorescence microscopy analysis. Control C2C12 cells showed the expected actin distribution (Fig. 2a): high expression in the perinuclear area, a clearly distinguishable ''actin ring'' close to the plasma membrane (arrow) and some stress fibers. After a cycle of three laser treatments the ''actin ring'' delimiting each individual cell disappeared. The cells tended to align and fuse to form tubes, filaments running parallel to the axis of the tubes appeared (arrows), the perinuclear area with high actin expression was thicker (Fig. 2b). The specific staining for tubulin revealed, in control cells, a radial distribution of the microtubules starting from the organization centre close to the nucleus (Fig. 2c). In treated samples a redistribution of the microtubules was observed: they were oriented parallel to the major axis of the cells and passed from one cell to another without interruption (arrows) (Fig. 2d). The intermediate filaments were studied by immunofluorescence staining of vimentin, which is their major constituent. The analysis of protein expression and distribution in control cells showed that the network of filaments was more dense in the perinuclear area, where it had the shape of a ball (Fig. 2e). In the cells treated with MLS laser, the intermediate filaments were parallel to the longitudinal axis of the cells (arrows), which appeared elongated and aligned to form tube-like structures (Fig. 2f).

In summary, C2C12 cells, subjected to MLS laser treatment, showed elongated shapes and were aligned and fused to form structures with two or more nuclei among which were no longer recognizable interposed membranes (tubes). These cytoarchitectural changes support the occurrence of a differentiation process[36] since the formation of a longitudinal microtubule array is an early event in myogenic differentiation.[37] It is also known that the reorganization of intermediate filaments and shifts from one to the other of the two major components, vimentin and desmin, occur during myogenesis.[38–40] Remodelling of actin

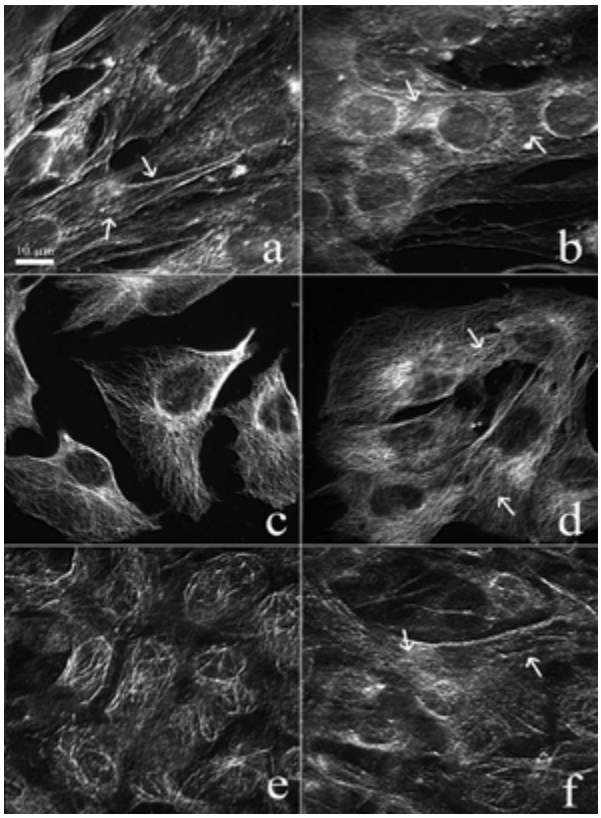

Fig. 2 Cytoskeleton organization in C2C12 cells after MLS laser treatment. a, b: actin immunostaining. c, d: tubulin immunostaining. e, f: vimentin immunostaining. In control C2C12 cells (a) actin resulted highly expressed in the perinuclear area and formed a distinguishable ''actin ring'' close to the plasma membrane (arrows). Some stress fibers were present. After a cycle of three laser treatments (b) the ''actin ring'' delimiting each individual cell disappeared. The cells tended to align and fuse to form tubes, filaments running parallel to the axis of the tubes appeared (arrows), the perinuclear area with high actin expression was thicker. In control cells (c), microtubules showed a radial distribution starting from the organization centre close to the nucleus. In treated samples (d) the microtubules oriented parallel to the major axis of the cells and passed from one cell to another without interruption (arrows). In control cells (e), the network of intermediate filaments was more dense in the perinuclear area, where it had the shape of a ball. In laser treated cells (f), the intermediate filaments were parallel to the longitudinal axis of the cells (arrows), which appeared elongated and aligned to form tube-like structures.

microfilaments with the formation of stress fiber like structures has a very important role leading to myofibrillogenesis and is regulated by actin binding proteins[41] and phospholipase D.[42,43]

Effect of MLS laser treatment on the proteome of C2C12 cells

In an attempt to identify the molecular changes induced by laser treatment of C2C12 cells, we have studied the protein expression pattern before and after laser treatment using 2-DE based proteomics. To ascertain the reproducibility of results 2-DE was performed three times for each protein sample. Fig. 3 shows a representative gel image. Image analysis using Progenesis Same Spot software allowed us to identify about 120 significantly (Anova p value, $p \leq 0.05$) and consistently up- or down-regulated protein spots with fold changes greater than 1.5 in terms of average normalised volumes in both triplicate gels of two independent experiments. Of these spots, 89 were up-regulated and 32 down-regulated in comparison to the control cells. Fig. 3 shows the significantly regulated spots, identified by MALDI-Tof mass spectrometry.

About eighty spots, corresponding to major protein variations, were cut from gels, destained, digested with trypsin and subjected to peptide mass fingerprinting followed by database searching. MALDI-TOF MS analysis allowed the unambiguous protein identification of 52 protein variations, corresponding to 42 proteins. Table 1 summarizes all the information obtained by protein identification. Protein numbering corresponds to that shown in Fig. 3. Swiss-Prot accession number and protein name are also included. The comparison between theoretical and measured molecular weight and pI values contributes to confirm the MASCOT search results in most cases. The MASCOT search results are reported in Table 1, showing experimentally measured peptide masses matching the theoretical ones from Swiss-Prot/UniProt entries, the percentage of the protein sequence covered by the matching peptides (sequence coverage), and the probabilistic score.

The identification of some protein spots, both selected as up-regulated proteins (spot No. 7, 13–15, 17, 40) and down-regulated ones (spot No. 4, 6, 37, 44), resulted in the same proteins: vimentin (spot No. 4, 13–15), actin γ (or β) (spot No. 17 and 44), tropomyosin α-3 chain (spot No. 6 and 40) and Rab GDP dissociation inhibitor β (spot No. 7 and 37). The position of these protein spots is clearly different. Fig. 4 shows details of the two-dimensional reference maps. Both tropomyosin α-3 chain and Rab GDP dissociation inhibitor β are associated with two spots with equal molecular weight but different isoelectric points. The observed shift in positions of these proteins could represent splice variants and/or post-translational modifications (*i.e.* phosphorylation) rather than an increase or decrease in the absolute amounts. Vimentin and actin γ (or β), identified by the analysis of protein spots up-regulated following laser treatment, have an apparent molecular weight higher than the theoretical one. This phenomenon was also observed for other proteins identified by the analysis of up-regulated protein spots. As we can note from Fig. 3 and Table 1, desmin (spot No. 16), pyruvate kinase isozymes M1/M2 (spot No. 22) and elongation factor 2 (spot No. 21) show a measured molecular weight higher than the theoretical one. We hypothesize that MLS laser treatment could induce the stabilization of pre-existing covalent polymeric species or their formation.

Classification and functional analysis of modulated proteins

In order to gain insight into the biological significance of the proteins identified by proteomic analysis, the 42 differentially expressed proteins, identified by MALDI-Tof peptide mass finger printing of 52 isolated protein spots, were categorized according to the DAVID bioinformatics tool. Concerning biological processes, the identified proteins were distributed into categories; we report here the results obtained with the PANTHER (Protein Analysis Through Evolutionary relationships) classification system (Table 2). The main biological process in which the identified

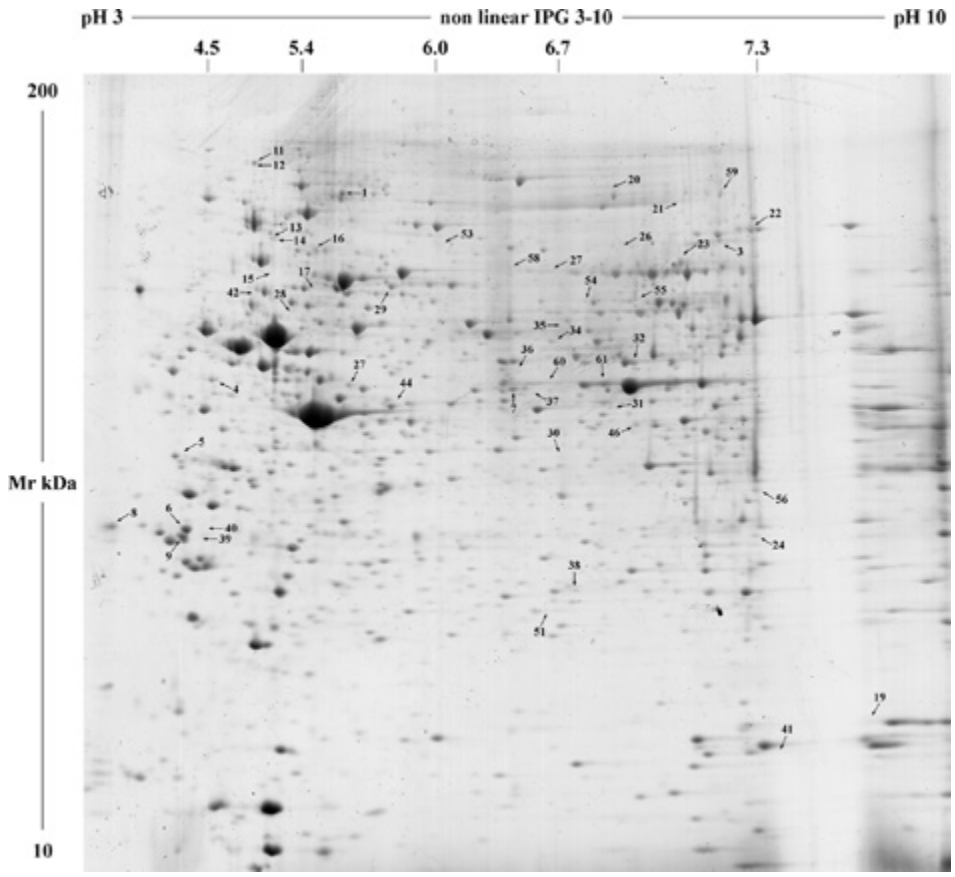

Fig. 3 Representative reference 2-DE gel of C2C12 cells. Cell lysates of control C2C12 cells and MLS-treated C2C12 cells were resolved by 2-DE. IEF was carried out on nonlinear wide-range IPGs (pH 3–10; 18 cm IPG strips) and achieved using the Ettan™ IPGphor™ system. Sample load, 800 μg per strip, was successively performed by cup loading in the IPGphor Cup Loading Strip Holders. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm). Protein spots were visualized by colloidal coomassie blue staining. 2-DE gels were analysed using the Progenesis SameSpot software package. The arrows point to differential protein spots identified with the peptide-mass fingerprinting.

proteins were involved was ''protein metabolism and modification'' (38.1%), followed by ''cell structure and motility'' (28.6%), ''carbohydrate metabolism'' (11.9%), and ''induction of apoptosis'' (7.1%). The four tropomyosin isoforms identified and assigned to the ''cell structure and motility'' class can be categorized also in the ''muscle contraction and development'' class (not shown). Similarly heat shock proteins can be classified also as ''stress response proteins''. It is well known that the main function of the heat shock proteins is to provide thermotolerance and cytoprotection. Hsp-β1, up-regulated after MLS laser treatment, belongs to the small heat shock protein group. It is overexpressed during different stages of cell differentiation and development and it is thought to have an essential role in the differentiation processes of numerous tissues.[44]

The membership of most of the identified proteins to the class ''protein metabolism and modification'' clearly indicates a cellular response toward specific anabolic events, probably related to cytoskeleton network remodelling, leading to the morphological changes observed and described above. It is also remarkable that about 30% of the changes shown by proteomic analysis in laser treated samples concern proteins involved in cell structure and motility, such as actin γ/β, tropomyosin α and β chains, vimentin, desmin, LIM domain and actin-binding protein, fascin, cofilin-1, many of which are actin-binding proteins and/or have been found to have a role in myogenesis. It has been demonstrated that the LIM domain and actin-binding protein increases when myoblasts are induced to differentiate and then progressively declines in myotubes.[45,46] Therefore, the increase in the LIM domain and actin-binding protein we observed fits with a scenario of differentiation at the early stages. Another interesting identified protein is α-enolase that results up-regulated after MLS laser treatment. Although it is clusterized into the class ''carbohydrate metabolism'', it is known that α-enolase is also involved in other cellular processes. It has been recently demonstrated that enolase isoforms interact with microtubules during muscle satellite cell differentiation contributing to the regulation of the cytoskeletal filaments dynamism that occurs during the transition from myoblasts to myotubes.[47,48] NLR family pyrin domain-containing protein 10 (NLRP 10), heterogeneous nuclear ribonucleoprotein K (HNRNP K) and galectin-3 following the PANTHER classification system were assigned to the ''induction of apoptosis'' class but they are also involved in other important processes.

Table 1 Differentially expressed proteins identified by 2-DE coupled to MALDI-Tof MS analysis

Spot no.	Accession no.	Protein names	MASCOT search results: No. of matched peptides	Sequence coverage	Score	Theoretical pI/Mr (kDa)	Measured pI/Mr (kDa)	Fold increase	ANOVA (p value)
1	Q02053	Ubiquitin-like modifier-activating enzyme 1	11	16	102	5.43/118.93	5.22/109.33	−1.5	0.011
3	Q8K1N2	Pleckstrin homology-like domain family B member 2	12	9	80	7.58/142.25	6.97/103.90	−1.6	0.025
4	P20152	Vimentin	12	32	105	5.06/53.71	4.76/49.61	−1.9	0.005
5	P58774	Tropomyosin β chain	11	32	123[a]	4.66/32.93	4.59/40.20	−4.6	0.048
6	P21107	Tropomyosin α-3 chain	13	39	104[a]	4.68/32.90	4.64/32.85	−4.6	0.048
7	Q61598	Rab GDP dissociation inhibitor β	20	46	157	5.93/51.02	5.75/48.36	−2.0	0.007
8	O35658	Complement component 1 Q subcomponent-binding protein	4	28	60	4.82/31.34	4.30/33.18	−2.9	0.008
9	Q6IRU2	Tropomyosin α-4 chain	14	50	144	4.65/28.56	4.62/31.86	−4.5	0.050
11	P07901	Heat shock protein, HSP 90-α	17	26	82	4.93/85.13	4.91/n.d.	2.9	0.050
12	P11499	Heat shock protein, HSP 90-β	29	43	226	4.97/83.61	4.91/n.d.		
13	P20152	Vimentin	26	57	226	5.06/53.71	5.03/105.51	5.7	0.048
14	P20152	Vimentin	26	59	291	5.06/53.71	5.06/106.09	2.5	0.050
15	P20152	Vimentin	7	21	71	5.06/53.71	5.03/81.78	2.5	0.031
16	P31001	Desmin	21	44	195	5.21/53.52	5.22/96.91	1.5	0.033
16	Q91VD9	NADH-ubiquinone oxidoreductase 75 kDa subunit	16	27	129	5.51/80.75	5.21/85.82	1.5	0.033
17	Q6ZWM3	Actin γ	10	38	108	5.29/42.05	5.24/67.30	8.1	0.032
	P63260	Actin β	10	38	108	5.31/42.11			
19	P18760	Cofilin-1	6	57	80	8.22/18.78	n.d./19.93	1.9	0.050
20	Q3TL91	Rho GTPase-activating protein 31	13	12	72	5.58/156.55	6.28/166.41	1.7	0.031
21	P58252	Elongation factor 2	20	42	203	6.41/96.22	6.74/151.03	1.9	0.031
22	Q91YI8	Pyruvate kinase isozymes M1/M2	12	32	138	7.18/58.38	7.17/120.36	3.4	0.022
23	Q9ERG0	LIM domain and actin-binding protein 1	23	27	133	6.18/84.66	6.70/93.27	1.8	0.034
24	P16110	Galectin-3	8	34	110	8.46/27.61	n.d./33.13	3.2	0.042
25	P56399	Ubiquitin specific peptidase 5	7	7	81	4.89/96.68	6.48/96.08	3.2	0.004
26	Q9ERG0	LIM domain and actin-binding protein 1	18	26	119	6.18/84.66	6.36/96.49	4.6	0.049
27	P60843	Eukaryotic initiation factor 4A-I	14	48	191	5.32/46.35	5.38/48.61	1.5	0.015
28	P61979	Heterogeneous nuclear ribonucleoprotein K	10	23	109	5.39/51.23	5.14/59.29	1.5	0.028
29	Q61696	Heat shock protein, HSP 70	12	25	123	5.53/70.32	5.48/69.42	2.6	0.001
30	P62141	PP1-β catalytic subunit	10	36	94	5.84/37.96	5.80/42.44	1.9	0.002
31	P50580	Proliferation-associated protein 2G4	14	51	169	6.41/44.01	6.57/48.61	2.9	0.049
32	Q61553	Fascin	12	34	148	6.44/55.22	6.64/52.38	1.6	0.033
34	P80314	T-complex protein 1 subunit β	15	35	171	5.97/57.78	6.29/55.47	1.5	0.050
35	P26638	Seryl-tRNA synthetase	21	45	239	5.95/58.86	6.37/57.87	1.8	0.005
36	Q62465	Synaptic vesicle membrane protein VAT-1 homolog	6	21	68	5.95/43.30	5.79/50.91	2.4	0.005
37	Q61598	Rab GDP dissociation inhibitor β	9	27	83	5.93/51.02	5.84/48.49	2.1	0.010
38	P14602	Heat shock protein, HSP β-1	10	50	122	6.12/23.06	6.08/27.93	4.1	0.004
39	P58771	Tropomyosin α-1 chain	15	45	165	4.69/32.72	4.75/32.39	3.5	0.049
40	P21107	Tropomyosin α-3 chain	9	21	157[a]	4.68/32.90	4.68/31.75	5.8	0.029
41	P17742	Peptidyl-prolyl *cis–trans* isomerase A	6	42	107	7.74/18.13	n.d./17.35	8.6	0.022
42	Q9JIG7	Coiled-coil domain-containing protein 22	9	18	71	5.71/71.26	5.00/67.81	−1.8	0.016
	Q6ZWM3	Actin γ	8	26	73	5.29/42.05	5.58/46.10	−2.3	0.008
44	P63260	Actin β	8	26	73	5.31/42.11			
46	Q61990	Poly(rC)-binding protein 2	7	28	81	6.33/38.60	6.66/43.85	−1.5	0.023
51	P43025	Tetranectin	5	24	74	5.50/22.64	6.27/26.31	3.1	0.007
53	Q8CCN1	NLR family, pyrin domain-containing protein 10	21	29	159	6.18/72.29	5.79/87.84	3.1	0.008
54	O08553	Dihydropyrimidinase-related protein 2	14	37	172	5.95/62.64	6.42/65.412	1.8	0.050
55	Q9CWJ9	Bifunctional purine biosynthesis protein	12	35	174	6.30/64.69	6.64/66.09	1.6	0.034
56	P06151	L-Lactate dehydrogenase A chain	13	34	131	7.62/36.82	7.36/36.55	2.4	0.033
58	Q8R4K2	Interleukin-1 receptor-associated kinase 4	9	25	100	5.23/51.41	5.78/83.51	5.2	0.012
59	P58252	Elongation factor 2	8	12	70	6.41/96.22	6.15/78.39	2.9	0.050
60	P17182	α-Enolase	13	45	153	6.37/47.45	6.28/49.47	4.4	0.031
61	P17182	α-Enolase	17	55	212	6.37/47.45	6.41/49.22	1.5	0.023

[a] The number of accepted missed cleavage sites was set to two.

NLRP 10 is one of 14 pyrin domain containing members of the NOD-like receptor family of cytoplasmic receptors. It is an intracellular protein involved not only in apoptosis but also in the immune system function. In fact it is believed that NLRP 10 helps to regulate the inflammatory response. NLRP 10 reduces inflammatory and innate immune responses by inhibiting the activity of two proteins associated with the inflammasome: caspase-1 and PYCARD.[49,50] Although the increase in NLRP 10 found after laser treatment does not seem to be connected to differentiation, it could represent one of the mechanisms underlying the anti-inflammatory effect attributed to the laser therapy.

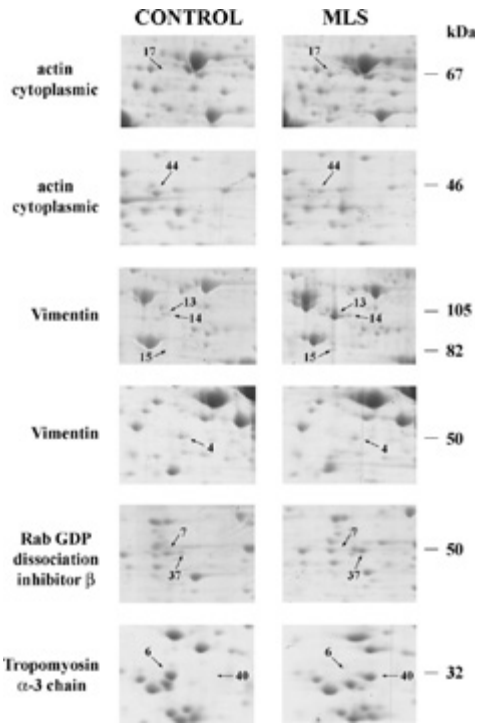

Fig. 4 Protein shift position of actin γ (or β), vimentin, tropomyosin α-3 chain, and Rab GDP dissociation inhibitor β upon MLS-treatment of C2C12 cells. The panels show the regions, selected by representative 2-DE gels, with actin γ (or β), vimentin, tropomyosin α-3 chain, and Rab GDP dissociation inhibitor β localization. The indicated proteins shift their position in response to MLS-treatment due, likely, to post-translational modification.

HNRNP K belongs to the subfamily of heterogeneous nuclear ribonucleoproteins (hnRNPs). These proteins are associated with pre-mRNAs in cell nucleus and are known to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. Experiments on animal models showed that HNRNP K is required for axonogenesis during development and several of its RNA targets are under strong post-transcriptional control during the regeneration process.[51] The increase in HNRNP K observed in laser treated cells could be an intriguing starting point for future research, since it has been shown that IR laser therapy promotes the regeneration of nerve fibers.[52] HNRNP K is also thought to have a role in cell cycle progression,[53] therefore the increase in expression could also be connected with the beginning of a differentiation process.

Galectin-3 plays a key role in several intracellular and extracellular processes. Documented intracellular functions are the regulation of cell growth, apoptosis and cell cycle.[54] Extracellular function consists in mediating/modulating cell to extracellular matrix adhesive interactions. Recent studies indicate galectin-3 as a mediator of signal transduction events on the cell surface as well as a mediator of a variety of extracellular processes such as angiogenesis, neuronal functions, endocytosis and possibly exocytosis.[55]

Finally, we would like to highlight the identification of PP1 as one of the up-regulated proteins in MLS laser treated cells. Interestingly the PP1 catalytic subunit protein is included in each of the three main classes. PP1 is a major eukaryotic protein serine/threonine phosphatase that regulates an enormous variety of cellular functions through specific associations with regulatory subunits. PP1, primarily known for its role in the carbohydrate metabolism, actually regulates functions such as actin and actomyosin reorganization, cell shape and cell adhesion, muscle contraction/relaxation.[56]

To assess the identity of PP1 we performed an immunoblot analysis using a specific anti-PP1 antibody. Actin and enolase were selected to undergo a confirmatory test as well. Fig. 5 shows immunoblot results in comparison with the colloidal coomassie staining. Western blot analysis also confirms the up-regulation of enolase and the up-regulation of PP1 in laser treated cells, according to 2-DE gel image analysis.

Another interesting aspect highlighted by the DAVID classification system as a function of the keywords is that 81% of the identified proteins are classified as ''phosphoproteins'' (Table 3). Some of these proteins actually are PP1 substrates or interact with it (*i.e.* cofilin,[57] heterogeneous nuclear ribonucleoprotein K,[58] enolase,[59] heat shock proteins,[60,61] peptidyl-prolyl isomerase,[62] vimentin[63,64]).

The overexpression of cofilin is notable. Cofilin is a protein associated with the cytoskeleton that binds actin and reversibly controls polymerization and depolymerization in a pH-sensitive manner; the ability of cofilin to control actin polymerization is known to be regulated by reversible phosphorylation.[65] Cofilin is phosphorylated by LIM kinase 1, which abolishes its ability to de-polymerize actin, and dephosphorylated by PP1 and PP2A.[66] PP1 and PP2A play an important role in myoblast differentiation. It has been shown that inhibition of PP1 and PP2A by okadaic acid blocks myogenesis by altering the MyoD binding activity[67] and depletion of PP1 abolishes the ability of myoblasts to differentiate into myotubes.[68] Proteomic analysis of samples exposed to MLS laser treatments for 3 consecutive days showed an increase in PP1 but no significant changes in PP2A. This finding completely agrees with the results of other authors, who examined PP1 and PP2A activities during various stages of myogenesis in rat skeletal muscle cells. PP1 activity increased progressively in cultures from 2 to 5 days, PP2A activities remained constant in days 2–4 cultures and increased sharply on day 5.[69] An indirect proof of the key role played by PP1 in muscle homeostasis is the decrease of PP1 levels, associated with a decrease in metabolic enzymes, observed in hypotrophic and sarcopenic muscles.[70]

Finally it is also relevant that 28.7% of the identified proteins are ''ATP-binding proteins'', 19% are ''coiled coil proteins'' and 16.7% ''actin-binding proteins''. The increased availability of ATP induced by exposure to the red-IR laser radiation[71] could be correlated with the net increase of proteins capable of binding ATP. Changes in the expression of actin-binding proteins, as well as those in typical intermediate filament proteins (here classified in part as ''coiled coil'' proteins), are events related to the general cytoskeletal rearrangement also observed by fluorescence microscopy (Fig. 2).

Table 2 Predominant biological processes associated with LMS-treatment when compared to control C2C12 cells as provided by DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov), using the PANTHER classification system. ↑: up-regulated proteins; ↓: down-regulated proteins; ↓/↑: proteins identified from multiple protein spots (both up-regulated and down-regulated). The italicized proteins are present in more than one functional class

Protein metabolism, modification 38.1%		Cell structure and motility 28.6%		Carbohydrate metabolism 11.9%		Induction of apoptosis 7.1%		Not classified 19%	
T-complex protein 1 subunit β	↑	LIM domain and actin-binding protein 1	↑	Pyruvate kinase	↑	NLR family, pyrin domain-containing protein 10	↑	Bifunctional purine biosynthesis protein	↑
Heterogeneous nuclear ribonucleoprotein K	↑	Cofilin-1	↑	L-Lactate dehydrogenase	↑	*Heterogeneous nuclear ribonucleoprotein K*	↑	Dihydropyrimidinase-related protein 2	↑
Interleukin-1 receptor-associated kinase 4	↑	Desmin	↑	*PP1-β*	↑	Galectin-3	↑	NADH-ubiquinone oxidoreductase	↑
Eukaryotic initiation factor 4 A-I	↑	Fascin	↑	Synaptic vesicle membrane protein VAT-1	↑			Rab GDP dissociation inhibitor β	↑
Peptidyl-prolyl *cis–trans* isomerase A	↑	Tropomyosin α-1	↑	α-Enolase	↑			Rho GTPase-activating protein 31	↑
Proliferation-associated protein 2G4	↑	*PP1-β*	↑					Coiled-coil domain-containing protein 22	↓
Seryl-tRNA synthetase	↑	Tropomyosin β	↓					Pleckstrin homology-like domain family B member 2	↓
Ubiquitin specific peptidase 5	↑	Tropomyosin α-4	↓					Complement component 1 Q subcomponent-binding protein	↓
HSP 90-α	↑	Vimentin	↓/↑						
HSP 90-β	↑	Actin γ	↓/↑						
HSP β-1	↑	Actin β	↓/↑						
HSP 70	↑	Tropomyosin α-3	↓/↑						
PP1-β	↑								
Elongation factor 2	↑								
Poly(rC)-binding protein 2	↓								
Ubiquitin-like modifier-activating enzyme 1	↓								

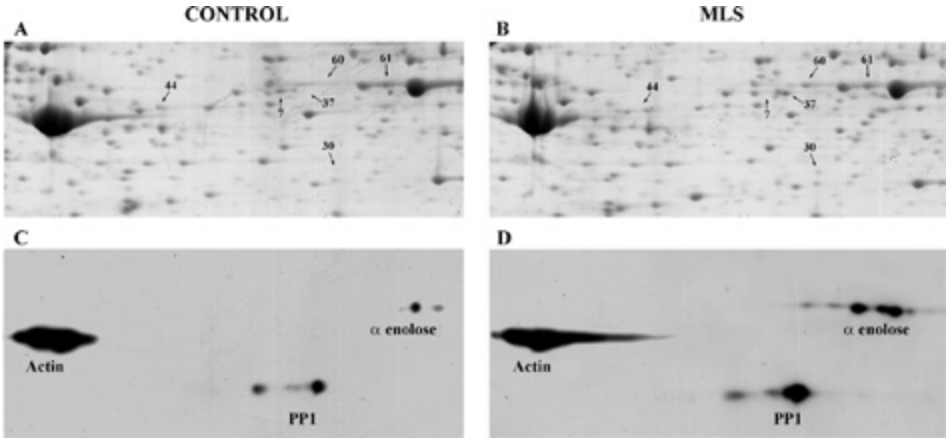

Fig. 5 Validation of the PP1 identity by immunoblot analysis. 100 μg of protein extracts from control and MLS treated C2C12 cells were separated by 2-DE and transferred on a PVDF membrane. The blots were incubated with anti-PP1 antibody. Anti-actin and anti-enolase antibodies were used as loading and position control. Blots were visualized by autoradiography (c, d). The panels represent the 2-DE selected regions in which localize PP1, actin and enolase. The upper panels (a, b) show the colloidal coomassie blue stained gels.

MLS laser treatment induces a significative PPPs activity increase

The identification of PP1 as one of the up-regulated proteins in response to MLS laser treatment and the observation that 81% of the identified proteins are proteins whose function depends on the phosphorylation status led us to investigate whether there were changes in the total cellular protein serine/threonine phosphatases activity. PPPs activity assays, performed on cell lysates obtained after MLS laser treatment of C2C12 cells, show a 1.8 fold increase with respect to untreated cells (Fig. 6) confirming the importance of specific phosphorylation/dephosphorylation events in maintaining the integrity of intermediate filaments[63–65,72] and other fundamental biological functions.[73–76] Fig. 6 shows the results obtained for other enzymes: lactate dehydrogenase, enolase and pyruvate kinase. All these proteins are overexpressed after MLS laser treatment and show an increase in their enzymatic activity.

Table 3 Predominant keywords associated with identified proteins as provided by DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov). ↑: up-regulated proteins; ↓: down-regulated proteins; ↓/↑: proteins identified from multiple protein spots (both up-regulated and down-regulated). The italicized proteins are present in more than one category

Phosphoprotein 81%				ATP-binding protein 28.6%		Coiled coil 19%		Actin-binding protein 16.7%		Stress-response 9.5%		Not classified 9.5%	
Eukaryotic translation elongation factor 2	↑	Ubiquitin specific peptidase 5	↑	NLR family, pyrin domain-containing protein 10	↑	*Coiled-coil domain containing 22*	↓	*LIM domain and actin binding 1*	↑	*HSP β-1*	↑	Rab GDP dissociation inhibitor β	↑
Rho GTPase-activating protein 31	↑	Heterogeneous nuclear ribonucleo-protein K	↑	*Eukaryotic translation initiation factor 4A1*	↑	Pleckstrin homology-like domain, family B, member 2	↓	*Cofilin 1*	↑	*HSP 70*	↑	Synaptic vesicle membrane protein VAT-1 homolog	↑
LIM domain and actin binding 1	↑	*Eukaryotic translation initiation factor 4A1*	↑	T-complex protein 1, subunit β	↑	*Tropomyosin β*	↓	*Fascin homolog 1*	↑	*HSP 90-α*	↑	NADH-ubiquinone oxidoreductase	↑
Complement component 1 Q subcomponent-binding protein	↓	Pleckstrin homology-like domain, family B	↓	*Seryl-aminoacyl-tRNA synthetase*	↑	*Tropomyosin α-4*	↓	*Tropomyosin α-1*	↑	*HSP 90-β*	↑	Tetranectin	↑
Interleukin-1 receptor-associated kinase 4	↑	Poly(rC) binding protein 2	↓	*Interleukin-1 receptor-associated kinase 4*	↑	*Tropomyosin α-1*	↑	*Tropomyosin β*	↓				
Ubiquitin-like modifier activating enzyme 1	↓	*Coiled-coil domain containing 22*	↓	*HSP 90-α*	↑	*Tropomyosin α-3*	↓/↑	*Tropomyosin α-4*	↓				
Seryl-aminoacyl-tRNA synthetase	↑	Bifunctional purine biosynthesis protein	↑	*HSP 90-β*	↑	*Vimentin*	↓/↑	*Tropomyosin α-3*	↓/↑				
Cofilin 1	↑	Dihydropyrimidinase-like 2	↑	*HSP β-1*	↑	Desmin	↑						
Fascin homolog 1	↑	*Tropomyosin α-3*	↑	*Pyruvate kinase*	↑								
Galectin-3	↑	PP1-β	↑	*Actin γ/actin β*	↓/↑								
L-Lactate dehydrogenase	↑	Peptidylprolyl isomerase A	↑	*Ubiquitin-like modifier activating enzyme 1*	↓								
α-Enolase	↑	*Pyruvate kinase*	↑										
Proliferation-associated 2G4	↑	*Tropomyosin α-1*	↑										
HSP β-1	↑	*Tropomyosin β*	↓										
HSP 90-α	↑	*Tropomyosin α-4*	↓										
HSP 90-β	↑	*Actin γ/actin β*	↓/↑										
Vimentin	↓/↑												

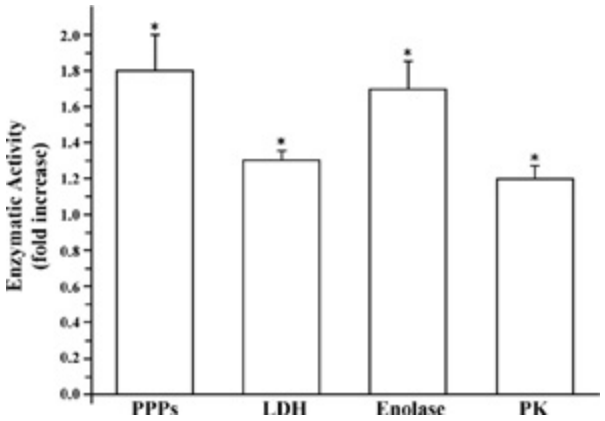

Fig. 6 Enzymatic assays of some overexpressed proteins and cellular protein phosphatases. The enzymatic activity of protein serine/threonine phosphatases (PPPs), lactate dehydrogenase (LDH), enolase, and pyruvate kinase (PK) was determined in cell lysates by specific tests. The figure shows the activity fold increase observed in MLS-treated C2C12 cells with respect to control cells. Results shown represent means of two experiments in duplicate (SEM ($*p \leq 0.05$)).

Experimental

Cell culture

Murine myoblasts (C2C12 skeletal muscle cell line) were routinely cultured in growing medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 100 μg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, 2 mM glutamine and 10% fetal bovine serum (FBS). Cells were incubated at 37 °C and 5% CO_2. All the reagents for cell culture were purchased from Sigma Chemical Co. (St Louis, MO, USA).

MLS laser treatment

The treatments have been performed with a Multiwave Locked System (MLS) laser (ASA Srl, Vicenza, Italy), a device already used for some years in clinics (FDA approved and CE certified instrument) and specifically applied in physical medicine and pain therapy. It is a high power (average power up to 1.1 W, class IV) IR laser with two synchronized sources (laser diodes). The two modules have different wavelengths, peak power and emission mode. The first one is a pulsed laser diode, emitting at 905 nm, with a peak optical power = 25 W; each pulse is composed of a pulse train (single pulse width = 100 ns, maximum frequency 90 kHz), thus varying the average power delivered to the tissue. The frequency of the pulse trains may be varied in the range 1–2000 Hz. The second laser diode (808 nm) operates in continuous mode (power 1.1 W) or in pulsed mode (pulses repetition rate 1–2000 Hz), mean optical power output = 550 mW, duty ratio 50% independent of the pulse repetition rate. The two propagation axes are coincident.

For the treatment, cells were seeded in the central 8 wells of a 24-multiwall plate. The plate was placed inside a plexiglass support, specifically designed and built. On the top of the support there was a central groove in which the laser handpiece slided. The plate was perfectly aligned with the handpiece, at a distance of 3 cm from it, so that the spot formed by the two superimposed laser beams had a diameter equal to that of a single well (13 mm). The support allowed us to perform a homogeneous scan of 8 samples at the same time, by moving the spot at a constant horizontal velocity above the 8 treated wells (5.6 cm s^{-1}: each scan of 8 wells lasted 20 s), in order to have the same radiant energy impinging into each well (~68 J for the whole treatment). Treatment parameters were 1500 Hz frequency and 8 min total scan time. The scan mode is also extensively used in clinics because it allows us to treat easily larger areas and, together with the other treatment parameters chosen, contributes to achieve the desired effects avoiding any side effects.

The treatment was repeated once a day, for 3 consecutive days under sterile conditions. The treated samples were compared with controls maintained under the same conditions, except for the laser exposure.

Viability and proliferation

Cell viability was assessed by the Trypan Blue assay after a single exposure and 3 exposures (once a day for 3 consecutive days) to MLS treatment. The treated samples were compared to untreated controls. After the MLS treatment, the samples were washed and cell detachment was obtained by treating with trypsin–EDTA for 3–4 minutes. Then the cells were centrifuged and resuspended in a solution of phosphate buffered saline (PBS) and Trypan Blue (dilution factor: 2). The dye is able to penetrate selectively into dead cells. After 5 min of incubation, cell counts were performed by using a Neubauer haemocytometer.

Immunofluorescence analysis

At the end of the experiments, cells were fixed for 5 min in cold acetone, then washed in phosphate buffered saline (PBS). After blocking unspecific binding with PBS containing 3% bovine serum albumin, cells were incubated overnight at 4 °C with the specific anti-MyoD (Santa Cruz Biotechnology, SC-32758), anti-α-actin (Millipore, MAB1501X), anti-tubulin (Upstate Biotechnology, 05829) and anti-vimentin (Chemicon, MAB1681) antibodies. The cells were then incubated with the fluorescein isothiocyanate (FITC) conjugated specific secondary antibodies (specifically: anti-mouse IgG (Chemicon Int, AP 124-T) for anti-tubulin antibody and anti-mouse IgM (Chemicon Int, AP 132-T) for anti-vimentin antibody). Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. Negative controls were obtained by omitting the primary antibodies. Samples were evaluated using an epifluorescence microscope (Nikon, Florence, Italy) at 100× magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy).

Image analysis was performed by extracting, for each cell image, the region of interest (ROI) by appropriate software (Image Pro Plus). Then, the mean pixel value (16 bit, gray level) related to the mean fluorescence intensity and therefore to the specific epitope detection was calculated.

Data analysis

Three different experiments were carried out in triplicate. For viability and proliferation assays at least 10 counts per sample were carried out and the mean value was calculated.

For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields per slide, and the data were expressed as mean ± SD. Statistical significance was determined using a Student's *t*-test. A p value lower than 0.05 was considered statistically significant.

Proteomic sample preparation and 2-DE

For 2-DE, MLS treated and control cells were harvested by centrifugation at room temperature. The pellet was washed twice in water and resuspended in 8 M urea, 4% CHAPS, and 10 mM DTT. After sonicating briefly, protein extracts were clarified by centrifugation at 14 000 × g for 10 min. The protein concentration of each purified sample was determined using the 2D Quant kit (GE Healthcare, USA). For each experimental condition 2-DE replicate gels (n = 3) were made using independent experiments, in order to assess biological and analytical variations. IEF (first dimension) was carried out on nonlinear wide-range IPGs (pH 3–10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the Ettan™ IPGphor™ system (GE Healthcare). IPG-strips were rehydrated with 350 μl of lysis buffer and 2% v/v carrier ampholyte, for 12 h at room temperature. Sample load, 800 μg per strip, was successively performed by cup loading in the IPGphor Cup Loading Strip Holders (GE Healthcare), with the sample cup system at the anodic side of IPG strips. IEF was then achieved according to the following voltage steps, at 20 °C: 30 V for 30 min, 200 V for 2 h, 500 V for 2 h, from 500 to 3500 V for 30 min, 3500 V for 5 h, from 3500 to 5000 V for 30 min, 5000 V for 4 h, from 5000 to 8000 V for 30 min, 8000 V until a total of 95 000 V h^{-1} was reached. After focusing, prior to the second-dimension separation, IPG strips were equilibrated in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS) containing 1% (w/v) DTT for 15 min and then in the same equilibration buffer containing 2.5% iodoacetamide for a further 15 min.

The second dimension separation was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 40 mA per gel constant current and 10 °C until the dye front reached the bottom of the gel.[77] Protein spots were visualized by colloidal coomassie blue staining.[78] The stained gels were scanned with the Epson Expression 1680 Pro image scanner.

Image analysis

Scanned images (16-bit grayscale) were processed and statistically evaluated with Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Both manual and automatic alignment was used to align the images. A control group and a "laser treated" group containing three technical replicates were created and only spots present in all the replicates were taken into consideration for subsequent analysis. The two groups were compared with each other and fold values as well as p-values of all spots were computed by the above-mentioned software using one way ANOVA analysis. All spots were prefiltered and manually checked before applying the statistical criteria (Anova $p \leq 0.05$ and fold ≥ 1.5). Normalized spot volumes, instead of spot intensity, were used in statistical processing. Protein identification involved only spots that fulfilled the statistical criteria. Experimental pI and M_w values were estimated using MW protein markers and some identified proteins selected as markers.

In-gel digestion and MALDI-TOF analysis

Protein spots were manually excised from gels and each sample was transferred to a 1.5 ml Eppendorf tube, washed twice in 50 mM ammonium bicarbonate (NH_4HCO_3)/CH_3CN 1/1 for 15 min and then de-hydrated in CH_3CN. Dried samples were re-swelled in NH_4HCO_3 containing 10 mM DTT (freshly prepared) and incubated for 30 min at 56 °C; the excess liquid was then removed and replaced with the same volume of freshly prepared 55 mM IAA in 25 mM NH_4HCO_3. After 30 min of incubation at room temperature in the dark, the gel particles were washed twice with NH_4HCO_3/CH_3CN 1/1 for 15 min, de-hydrated in CH_3CN and dried in a vacuum centrifuge. Each sample was incubated for 1 h at 37 °C in 20 μl of 20 μg ml^{-1} trypsin solution (Trypsin Proteomics Sequencing Grade T6567, SIGMA) in 40 mM NH_4HCO_3 with 10% CH_3CN. An additional 30 μl of 40 mM NH_4HCO_3 with 10% CH_3CN were added to each sample and incubated overnight at 37 °C. The reaction was stopped by adding a final concentration of 0.1% trifluoroacetic acid. The supernatant was collected and the gel was further extracted with 0.1% trifluoroacetic acid in 50% CH_3CN.[79,80] The extracts were combined and then analysed on a MALDI-TOF/TOF mass spectrometer Ultraflex III (Bruker Daltonics, Bremen, Germany) by using Flex Control 3.0 as data acquisition software. A 0.75 μl volume of the sample was mixed with 0.75 μl of the matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) CH_3CN and 0.5% (v/v) TFA) on the anchorchip target plate and allowed to dry. Spectra were acquired in the reflectron mode over the m/z range 860–4000 for a total of 500 shots. The instrumental parameters were chosen by setting the ion source 1 at 25 kV, ion source 2 at 21.5 kV, the pulsed ion extraction at 20 ns and the detector gain at 7.7×. The instrument was externally calibrated prior to analysis using the Bruker Peptide Calibration standard kit. All the resulting mass lists were cleaned up from eventually present contaminant masses, such as those from matrix, autodigestion of trypsin and keratins. Mass fingerprinting searching was carried out in Swiss-Prot/TrEMBL databases using MASCOT (Matrix Science Ltd., London, UK, http://www.matrixscience.com) software. The taxonomy was restricted to *Mus musculus*, a mass tolerance of 50 ppm was allowed, and the number of accepted missed cleavage sites was set to one. Alkylation of cysteine by carbamidomethylation was assumed as fixed modification. The experimental mass values were monoisotopic. No restrictions on protein molecular weight and pI were applied. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides and probabilistic score sorted by the software.

Western blot

Western blot analysis was performed to validate the identity and the differential expression of PP1, enolase and actin. 100 μg of protein extracts from control and MLS treated C2C12 cells were separated by 2-DE as previously described and transferred

onto a PVDF membrane (Millipore). The blots were incubated with anti-actin (Santa Cruz Biotechnology, SC-1615), anti-enolase (Santa Cruz Biotechnology, SC-7455) and anti-PP1 antibodies (Santa Cruz Biotechnology, SC-7482) in blocking buffer (PBS, 2% nonfat dry milk, 0.1% v/v Tween-20). After incubation with secondary antibodies, the blotting was developed by using the ECL plus immunodetection system ECL (GE Healthcare) and visualized by autoradiography.

Cluster analysis

The differentially expressed proteins were subjected to functional pathway analysis using DAVID database (http://david.abcc.ncifcrf.gov/home.jsp)[81] for better understanding of the biological context of the identified proteins and their participation in various physiological processes. UniProt accession numbers of the 42 differentially expressed proteins identified in our study were uploaded and mapped against the *Mus musculus* reference dataset to extract and summarize functional annotation associated with individual or group of genes/proteins and to identify gene ontology terms, molecular function, biological process and important pathways for each dataset.

Determination of protein serine phosphatases, pyruvate kinase, enolase and lactate dehydrogenase activities

Protein Serine Phosphatases (PPPs) activity was determined in C2C12 cell lysates from three independent experiments. MLS-treated and not treated C2C12 cells were quickly rinsed in ice-cold phosphate-buffered saline (PBS, 10 mM sodium phosphate and 0.15 M NaCl, pH 7.2), and freezed. After thawing the material at room temperature, the lysis was performed at 4 °C in 50 mM Tris, pH 7.4, containing 5 mM dithiothreitol and Sigma protease inhibitors mix (1/100, v/v). After 30 min of incubation on ice, lysates were sonicated (three short bursts) and centrifuged at 12 000*g* in a microcentrifuge at 4 °C for 30 min. Supernatants were quantified with respect to proteins content by the Bradford method. PPPs activity was determined using *p*-nitrophenyl phosphate as the substrate. All enzymatic activity tests were performed in duplicate. The substrate (4 mM) was dissolved in 25 mM Tris–HCl buffer, pH 7.2, containing 5 mM dithiothreitol, 20 mM sodium–potassium DL-tartrate and 0.1 mM sodium orthovanadate. Tartrate and orthovanadate were added in order to inhibit protein tyrosine phosphatases, lysosomal acid phosphatases and non-specific phosphatases.[82,83] The reaction was stopped with 0.1 M KOH and the released *p*-nitrophenolate ion was measured by reading the absorbance at 400 nm (ε = 18 000 M^{-1} cm^{-1}). The activity measured under these conditions was completely inhibited by 0.01 mM cantharidic acid, a specific and strong inhibitor of all PPPs.[84] Statistical significance was determined using a Student's *t*-test. A *p* value lower than 0.05 was considered statistically significant.

Pyruvate kinase (PK) activity was determined at 30 °C according to Bergmeyer,[85] with slight modifications, continuously following the NADH oxidation at 340 nm, using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD). The assay mixture contained in 1 ml final volume consisted of 50 mM triethanolamine (pH 7.6), 8 mM $MgSO_4$, 5 mM EDTA, 75 mM KCl, 1.5 mM ADP, 0.15 mM NADH, 60 units of lactate dehydrogenase. The reaction was started by adding substrate (0.8 mM phosphoenolpyruvate). Enolase activity was determined at 30 °C according to Bergmeyer,[86] with slight modifications, continuously following the NADH oxidation at 340 nm, using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD). The assay mixture contained in 1 ml final volume consisted of 80 mM triethanolamine (pH 7.6), 3.3 mM $MgSO_4$, 1.1 mM ADP, 0.2 mM NADH, 20 units of lactate dehydrogenase, 3 units of pyruvate kinase. The reaction was started by adding substrate (0.9 mM phosphoglycerate). Lactate dehydrogenase (LDH) activity was determined at 30 °C according to Bergmeyer,[87] with slight modifications, continuously following the decrease of NADH at 340 nm, using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD). The assay mixture, contained in 1 ml final volume, consisted of 100 mM phosphate buffer pH 7.0 and 0.2 mM NADH. The reaction was started by adding substrate (0.77 mM pyruvate). The value of 6.22 mM^{-1} cm^{-1} is considered to be the NADH molar extinction coefficient. One unit of activity is defined as that quantity of enzyme which transforms one μmole of substrate in one minute at 30 °C.

Conclusions

The aim of this study was to investigate the response of myoblasts to IR laser treatment in order to get further insights into the cellular and molecular mechanisms underlying the effects of laser therapy on muscle tissue described in clinical studies and on animal models. Our results show that laser treatment, with the source and parameters chosen, did not affect cell viability but induced a decrease in cell proliferation and increase in expression of the early differentiation marker MyoD, associated with changes of cell morphology and cytoskeletal architecture leading to the formation of tube-like structures. Taken together, these findings suggest that the exposure to IR laser triggers a differentiation process in myoblasts.

The analysis of differential expression in the proteomic profile of laser treated and untreated cells, which to the best of our knowledge had never been performed before, further confirmed in treated cells a scenario of differentiation process in its early stages. In fact, following laser exposure, numerous proteins known to be involved in cell cycle regulation, cytoskeleton organization and differentiation showed a significant increase or modulation. The fact that IR laser treatment seems to be able to promote myoblast differentiation *in vitro* could in part explain the regenerative and reparative effects attributed to laser therapy when applied to muscle injury in clinics. Very interestingly, the proteomic analysis also revealed the increase of numerous ATP-binding proteins and proteins involved in the regulation of muscle metabolism, as PP1, establishing a connection with the well-known effect of red-IR laser radiation on the activity of cytochrome oxidase and ATP synthesis.[4] Moreover, among the proteins overexpressed in the treated cells there were NLRP 10 and other proteins which regulate the inflammatory response and could contribute to the anti-inflammatory action attributed to laser therapy. Finally, the increase of proteins involved in cell adhesion/migration, angiogenesis and axonogenesis fits with the

possibility to induce by laser treatment mechanisms related to tissue repair processes.

In conclusion, this study reports for the first time a proteomic analysis of IR laser treated myoblasts, thus contributing with original results to shed light on molecular and cellular mechanisms underlying the effect of laser therapy in muscle repair and recovery.

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Key words: MLS laser therapy, cell differentiation, myoblasts, extracellular matrix

Effects of MLS laser on myoblast cell line C2C12.

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ABSTRACT

Laser is widely used in many medical fields and its effects are reported by several studies in literature. Very important are the applications in sports medicine, physical medicine and rehabilitation, based on the analgesic, anti-inflammatory and anti-oedema effects of laser therapy, as well as the stimulating action on tissue repair processes. In our study, we analyzed the effects of an advanced laser system, the Multiwave Locked System (MLS), on myoblasts in order to evaluate the effectiveness of this laser in promoting recovery of damaged muscle tissue. The MLS device consists of two synchronized diodes emitting at 808 and 905 nm, respectively. C2C12 murine myoblasts cell line was used as experimental model since it is a widely accepted model in muscle cells behavior studies.

Viability and proliferation was assessed after a single treatment as well as after 4 consecutive treatment (1 treatment/day). No significant changes were observed in viability, while proliferation decreased after 4 treatments. Moreover, we found an increased expression of MyoD, a key factor in myoblasts maturation. Changes in cytoskeleton organization, in particular the networks of actin microfilaments and microtubules, were also observed. Decresed proliferation rate, increased MyoD expression and cytoskeleton rearrangement are consistent with myoblast differentiation.

Finally the expression of molecules involved in the regulation of extracellular matrix (ECM) turnover (collagen I, MMP-2, MMP-9) was analyzed. After 4 treatments, collagen I expression showed a 14% increase while MMP-2 and MMP-9 decreased of 33% and 18%, respectively. These results suggest that MLS treatment could affect ECM turnover shifting the balance toward the production rather than to the degradation.

In conclusion, our findings demonstrate that MLS treatment induces in muscle cells a biological response that could favour muscle cell differentiation and the recovery of diseased muscle tissue. A deeper knowledge of the mechanisms underlying the effects described above and a greater understanding of the changes in the biological response to variations in instrumental parameters setting can lead to concrete improvements in treatment protocols.

INTRODUCTION

Lasers are widely used in biomedicine. Sport medicine, physiatrics and rehabilitation are among the most important fields of application. Here the analgesic, antiinflammatory, anti-oedema and stimulating effects of laser therapy are used to favour tissue repair and function recovery.

According to the literature, many factors can contribute to the stimulating effect.

The moderate vasodilation increases the supply of nutrients and growth factors. For example, it has been demonstrated that low-level laser (LLL) irradiation (Ga-Al-As laser) promotes expression of fibroblast growth factor (FGF) in rat gastrocnemius muscle recovering from disuse muscle atrophy [1]. FGF promotes angiogenesis and lead to fibroblasts activation [2,3] which determines an increase of collagen synthesis, essential for tissue repair and regeneration [4-6]. Neoangiogenesis is crucial for ensuring oxygen and nutritional substances to new tissues and has a very important role in muscle recovery [7,3]

Effects that induce a local increase of nutrients, promote angiogenesis and influence the development of inflammation can strongly affect the healing process and functional recovery of the injured tissues.

Another factor widely recognized as fundamental to the stimulating effect is the red/infrared (IR) laser-induced increase in ATP production in mitochondria [7-9]. After treatment with He-Ne laser, an increase in membrane potential and consequent ATP production have been observed in isolated mitochondria [10]. Moreover, many authors found that red/IR lasers may promote cell proliferation [4,11-13].

All these effects are consistent with the hypothesis that the recovery of injured tissues can be accelerated through the application of suitable laser therapy.

Studies on nerve fibers regeneration [14] showed that reconnection process of nerve cells is accelerated after laser

treatment, leading to the regeneration of insensitive areas [15-17]. Other studies have demonstrated a faster recovery of wound healing [18] and bone fractures [19], as well as a marked reduction in infarct size and myocardial infarct [20].
Many studies report on effects of laser radiation on muscle homeostasis and repair mechanisms in this tissue. In a recent study, using mice as experimental model, the anterior tibial muscle previously damaged by a cryolesion has been exposed to LLLT (GaAlAs Laser, 660 nm). Although a significant reduction in recovery time was not recorded, an increase of collagen IV was found in the treated muscles [21].
Another study on mice demonstrated that He-Ne laser irradiation (632.8 nm), associated with physical exercise, reduced skeletal muscle inflammation, improved the activity of superoxide dismutase and diminished the activity of creatine kinase [22].

Some authors found an increase in proliferation of muscle satellite cells [1, 23-25]. These cells, usually quiescent, can be activated by factors released by cells of the injured muscle [26-28]. The satellite cells have the function of creating new fibers and replacing the necrotic ones [27].

In the frame of studies aimed at understanding the mechanisms by which laser therapy can promote the repair and functional recovery of skeletal muscle, here we report the results obtained investigating the effect of IR laser radiation on myoblasts.

As for any other radiation source, the main parameters for characterizing laser emission are: power, frequency and wavelength. These ones, together with the features of the irradiated tissues or samples, strongly affect the way the radiation propagates into the tissue/sample and the consequent effects.
In our experiments, we chose as the laser source a Multiwave Locked System (MLS) because we hypothesized that this laser system could be particularly suitable for the treatment of skeletal muscle. In fact the system is characterized by two synchronized emissions with wavelengths 808 and 904, respectively. The two emissions are absorbed by different mitochondrial complexes, therefore the MLS treatment can affect cellular energy metabolism by acting on multiple sites in the respiratory chain at the same time. Radiation with λ = 808nm is absorbed by the cytochrome oxidase (complex IV) which is considered as a principal photoacceptor in mammalian cells [29,30]. It is know that the activation of this mitochondrial enzyme after absorbing a radiation in red/near infrared (IR) promotes the production of ATP [31,32]. The radiation with λ= 905 nm interacts with the complexes I, II, III, IV of the respiratory chain and succinate dehydrogenase [33].

Considering the emission wavelengths and tissue type (muscular tissue) optical properties, it is possible to estimate MLS radiation which is expected to propagate within the tissue a penetration depth of about 10 mm in this kind of tissue; this means that still about 13% of initial power reaches a 20 mm depth. Therefore it is possible to affirm that MLS radiation can interact with deep-located muscle tissue.
Moreover, since our previous data (not yet published) demonstrated that MLS radiation is absorbed by collagen and polysaccharide biogels, which are models of extracellular matrix, we hypothesized that the MLS treatment could also affect cell behaviour by modification of the extracellular microenvironment.

MATERIAL AND METHODS

Cell Cultures

Murine myoblasts have been cultured in Dulbecco's Modified Eagle's Medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 10% fetal bovine serum (FBS).
Cells were incubated at 37°C in humidified atmosphere containing 95% air and 5% CO2 in order to maintain a pH value between 7.3 and 7.5. When confluence has been reached, cells have been washed twice with PBS, then treated with a 0,05% trypsin solution and plated on 55 cm2 plates. All the reagents have been purchased from Sigma (Chemical Co St Louis, MO, USA).

MLS Treatment

The laser source was a Multiwave Locked System (MLS) provided by ASA s.r.l. (Arcugnano, Vicenza, Italy). The instrument consists of two assembled laser diodes, with synchronized emissions at 808 and 905 nm, respectively.
The diode with λ = 808nm may emit in continuous mode, with a power P = 1.1W, or pulsed mode with an average power Pa = 0.55W and a maximum frequency of 2000Hz.

The diode λ = 905 nm is characterized by a pulsed emission with a maximum frequency of 2000Hz and an average power Pa = 60mW.
Therefore, the MLS emission can occur in different modes, according to the operator's choice:
Continuous Mode (Continuous Mode Operation, CW): diode with λ = 808 nm, continuous emission and diode with λ = 905 nm, pulsed emission. Pulsed mode (Pulsed Mode operation): diode with λ= 808nm, pulsed emission with pulses repetition frequency f808 (Max value 2000Hz) and diode with λ = 905nm, pulsed emission with pulses repetition frequency f905 = f808.

When frequency changes, the emission features allow the average power of the 905nm diode emission to change, while the average power of the 808nm diode emission does not change. In fact, when the frequency changes the 808nm diode emission duration changes in proportion, in this way the average power remains the same. It is the temporal distribution of the released energy which changes. With the

same emission time (and spot sizes), the whole energy (808nm + 905nm) changes when the set frequency changes.
For our experiments, cells have been plated on slides Ø of 13mm (5000 cells per slide) previously sterilized and put in multiwell (plates of 24 wells) to carry out the treatment. Each plate has been put in a holder which allowed an easy scanning of the samples. Each scanning lasted 20s. The treatment was repeated once a day for 4 consecutive days in sterile conditions. The treated samples have been compared with controls maintained in the same conditions, except for the exposure to MLS laser device.
The following treatment parameters have been applied: 8 min exposure to 1500Hz emission frequency. To calculate the energy given to each sample during a single treatment (E) it has been considered the following relation:

$$E = P_t \cdot (t_t / n) \quad (1)$$

where n is the number of samples (8 in our experiment), t_t is the treatment time, P_t is the average power, estimated on the slide surface (132 mm^2), equal to the sum of the two laser sources contribution (P_t ~ 200mW). Entering the data in the formula (1), we obtain E ~ 12.0 J.

Cell viability
Cell viability after exposure to MLS was determined by a Trypan Blue assay. The dye is capable of selectively penetrate into dead cells. After treatment, cells are washed and detached with trypsin/EDTA for a few minutes. Then cells are centrifuged and resuspended in a solution of PBS and Trypan Blue (dilution factor: 2) and counted, after 5 min of incubation, using Neubeuer emocytometer.

Immunofluorescence
After treatment the cells were fixed in cold acetone for 5 minutes and then washed with PBS without Ca and Mg. After blocking unspecific binding with PBS containing 3% bovine serum albumin (BSA), cells were incubated overnight at 4°C with the specific antibodies: anti-α actin, anti-collagen I, anti-α tubulin and anti-vimentin antibodies (Chemicon Int, Temecula, CA), anti-Myo D antibody (Santa Cruz Biotechnology, Heidelberg, Germany), anti-MMP-2 and anti-MMP-9 antibodies (Abcam, Cambridge, UK). The cells were then incubated with the FITC (fluorescein isothiocyanate) conjugated specific secondary antibodies (specifically: anti-mouse IgG for tubulin and Myo D antibodies, anti-rabbit IgG for collagen I and MMP-2 antibodies, anti-mouse IgM for vimentin antibody and anti-goat for MMP-9 antibody) (Chemicon Int, Temecula, CA). Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an inverted epifluorescence microscope (Eclipse TE2000-E, Nikon, Italy) with oil immersion objective (CSI S fluor 100x, N.A. = 1.3) at 100x magnification and imaged by a HiRes IV digital CCD camera (DTA, Italy). Fluorescence excitation has been achieved by selecting the 365nm emission line of a mercury vapor lamp (HBO 100W, Osram). About 30 cells from different fields have been imaged for each slide.

Image processing
The image processing has been performed by using a specific program written in the LabVIEW language (National Istruments). By first obtaining a binarized image, in which pixels corresponding to cells and those corresponding to the background have been given the value of 1 and 0 respectively, the program is able to distinguish the cell signal from the background; as a second step, it calculates the average cell intensity by applying the binarized images to the original gray-scale ones. It is then possible to compare the average fluorescence intensity of a first images set (control samples) with the intensity of a second one (treated samples).

Data Processing
The experiment has been made three times to confirm the results. For each slide 30 images have been acquired and selected in a random way. The fluorescence intensity of each field (analyzed with previously described method) has been expressed as the average pixel intensity corresponding to the visualized cells. Intensities corresponding to the 30 acquired fields have been further mediated to give a final value, whose error has been calculated as Standard Deviation (SD). The statistical significance has been determined using the T-Student's test (chosing $p<0.05$).

RESULTS
The aim of this study was to evaluate the effects of MLS treatment on muscle cells and to identify mechanisms possibly involved in the stimulation of tissue repair. For our experiments, we used a murine myoblasts cell line (C2C12) widely accepted as a model in muscle cells behavior studies. In particular, the research focused on cell viability and proliferation, organization of cell cytoskeleton, expression of MyoD, an early marker of muscle differentiation, and proteins involved in the extracellular matrix turnover (collagen I, MMP2, MMP9).

Viability and proliferation
In order to verify the effect of the exposure to MLS emission on cell viability and proliferation, Trypan blue assays were carried out 24 h after the first treatment and 24 h after the fourth treatment.
As shown in Fig.1, in both cases, no significant differences were observed between treated samples and controls as regards cell viability, which resulted higher than 97.5% in all the samples.
Cell proliferation did not change significantly after the first treatment, but showed a decrease of the 25% after four treatments (Fig.2)

Cytoskeleton
The cytoskeleton is an important structure for the cell since it allows both movement and shape modifications and

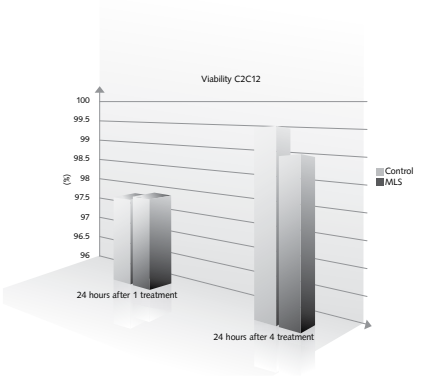

Fig.1. C2C12 Cell viability assessed 24 h after MLS treatment and 24 h after the fourth MLS treatments. (Control vs. MLS). Data were obtained by Trypan Blue assay.

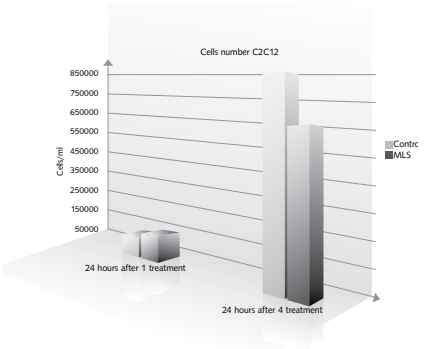

Fig. 2. C2C12 Cell proliferation assessed 24 h after MLS treatment and 24 h after the fourth MLS treatment. (Control vs. MLS). Data were obtained by Trypan Blue assay.

has an important role in intracellular transport and signalling. The cytoskeleton is mainly composed of three elements: actin microfilaments, microtubules and intermediate filaments made of tubulin and vimentin, respectively.

The distribution of actin, tubulin and vimentin in myoblasts exposed to MLS treatments was studied by immunofluorescence microscopy and image processing.

Actin is modified by mechanical stimulation, in particular by physical stimulation. It can be used as a sensitivity marker of the cells when exposed to physical factors [34]. Moreover, it is considered an important marker for muscle cells differentiation [35].

As shown in Fig.3 (a,b), after MLS treatments, actin expression decreased by about 13% and cleary changed the organization of the microfilament network. The microfilaments appeared more concentrated in perinuclear area. The treated samples showed also changes

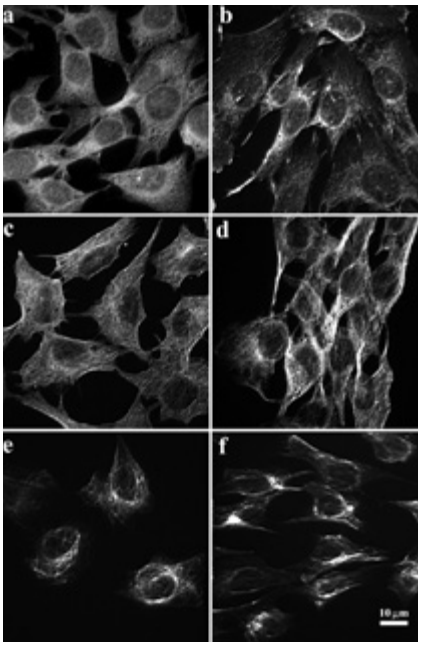

Fig. 3. Expression of cytoskeleton components assessed by immunofluorescence microscopy. Actin expression in control (a) and cells exposed to MLS treatment (b). Tubulin expression in control (c) and cells exposed to MLS treatment (d). Vimentin expression in control (e) and cells exposed to MLS treatment (f).

in the cell morphology, which resulted elongated, when compared with control samples. From a quantitative point of view, the expression of tubulin, which is the main constituent of microtubules, did not change following laser treatment. However, as observed in the case of actin, a different organization of the microtubule network has been observed: in fact, in control cells microtubules were organized radially while in treated cells appeared randomly distributed. See Fig.3 (c,d).

We did not find any significant effect of the treatment on vimentin, the protein which form the intermediate filaments [Fig.3 (e,f)].

Extracellular matrix

The extracellular matrix (ECM) is the non-cellular component of a tissue. It has many functions depending on the composition. For example, it acts as support and anchorage for cells and is a reservoir of growth factors. Cells bind to ECM via membrane proteins called integrins. Through these molecular "bridges", ECM

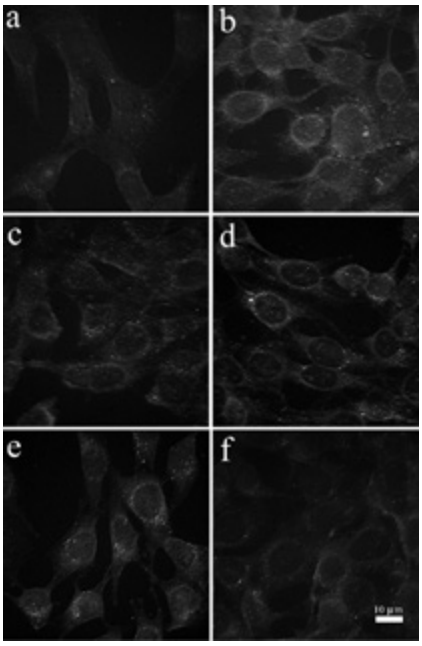

Fig. 4. Expression of extracellular matrix components assessed by immunofluorescence microscopy. Collagen I expression in control (a) and cells exposed to MLS treatment (b). MMP-2 expression in control (c) and cells exposed to MLS treatment (d). MMP-9 expression in control (e) and cells exposed to MLS treatment (f).

deformations can transmit mechanical stresses to the cells and affect cytoskeleton organization; in the same way cells can induce changes in ECM [36,37].

The ECM turnover is a key factor in the repair process of traumatized muscle.

The main ECM protein is collagen, which forms very dense fibres. Different types of collagen are present in the various tissues. Collagen I is the most abundant in the human body. It can be found in tendon, muscle, endomysial fibrils, the organic part of the bone tissue [38,39] and in the scar tissue. After exposure to MLS, myoblast cultures showed a moderate (14%) but significant increase ($p < 0,025$) in collagen I expression. Fig.4 (a,b)

The homeostasis of the ECM is also regulated by proteins belonging to metalloprotease family (MMP), which are involved in ECM degradation and repair during normal physiological processes [40,41]. These proteins are also involved in pathological conditions like arthritis [42]. In myoblast cultures treated with MLS we analyzed the expression of

matrix metalproteinase-2 (MMP-2) and matrix metalproteinase-9 (MMP-9), which degrade collagen IV, one of the most abundant types of collagen in skeletal muscle. In comparison with control samples we found a decrease of expression of 33% and 18% respectively Fig. 4 (c,d and e,f).

Differentiation markers

As above described, the data of our experiment revealed a decrease in proliferation but no significant changes in viability. Since this means that the MLS treatment does not induce cell damage, we hypothesized that the reduction in the growth rate could be due to the triggering of a differentiation process. Therefore, we analysed in the treated cells the expression of the differentiation marker MyoD. The differentiation markers are molecules which are expressed when cells pass from proliferation to maturation. Each tissue has its own differentiation markers. MyoD, an early marker of myogenesis, belongs to a protein family known as myogenic regulatory factors (MRFs). The main MyoD function is removing cells from cellular cycle and blocking proliferation. It is mainly expressed in muscle cells, where it has an important function in regulating muscle differentiation [43,44]. Our results demonstrate that MLS treatment induced an increase of the 26% in MyoD expression (Fig. 5).

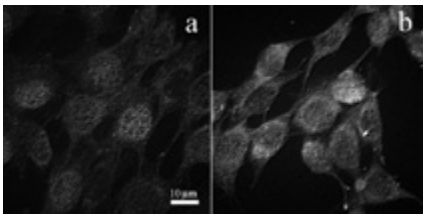

Fig. 5. MyoD expression assessed by immunofluorescence microscopy. Control (a) and cells exposed to MLS treatments (b).

DISCUSSION

The analysis of the data obtained by our experiments shows that the exposure to MLS treatment, even if repeated over time, did not produce significant changes in cells viability, which never fell below 97.5%. The proliferation decreased moderately, but significantly, after 4 treatments.

In literature there are many studies concerning the effect of laser radiation on cell viability. The results are often controversial and depends on laser type and experimental models used. However our results are in accordance with those reported by Ferreira et al. in a study on the effect of red/IR lasers on C2C12 cells, the same as our experimental model [45]. Recent studies carried out on different cell types showed that proliferation increased after exposure to wavelengths ≤ 780 nm, while it decreased by irradiation at 810 nm [12,46].

Since the unchanged cell viability demonstrated the absence of acute cell damage, the slower rate of growth induced us to hypothesize that MLS treatment could promote muscle cell differentiation. This hypothesis was indeed confirmed by the increase in MyoD that we found in treated myoblasts. As above explained, MyoD is an early marker of myoblast differentiation and plays a key role in the maturation of muscle cells [47].

The analysis of cytoskeleton organization, made through immunofluorescence microscopy, has shown that MLS treatment induced a considerable reshape both in microtubules distribution and in the network of actin microfilaments.

These data are in agreement with results we obtained previously in chondrocytes and fibroblasts exposed to IR laser treatment [48] and also with the studies of Ricci et al [49], where changes in organization of actin filaments and stress fibers formation in endothelial cells of rabbit aorta (REAC) subjected to LLLT are described.

It is well know that important changes of the cytoskeleton can be inducted by physical stimulation and laser radiation is not an exception. These changes can determine important effects on cells behavior, since microtubules have a primary function in regulating distribution and positions of intracellular organelles and actin is involved in cell shape determination, and regulates the adherence/migration processes [50]. Moreover, in muscle cells, actin has a very important and significant function. Finally, the transition from proliferation to differentiation, such as that observed after MLS treatments, involves changes in cell morphology and therefore in cytoskeleton organization.

Indeed, it has been demonstrated that substances like phospholipase D induce myogenic differentiation through a remodeling of actin cytoskeleton [51].

MLS treated samples showed also changes in expression of molecules which have important functions in reshaping the ECM. Collagen I expression increased, in agreement with what other authors have found recently in tissues exposed to GaAlAs laser (λ = 808nm) [52].

On the contrary, the expression of MMP-2 and MMP-9, involved both in migration and myoblasts differentiation [53], diminished. The moderate increase in collagen and reduction in MMP-2 and MMP-9 could affect myoblasts migration and ECM formation.

In conclusion, the results we obtained on cell viability and proliferation, structural changes of the cytoskeleton, MyoD, collagen I, MMP-2 and MMP-9 expression demonstrate that MLS treatment does not affect myoblast viability but can affect migration, differentiation and production of ECM molecules.

These results indicate that MLS treatment is able to induce, in muscle cells, a biological response that can affect muscle function. This response is consistent with therapeutic effects observed at systemic level and suggest that MLS therapy could be effective in treating muscle diseases by direct action on myoblast behaviour. Additional studies to further understand the molecular mechanisms underlying the observed effects are needed, since a better understanding of mechanisms and biological responses evoked by use of different instrumental parameters can lead to significant improvements in therapeutic protocols.

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ORIGINAL ARTICLE

Effect of IR Laser on Myoblasts: Prospects of Application for Counteracting Microgravity-Induced Muscle Atrophy

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Abstract Microgravity-induced muscle atrophy is a problem of utmost importance for the impact it may have on the health and performance of astronauts. Therefore, appropriate countermeasures are needed to prevent disuse atrophy and favour muscle recovery. Muscle atrophy is characterized by loss of muscle mass and strength, and a shift in substrate utilization from fat to glucose, that leads to a reduced metabolic efficiency and enhanced fatigability. Laser therapy is already used in physical medicine and rehabilitation to accelerate muscle recovery and in sports medicine to prevent damages produced by metabolic disturbances and inflammatory reactions after heavy exercise. The aim of the research we present was to get insights on possible benefits deriving from the application of an advanced infrared laser system to counteract deficits of muscle energy metabolism and stimulate the recovery of the hypotrophic tissue. The source used was a Multiwave Locked System (MLS) laser, which combines continuous and pulsed emissions at 808 nm and 905 nm, respectively. We studied the effect of MLS treatment on morphology and energy metabolism of C2C12 cells, a widely accepted myoblast model, previously exposed to microgravity conditions modelled by a Random Positioning Machine. The MLS laser treatment was able to restore basal levels of serine/threonine protein phosphatase activity and to counteract cytoskeletal alterations and increase in glycolytic enzymes activity that occurred following the exposure to modelled microgravity. In conclusion, the results provide interesting insights for the application of infrared laser in the treatment of muscle atrophy.

Keywords Muscle atrophy · Microgravity · Myoblasts · IR laser

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Introduction

Aging and disuse, as occurs in bed rest and spaceflights, induce in skeletal muscle a reductive remodelling and may lead to atrophy. The mechanisms underlying muscle atrophy caused by disuse and muscle aging have some similarities: in both the lack of mechanical stimuli plays a relevant role. In this aspect, conditions associated with muscle disuse, such as the exposure to a weightless environment, are considered a model for studying aging processes in skeletal muscle (Biolo et al. 2003), although other important factors such as changes in the innervation (Doherty 2003) and levels of cytokines and growth factors (Degens 2010) are involved in aging.

Disuse atrophy has been widely studied and is considered a problem of utmost importance in manned spaceflights. It is characterized by loss of muscle mass, force and power, changes in fiber type composition and increased muscle fatigue due to reduced metabolic

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efficiency: a shift in substrate utilization from fat to glucose occurs, leading to an enhanced fatigability (Fitts et al. 2000; Stein and Wade 2005; Blaauw et al. 2010). Hexokinase (HK) activity, considered a marker of glycolytic metabolism, significantly increases (Manchester et al. 1990; Chi et al. 1992). The susceptibility of skeletal muscle to damage increases and becomes particularly evident during postflight reloading (Fitts et al. 2000).

In the future, the increase of mission duration from one side and, from the other side, the expected increase of extravehicular activities, which could require sustained work output, will further exacerbate the problem of managing muscle atrophy during spaceflights and postflight. Therefore, appropriate countermeasures are needed to prevent disuse atrophy and/or favour muscle recovery.

Several studies demonstrated the utility of physical protocols as vibration or electrical stimulation (Chopard et al. 2009; Guo et al. 2012), but too few data regarding the effects of different physical countermeasures on the processes involved in skeletal muscle disuse atrophy and recovery are available.

Due to the proven ability of red-infrared (IR) radiation to enhance cell energy metabolism (Silveira et al. 2009) and reduce inflammation (Rizzi et al. 2006), laser therapy is already used in physical medicine, rehabilitation and sports medicine to accelerate muscle recovery (dos Santos et al. 2010) and to prevent damages produced by metabolic disturbances and inflammatory reactions after heavy exercise (Leal Junior et al. 2009).

This paper reports the results of a study aimed at investigating the effects of IR laser radiation on myoblasts and considering the possibility to apply IR laser therapy to promote muscle regeneration and recovery in disuse atrophy. We used as an experimental model the C2C12 skeletal muscle cell line, derived from satellite cells. The C2C12 cells are widely accepted as a model to study the behaviour of satellite cells (Burattini et al. 2004), which play a crucial role in skeletal muscle regeneration and repair (Wang and Rudnicki 2012) and are capable to repopulate atrophied muscle (Hawke and Garry 2001). Moreover, C2C12 cells have been already used in previous studies regarding the effects of microgravity on mechanical signalling mechanisms in muscle plasticity (Torgan et al. 2000) and myoblast behaviour (Slentz et al. 2001; Pache et al. 2010).

Recently, studies in progress in our laboratory, aimed at understanding the cellular and molecular mechanisms underlying the effects of IR laser radiation on repair processes in muscle tissue, demonstrated that C2C12 cells exposed to IR laser radiation show enhanced cell energy metabolism and a significant increase in serine/threonine protein phosphatases (PSPs), in particular serine/threonine protein phosphatase 1 (PP1), which plays a crucial role in the regulation of glycogen metabolism and is involved in myosin dephosphorylation, thereby controlling muscle contraction/relaxation (Cohen 2002; Ceulemans and Bollen 2004). Moreover, we observed that proteins involved in cytoskeleton organization/cell shape regulation and muscle contraction, such as vimentin, actin and tropomyosin, also increased (Monici et al. 2012).

Following these findings, we hypothesized that IR laser radiation could be a useful tool to counteract the microgravity-induced impairment of energetic metabolism (Fitts et al. 2000), cytoarchitectural alterations (Pache et al. 2010) and decrease in the levels of contractile proteins (Torgan et al. 2000) observed in myoblasts exposed to microgravity.

Therefore, in myoblasts previously exposed to modelled microgravity, we tested the effect of IR laser treatment on cell metabolism and morphology.

Materials and Methods

Cell Culture

Murine myoblasts (C2C12 cell line) were routinely cultured in growing medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 10 % fetal bovine serum (FBS). Cells were incubated at 37 °C and 5 % CO_2. All the reagents have been purchased from Sigma Chemical Co. (St Louis, MO, USA).

MLS Laser Treatment

The treatments have been performed with an advanced Multiwave Locked System (MLS) laser (ASA Srl), that is a high power (average power up to 1.1 W, class IV) IR laser with two synchronized sources (laser diodes). The two modules have different wavelengths, peak power and emission mode. The first one is a pulsed diode laser, emitting at 905 nm, with peak optical power = 25 W; each pulse is composed of a pulse train (single pulse width = 100 ns, maximum frequency 90 kHz), thus varying the average power delivered to the tissue. Frequency of the pulse trains may be varied in the range 1–2000 Hz. The second laser diode (808 nm) operates in continuous mode (P 1.1 W) or in pulsed mode (pulses repetition rate 1–2000 Hz), mean optical power output = 550 mW, duty ratio 50 % independently of

the pulse repetition rate. The two propagation axes are coincident.

For the treatment, cells were seeded in the central 8 wells of a 24-multiwell plate. The plate was placed inside a plexiglass support, specifically designed and built. On the top of the support there was a central groove in which laser handpiece slided. The plate was perfectly aligned with the handpiece, at a distance of 3 cm from it, so that the spot formed by the two superimposed laser beams had a diameter equal to that of a single well (13 mm). The support allowed us to perform an homogeneous scan of 8 samples at the same time, by moving the spot at a constant horizontal velocity above the 8 treated wells (5.6 cm/s: each scan of 8 wells lasted 20 s), in order to have the same radiant energy impinging into each well (~68 J for the whole treatment). Treatment parameters were: 1500 Hz frequency, 8 min total scan time. The scan mode is now extensively used also in clinics because it allows to treat large areas and further contributes to avoid photothermal damage. The treatment was repeated once a day, for 3 consecutive days in sterile conditions. The treated samples were compared with controls maintained in the same conditions, except for the laser exposure.

Random Positioning Machine

A Random Positioning Machine (RPM) (Dutch Space, Leiden, The Netherlands) was used in order to model unloading conditions.

In the RPM, introduced by Hoson et al. (1997), samples are fixed close to the centre of two frames rotating one inside the other, driven by separate motors. The rotation of each frame is random and autonomous under computer control. The low g conditions are modelled by averaging the gravity vector via the independent rotation of the two frames.

In our experiments, the speed of rotation was 60°/s (about $10^{-3} \times g$), and direction and interval were set at random. Temperature was maintained at 37 °C. The cells were placed in suitable T25 flasks, which were completely filled with culture medium in order to avoid shear stress, and exposed to the RPM for 72 h.

Study Design

The following samples were prepared, analyzed and compared:

- Samples exposed to the RPM were compared with the corresponding ones non exposed to the RPM ($1 \times g$ controls).
- Samples exposed to the RPM and then treated with MLS laser were compared with samples exposed to the RPM but untreated with MLS laser.
- $1 \times g$ controls untreated and treated with MLS laser.

$1 \times g$ controls were placed on the fixed base of the RPM, facing the same vibrations and temperature as the rotating ones. At the end of the treatments, the cells were recovered and prepared for analytical tests.

Immunofluorescence Analysis

At the end of the experiments, cells were fixed for 5 min in cold acetone, then washed in phosphate buffered saline (PBS). After blocking unspecific binding with PBS containing 3 % bovine serum albumin, cells were incubated overnight at 4 °C with the specific antibodies: anti-α actin, anti-tubulin and anti-vimentin. The cells were then incubated with the fluorescein isothiocyanate (FITC) conjugated specific secondary antibodies (specifically: anti-mouse IgG for anti-tubulin antibody and anti-mouse IgM for anti-vimentin antibody). Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. All antibodies were purchased from Chemicon Int, (Temecula, CA). Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an epifluorescence microscope (Nikon, Florence, Italy) at 100× magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy).

Cell Lysis

Cells were quickly rinsed in ice-cold phosphate-buffered saline (PBS, 10 mM sodium phosphate and 0.15 M NaCl, pH 7.2), and frozen at −80 °C. After thawing the material at room temperature, the lysis was performed at 4 °C in 50 mM Tris, pH 7.4, containing 5 mM ditiothreitol and Sigma protease inhibitors mix (1/100, v/v). After 30 min of incubation on ice, lysates were sonicated (three short bursts) and centrifuged at 12,000 g in a microcentrifuge at 4 °C for 30 min. Supernatants were quantified with respect to protein content by Bradford method.

Determination of Total Protein Concentration

Total protein concentration was determined according to the Bradford's method (1976), using a kit (Bradford

Reagents) produced by Sigma Chemical Co. (St Louis, MO, USA).

Determination of Pyruvate Kinase Activity

Pyruvate kinase (PK) activity was determined at 37 °C according to Hess and Wieker (1974), with slight modifications, continuously following NADPH oxidation at 340 nm, by using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD). The assay mixture contained in 1 ml final volume consisted of 50 mM triethanolamine (pH 7.6), 8 mM $MgSO_4$, 5 mM EDTA, 75 mM KCl, 1.5 mM ADP, 0.15 mM NADH, 5 mg/ml lactate dehydrogenase.

The reaction was started by adding the substrate (0.8 mM phosphoenolpyruvate). The value of 6.22 mM^{-1} cm^{-1} is considered to be the NADH (or NADPH) molar extinction co-efficient. One unit of activity is defined as the quantity of enzyme which transforms 1 μmole of substrate in 1 min, at 30 °C.

Determination of Hexokinase Activity

HK activity was determined at 37 °C according to Bergmeyer (1974), with slight modifications, continuously following the formation of NADPH at 340 nm, by using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD). The assay mixture contained in 1 ml final volume consisted of 50 mM triethanolamine (pH 7.6), 8 mM $MgSO_4$, 5 mM EDTA, 1.5 mM ATP, 0.2 mM NADP, 2 mg/ml glucose-6-phosphate dehydrogenase. The reaction was started by adding the substrate (0.4 mM glucose).

Determination of Serine/Threonine Protein Phosphatase Activity

Protein serine/threonine phosphatase (PSPs) activity was determined using *p*-nitrophenyl phosphate as a substrate. The substrate (4 mM) was dissolved in 25 mM Tris-HC1 buffer, pH 7.2, containing 5 mM dithiothreitol, 20 mM sodium-potassium DL-tartrate, and 0,1 mM Sodium orthovanadate. Tartrate and orthovanadate were added in order to inhibit protein tyrosine phosphatases, lysosomal acid phosphatases and non-specific phosphatases (Walton and Dixon 1993). The reaction was stopped with 0.1 M KOH and the released p-nitrophenolate ion was measured by reading the absorbance at 400 nm ($\varepsilon = 18{,}000$ M^{-1} cm^{-l}). The activity measured in these conditions was completely inhibited by 10 μM cantharidic acid, a specific and strong inhibitor of all PSPs (Knapp et al. 1998).

Statistics

Experiments were carried out in triplicate. For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide, and the data were expressed as mean ± SD. Statistical significance was determined using a Student's t test. A p value lower than 0.05 was considered statistically significant.

Results

In all the samples, the total protein content was analyzed and then used to normalize the measurements of enzymatic activities. The results show that the 1 × g control samples, both laser-treated and untreated, had a similar protein content. The protein content was reduced of about 40 % in the samples exposed to the RPM, but increased fivefold in samples exposed to the RPM and then treated with MLS laser (Fig. 1).

The HK activity, considered a marker of glycolytic metabolism (Grichko et al. 2000), did not show significant changes in 1 × g controls exposed to MLS laser, in comparison with untreated 1 × g controls. Myoblasts kept on the RPM showed a HK activity sevenfold higher than 1 × g controls, but in the samples exposed to modelled microgravity and then to MLS laser irradiation the enzyme activity strongly decreased (Fig. 2a). Also PK activity increased (30 %) in modelled microgravity and decreased (84 %) after the laser treatment (Fig. 2b). Serine/threonine protein phosphatase (PSPs)

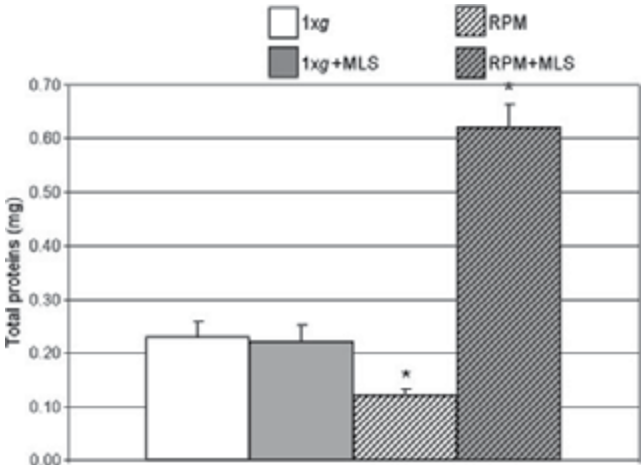

Fig. 1 Analysis of total protein content—1 × g control samples, both laser-treated and untreated, showed a similar protein content. It decreased significantly in the samples exposed to the RPM for 72 h, but strongly increased in samples exposed to the RPM and then treated with MLS laser. The symbol "*" indicates a p value lower than 0.05

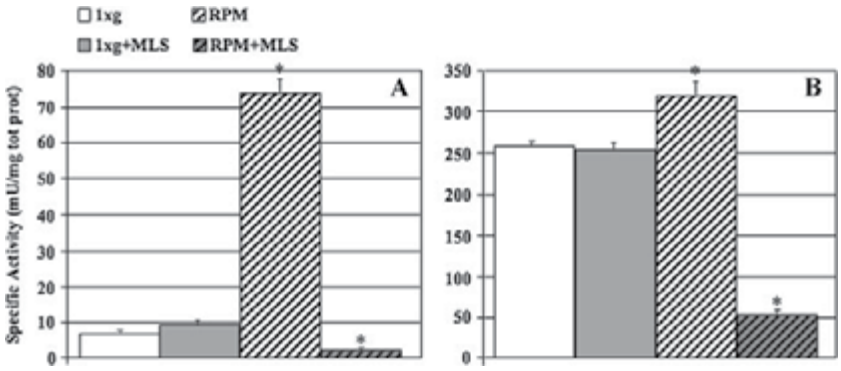

Fig. 2 **a** Hexokinase activity—The HK activity did not change significantly in the 1 × *g* controls exposed to MLS laser, in comparison with the untreated ones. Myoblasts exposed to modelled microgravity (RPM) showed an impressive increase in HK activity. In the samples exposed to modelled microgravity, and then to MLS laser irradiation, the enzyme activity decreased to values lower than 1 × *g* controls. **b** Pyruvate Kinase activity—Also PK activity increased (30 %) in modelled microgravity and decreased (84 %) after the laser treatment. No differences were observed between the 1 × *g* controls untreated and treated with MLS laser. The symbol "*" indicates a *p* value lower than 0.05

activity appeared more than doubled in laser-treated 1 × *g* controls, in comparison with the untreated ones. In C2C12 cells exposed to gravitational unloading PSPs activity dramatically fell down, but went up significantly in cells treated with MLS laser after the exposure to modelled microgravity conditions (Fig. 3).

The morphological analysis of the three major components of cytoskeleton, actin microfilaments, microtubules and intermediate filament network, which was performed by immuno-fluorescence microscopy, showed evident architectural alterations of all the three cytoskeletal structures examined in the samples kept in the RPM: in comparison with 1 × g controls, the actin expression in the cytoplasm decreased while stress fibers became more evident and microspikes with high actin expression appeared on the cell surface (Fig. 4c); the intermediate filaments lost the orderly perinuclear arrangement and concentrated at the center of the cell partially covering the nucleus (Fig. 4g), microtubules lost the usual radial distribution starting from the organization centre but formed a tangled network (Fig. 4k). No significant differences were found between laser-treated and untreated 1 × *g* controls (Fig. 4a, b, c, f, i, j). The samples exposed to modelled microgravity and then to the laser treatment showed a cytoskeletal structure restored and more similar to the 1 × g controls than the samples kept in RPM without subsequent laser treatment (Fig. 4d, h, l).

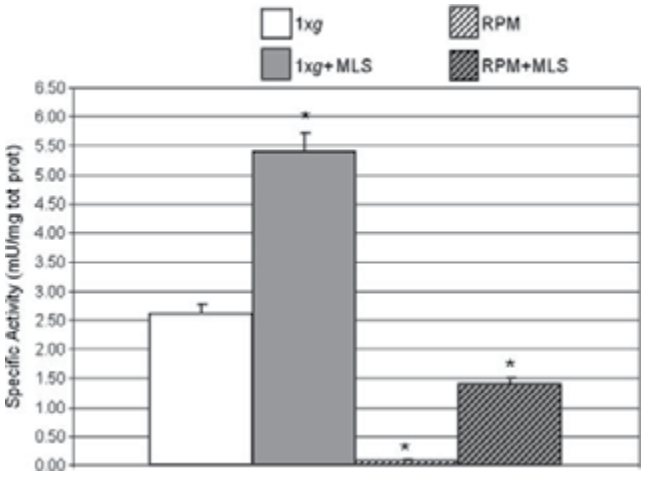

Fig. 3 Serine/threonine protein phosphatase activity—PSPs activity increased in laser-treated 1 × *g* controls, in comparison with the untreated ones. In C2C12 cells exposed to gravitational unloading, PSPs activity dramatically decreased, but increased significantly in cells treated with MLS laser after the exposure to modelled microgravity conditions. The symbol "*" indicates a *p* value lower than 0.05

Discussion

In literature, the data on protein content in muscle cells exposed to weightlessness are controversial and very difficult to compare because the authors used different times of exposure (from minutes to days), different models (myoblasts, muscle fibers, 2D cultures or 3D cultures), real or modelled microgravity and, in this last case, different devices for modelling microgravity (random positioning machine, rotating wall vessel). Likely, the different results depend on the different experimental conditions and models used.

The decrease in total protein content we observed in the samples exposed to modelled microgravity conditions (Fig. 1) could be due to many processes: an

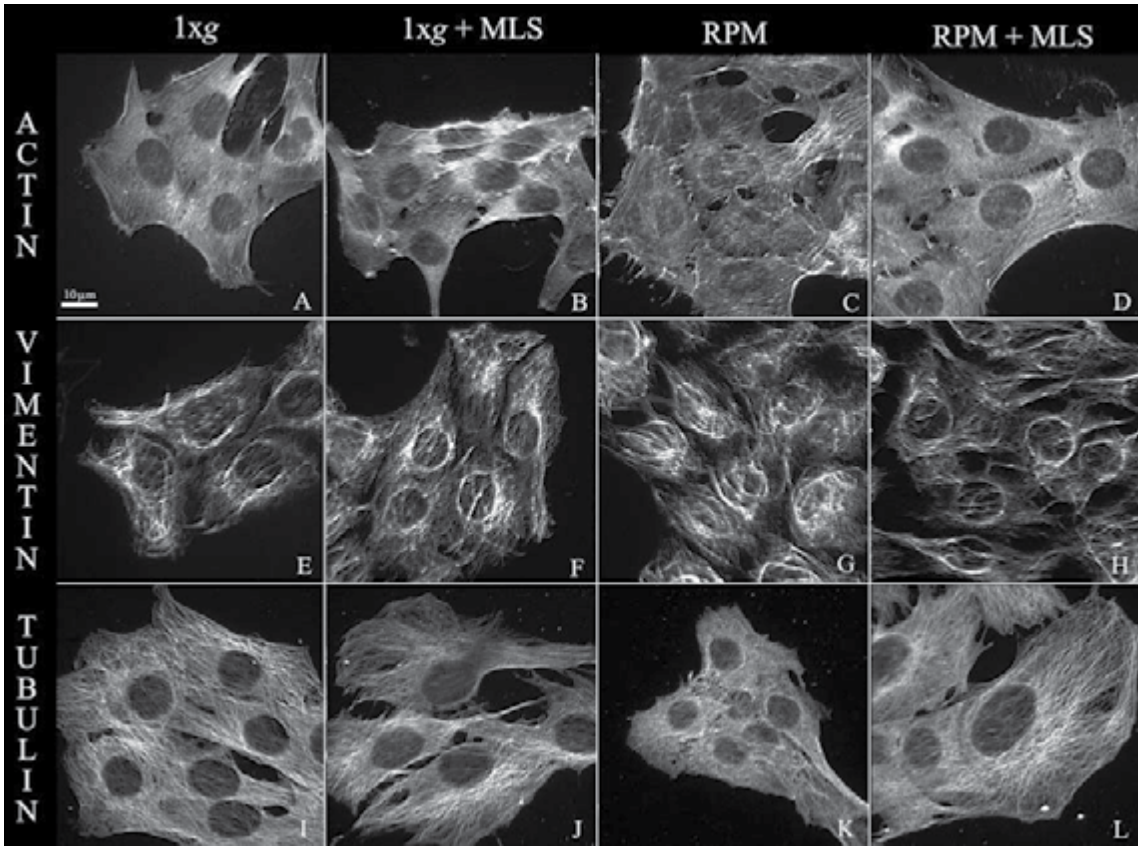

Fig. 4 Cytoskeleton components analyzed by immunofluorescence microscopy—Samples exposed to modelled microgravity (**c**, **g**, **k**), compared to 1 × *g* controls, showed evident architectural alterations of the three major cytoskeleton components: actin microfilaments, intermediate filaments and microtubules. Actin stress fibers became more evident and microspikes with high actin expression appeared on the cell surface (**c**); the intermediate filaments lost the orderly perinuclear arrangement and concentrated at the center of the cell partially covering the nucleus (**g**), microtubules lost the usual radial distribution starting from the organization centre but formed a tangled network (**k**). No significant differences were found between untreated and laser-treated 1 × *g* controls (**a**, **b**, **c**, **f**, **i**, **j**). The samples exposed to modelled microgravity and then to the laser treatment showed a cytoskeletal structure restored and similar to the 1 × *g* controls (**d**, **h**, **l**)

increase in apoptosis, which often occurs in cell cultures exposed to weightless conditions (Uva et al. 2002; Monici et al. 2006), a microgravity-induced decrease in myoblast proliferation, which has been recently found by other authors (Pache et al. 2010), altered protein synthesis/degradation (Moriggi et al. 2010), or a combination of these effects.

In agreement with data presented by other authors (Shimkus et al. 2011), preliminary experiments we performed did not reveal significant changes in proliferation and apoptosis induced by exposure to modelled microgravity. Some authors have found an increase (Slentz et al. 2001) or a decrease (Pache et al. 2010) in proliferation using different exposure times and conditions for simulating microgravity. Thus, in our experimental conditions, we are inclined to think that the decrease in total protein content depends on an altered protein synthesis/degradation.

As expected, a strong increase in HK activity was found in RPM-exposed cells and also PK activity increased (Fig. 2a and b). These results fit very well with data reported in literature: after spaceflights and bed rest, an upregulation of glycolytic enzymes, in particular HK and PK levels, has been described by several authors (Chi et al. 1992; Stump et al. 1997; Stein et al. 2002) and represents a clear sign of microgravity-induced metabolic impairment. In myoblasts exposed to modelled microgravity, besides the decrease in total protein content and increase in glycolytic enzyme levels, the PSPs activity dramatically fell down (Fig. 3).

Despite the crucial role PSPs have in signal transduction and biological functions, the effect of the gravitational conditions on their expression and activity has been relatively little studied. PSPs, by opposing the action of protein kinases, regulate protein phosphorilation and therefore control metabolism and basical

processes such as protein-protein interactions, gene transcription and translation, cell-cycle progression and apoptosis, cytoskeleton dynamics and cell movement (Berridge 2009).

Our results suggest that the dysregulation of HK (and other glycolytic enzymes) could be related to the impressive reduction of PSPs activity, since it has been recently demonstrated in skeletal muscles of freeze-tolerant frogs that HK can be dephosphorilated by PP1 and, in this form, displays lower substrate affinity and lower activity (Dieni and Storey 2011).

The deficit in PSPs activity could be also involved in the cytoskeletal alterations induced in myoblasts by weightlessness (Fig. 4c, g, k) and described also by other authors (Pache et al. 2010). In fact it is known that PSPs, and particularly PP1, are involved in actin and actomiosin reorganization, regulation of cell shape and cell adhesion (Cohen 2002; Ceulemans and Bollen 2004).

Finally, a low PSPs activity could have altered protein turnover (Ceulemans and Bollen 2004), thus affecting protein content.

MLS laser irradiation induced in $1 \times g$ controls a significant increase in PSPs activity (Fig. 3), confirming the results we obtained in preliminary studies on the effect of MLS emission on muscle cells, where we found an significant increase in PSPs expression, foremost the expression of PP1 (Monici et al. 2012). In the same samples, protein content, HK and PK activities did not change significantly.

In myoblast cultures previously exposed to microgravity, the laser treatment was able to restore, at least partially, the level of PSPs activity (Fig. 3). Moreover, protein content strongly increased (Fig. 1) and both HK and PK activities returned to levels comparable to those expressed by the $1 \times g$ controls, or even lower (Fig. 2a and b).

These results support the hypothesis that, in myoblasts exposed to modelled microgravity conditions, a relationship could exist between the downregulation of PSPs activity and alteration of metabolism markers. Moreover, as we hypothesized, in myoblasts previously kept in the RPM, MLS laser treatment, through the increase of PSPs activity, was able to reverse the metabolic alterations induced by modelled microgravity.

Our findings completely fit with the actual knowledge on the function of PSPs and, in particular, the role of PP1. In a recent review PP1 has been defined a "green" enzyme that promotes the rational use of energy and a reversal of the cell to a basal and/or energy-conserving state, the recycling of protein factors and the return to basal patterns of protein synthesis; in addition, PP1 plays a key role in the recovery from stress (Ceulemans and Bollen 2004).

The results obtained by analyzing cell morphology further confirm our hypothesis. In fact, as expected, C2C12 cells exposed to modelled microgravity showed an evident reorganization of cytoskeleton, with architectural features of the networks of microfilaments, microtubules and intermediate filaments very different from $1 \times g$ controls. Conversely, myoblasts treated with MLS laser after RPM exposure exhibited a cytoskeletal architecture very similar to that of the $1 \times g$ controls. This faster return to the basic morphological pattern fits very well with the widely recognized role of PSPs in cytoskeletal rearrangement.

To the best of our knowledge this is the first time that a decrease in PSPs activity in myoblasts exposed to weightlessness is described and the ability of IR laser radiation to reverse the effect is demonstrated.

In conclusion, the MLS laser treatment was able to restore the level of PSPs activity and to counteract increase in glycolytic enzymes activity and cytoskeletal alterations that occurred in C2C12 cells following the exposure to modelled microgravity.

While taking into account the limitation of the present data on myoblasts with respect to the interpretation for mature muscle fibers, however the results suggest that changes in PSPs activity could underlie some effects induced by microgravity in skeletal muscle cells. Moreover, the possibility of reversing the effects by laser treatment opens the way to considerations about the usefulness of laser therapy to favour muscle recovery in disuse atrophy.

The findings of this "in vitro" study represent only a preliminary step in exploring the effectiveness of laser therapy as a countermeasure to disuse atrophy. However, in our opinion, they provide original insights that encourage further research in this field.

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Effect of NIR laser therapy by MLS-MiS source against neuropathic pain in rats: *in vivo* and *ex vivo* analysis

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Neuropathic pain is characterized by an uncertain etiology and by a poor response to common therapies. The ineffectiveness and the frequent side effects of the drugs used to counteract neuropathic pain call for the discovery of new therapeutic strategies. Laser therapy proved to be effective for reducing pain sensitivity thus improving the quality of life. However, its application parameters and efficacy in chronic pain must be further analyzed. We investigated the pain relieving and protective effect of Photobiomodulation Therapy in a rat model of compressive mononeuropathy induced by Chronic Constriction Injury of the sciatic nerve (CCI). Laser (MLS-MiS) applications started 7 days after surgery and were performed ten times over a three week period showing a reduction in mechanical hypersensitivity and spontaneous pain that started from the first laser treatment until the end of the experiment. The *ex vivo* analysis highlighted the protective role of laser through the myelin sheath recovery in the sciatic nerve, inhibition of iNOS expression and enhancement of EAAT-2 levels in the spinal cord. In conclusion, this study supports laser treatment as a future therapeutic strategy in patients suffering from neuropathic pain induced by trauma.

Neuropathic pain is the result of damage (due to injury or disease) to the nervous system (including nerves), spinal cord and other central nervous system regions[1–3]. Neuropathy patients suffer from spontaneous pain, allodynia (pain response to normally innocuous stimuli) and hyperalgesia (aggravated pain evoked by noxious stimuli) that interferes with their quality of life[4–6]. Several experimental models have been developed to better understand neuropathy. The chronic constriction injury (CCI) model, developed by Bennett and Xie[7], is a widely used model of mononeuropathy that replicates in rats most of the symptoms occurring in patients[7–10].

Currently the most common way to treat pain is the administration of pain relief medications, although they have proved to be effective in only 30% of neuropathy patients which makes the research for new and effective treatments an ongoing challenge[11–13].

The history of investigation and clinical use of laser therapy in medicine goes back to the late 1960s[14]. Since then, laser irradiation has been acknowledged as one of the most important non-pharmacological therapies. Nowadays laser therapy use has become increasingly widespread because it is a non-invasive approach with few contraindications, rare side effects and relatively low costs, thus well accepted by patients[15,16]. The literature on laser therapy action mechanisms is extremely wide although with controversial findings that are difficult to compare and interpret, due to the very different experimental conditions (in particular the type of laser source and treatment parameters, that are wavelength, power, fluence, exposure time, etc…) used in the studies. However, through the years laser therapy has been has demonstrated its effectiveness in treating a number of different

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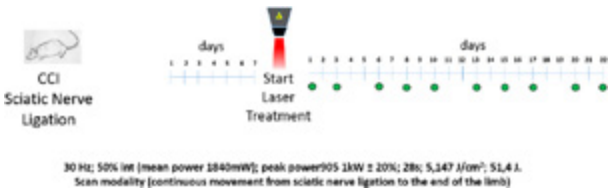

Figure 1. Laser treatment protocol with time schedule and parameters used.

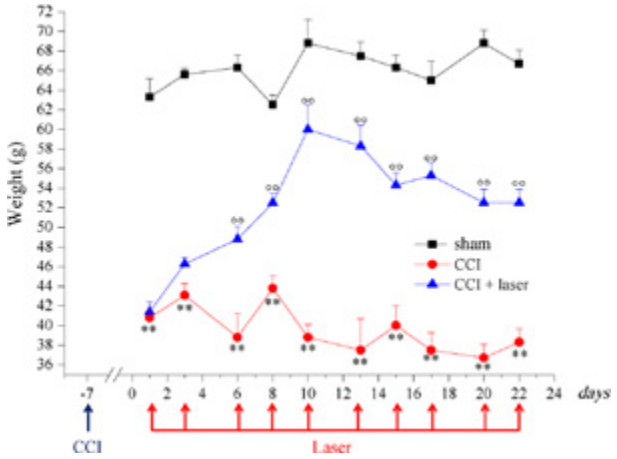

Figure 2. Monolateral neuropathy model induced by CCI. Sciatic nerve ligation was performed 7 days before the beginning of the test (day −7). Laser treatment [28 s, 30 Hz; 50% int (mean power 1840 mW); peak power$_{905}$ 1 kW ± 20%; 5,147 J/cm^2; 51,4 J] was applied on days 1; 3; 6; 8; 10; 11; 13; 15; 17; 20; 22 at 0 min and 30 min after laser application. The response to a noxious mechanical stimulus was measured by Paw pressure test. Values reported in the graph are referred to measurements conducted before treatments. Each value represents the mean ± S.E.M of 6 rats per group performed in two different experimental sets. **P < 0.01 vs sham group; °°P < 0.01 vs CCI group.

pathological conditions[17–24]. The possibility to apply laser therapy in so many different pathological states depends on the effects that radiation has on important biological processes.

A number of studies in literature have reported that laser radiation is effective in improving cell energy metabolism through ATP synthesis increase[25–27]. Further insights into the action mechanisms underlying enhanced cell energy metabolism were provided in a proteomic study, carried out on myoblasts exposed to near infrared (NIR) laser radiation (808 and 905 nm), where an increase in ATP-binding proteins and Protein Phosphatase 1 (PP1) was observed[28]. The same study, showed that laser irradiation induced a significant increase in NLRP10, an anti-inflammatory protein that inhibits the production of interleukin 1β[28]. The analgesic effect is also due to other mechanisms acting on the production of anti-nociceptive substances (endorphins), peripheral nerve conduction and the transmission of nociceptive stimuli, as demonstrated by the rapid analgesic effect evoked by laser radiation in animal models of persistent pain[18,29–31].

Preliminary studies showed that the anti-hypersensitivity effect and its persistence depended on treatment protocols and parameters (irradiation mode, treatment frequency, source wavelength and power, pulse frequency)[31].

The aim of the present study was to investigate the effectiveness of a high power, dual wavelength NIR laser source (Multiwavelength Locked System laser, MLS-MiS) in producing a persistent anti-hypersensitivity effect in CCI-induced neuropathy caused by compressive damage in the rat. The laser therapy action mechanism were also assessed with *ex vivo* evaluations of the central and the peripheral nervous system aimed to highlight the regeneration of the sciatic nerve and the reduction of the inflammatory processes in the spinal cord.

Results

Effect of laser treatments on CCI-induced hypersensitivity. Behavioural measurements were performed to evaluate the anti-hypersensitivity effect of repeated laser treatments on CCI-induced peripheral mononeuropathy in the rat. Laser treatment started one week after surgery and consisted of 10 sessions every other day, until the 3rd week (Fig. 1). The evaluation of hypersensitivity (Paw pressure test) was performed immediately before and 30 min after each laser application. Figure 2 shows the mean values monitored in the 3 groups of animals (sham, CCI, CCI + laser) before each of the 10 laser sessions.

The measurement performed before the first laser application (day 1) demonstrated that sciatic nerve ligation decrease the response to a mechanical noxious stimulus (Paw pressure test) from a value of 63.3 ± 1.9 g (sham

Laser applications	Weight (g)	
	before treatment	after treatment
	0 min	30 min
1	41.8 ± 0.6**	49.6 ± 2.6
2	46.3 ± 0.7**	50.3 ± 3.6
3	48.8 ± 1.3*	50.0 ± 1.0
4	52.5 ± 1.0°°	51.3 ± 1.2
5	60.0 ± 2.5°°	58.3 ± 2.9
6	58.3 ± 2.1°°	55.0 ± 2.5
7	54.3 ± 1.3°°	52.5 ± 2.5
8	55.3 ± 1.3°°	52.5 ± 1.4
9	52.5 ± 1.4°°	53.8 ± 2.4
10	52.5 ± 1.4°°	56.3 ± 1.3

Table 1. Response to a mechanical noxious stimulus of CCI + laser treated animals, Paw pressure test. Paw pressure test performed on CCI + laser treated animals, 30 min after the daily laser treatment. Each value represents the mean ± s.e.m of 6 rats performed in two different experimental sets.

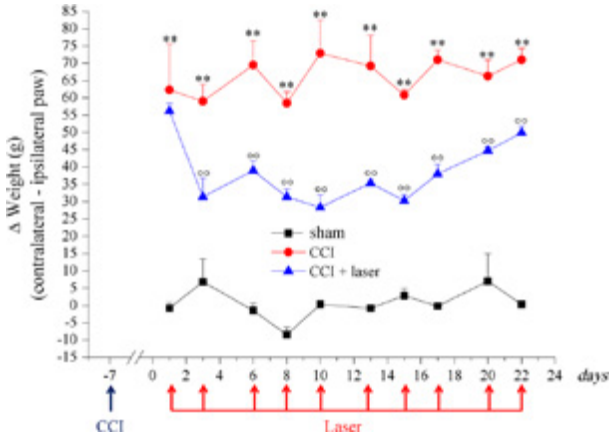

Figure 3. Monolateral neuropathy model induced by CCI. Sciatic nerve ligation was performed 7 days before the beginning of the test (day −7). Laser treatment [28 s, 30 Hz; 50% int (mean power 1840 mW); peak $power_{905}$ 1 kW ± 20%; 5,147 J/cm^2; 51,4 J] was applied on days 1; 3; 6; 8; 10; 11; 13; 15; 17; 20; 22 at 0 min and 30 min after laser application. The hind limb weight bearing alteration was measured by Incapacitance test. Values reported in the graph are referred to measurements conducted before treatments. Each value represents the mean ± S.E.M of 6 rats per group performed in two different experimental sets. **P < 0.01 vs sham group; °°P < 0.01 vs CCI group.

animals group) to 40.8 ± 0.7 g (CCI and CCI + laser groups). In the CCI group this condition lingered for 3 weeks, until the end of the experiment (day 22). On the contrary, in the CCI + laser group two laser applications were enough to significantly increase the weight tolerated on the ipsilateral paw compared to the untreated CCI animals (48.8 ± 1.3 g and 38.8 ± 1.4 g, respectively, on day 6). The higher anti-hypersensitivity effect was recorded after four laser applications (day 10), with a value of 60.0 ± 2.5 g on the ipsilateral paw. Subsequent laser applications did not increase the paw threshold that remained stable (about 55 g) until the end of the experiment (day 22). In each laser session, the measurement performed 30 min after laser irradiation did not show any significant change in comparison to the pre-irradiation measurement (Table 1).

Monolateral pain as CCI induced alteration of hind limb weight bearing has been shown by the Incapacitance test (Fig. 3). Also in this case, measurements were performed immediately before each laser application and 30 min later. The values monitored before each laser session are reported in Fig. 3. Before the first laser treatment (day 1, 7 days after surgery), the difference between the weight burdened on the contralateral and the ipsilateral paw (Δ g) was significantly increased in both CCI and CCI + laser groups (about 60 g) compared to the sham group (−0.8 ± 1.7 g). In the CCI group this difference remained constant until the end of the experiment (day 22), while in the CCI + laser group the gap has been reduced by about 50% on the first laser application (31.3 ± 5.5 g, day 3). The pain relieving effect remained stable throughout the following laser treatments (days 6–16) to then slightly decrease from day 20. The measurements performed 30 min after each laser session did not show significant changes compared to the pre-irradiation measurements (Table 2).

Laser applications	Δ Weight (g)	
	before treatment	after treatment
	0 min	30 min
1	56.2 ± 2.3**	47.9 ± 5.0
2	31.3 ± 5.5°°	27.9 ± 5.0
3	39.0 ± 2.7°°	33.3 ± 4.6
4	31.3 ± 2.4°°	30.3 ± 7.0
5	28.3 ± 3.6°°	33.9 ± 3.9
6	35.3 ± 1.0°°	32.5 ± 1.5
7	30.2 ± 1.8°°	35.8 ± 2.5
8	38.0 ± 2.7°°	47.8 ± 1.7
9	44.7 ± 1.2°°	48.4 ± 3.1
10	49.9 ± 1.9°°	51.2 ± 0.7

Table 2. Hind limb weight bearing alterations of CCI + laser treated animals, Incapacitance test. Incapacitance test performed on CCI + laser treated animals, 30 min after the daily laser treatment. Each value represents the mean ± s.e.m of 6 rats performed in two different experimental sets.

Effect of laser treatments on CCI-induced sciatic nerve damage: histological evaluation. The morphometric assessment by Luxol Fast Blue (LFB) tissue staining allowed to characterize the laser-dependent effect in comparison to the CCI-dependent alteration in the myelin sheath thickness. The histological examination of the specimens revealed a normal sciatic nerve appearance in the sham group with a regular distribution of small and large diameter nerve fibers as well as a normal proportion between myelin sheath thickness and fiber diameter (Fig. 4, sham). As expected, the CCI group presented a wide distribution of very thinly myelinated nerve fibers (Fig. 4, CCI – black arrows), Wallerian degeneration (Fig. 4, CCI – black arrowhead) and unmyelinated fibers in the tissue sections of the sciatic nerve 900 μm proximal to the ligation site, compared to sham rats. In contrast, nerves of animals treated with laser radiation (CCI + laser treated group) showed a remarkable myelin regeneration, as demonstrated by the presence of a greater number of nerve fibers that were surrounded by much more myelin compared with the CCI group (Fig. 4, CCI + laser). Table 3 shows the measurements of fiber and axonal diameters and myelin thickness. Laser treatment significantly increased (°°$P < 0.01$ vs CCI) the myelin thickness in comparison to CCI animals (2.24 ± 0.18 *vs* 1.81 ± 0.20) whereas fiber and axonal diameter measurements did not reveal any significant laser-dependent improvement.

Based on the results described above, to further investigate the effectiveness of NIR laser radiation in nerve protection and myelin sheath regeneration, Myelin Basic Protein (MBP) expression was evaluated by immunocytochemistry (Fig. 5). MBP is a major constituent of the myelin sheath produced by Schwann cells in the peripheral nervous system. On day 22 (end of laser treatments, 30 days post-injury), MBP was significantly lower in the CCI group compared to the sham one (*$P < 0.01$ vs sham). Laser treatment partially restored the MBP in the sciatic nerve of CCI + laser group (°$P < 0.01$ vs CCI). These data further confirm the laser-dependent neuroprotection, in particular the restoration of the myelin sheet, revealed by morphometric analysis.

Effect of laser treatments on inflammatory markers. On day 22 (end of laser applications, 30 days post-injury), the nervous tissue (spinal cord) was evaluated for the expression of the glutamic acid transporter EAAT-2 and the inflammatory markers iNOS, COX-2 and mPGES-1. Results showed that while EAAT-2 was scarcely expressed in the spinal cord of sham animals, the CCI group (laser-untreated) presented an increased expression, that was even higher in laser treated animals (CCI + laser group) (Fig. 6A).

A panel of inflammatory markers was then evaluated as the inducible isoform of NOS (iNOS) and the prostanoid pathway key enzymes COX-2 and mPGES-1. While COX-2 and mPGES-1 were not detected under any experimental condition (data not shown), the CCI procedure induced an increase in iNOS expression, whose levels were significantly blunted by laser application, with value even below the sham group (Fig. 6B).

Discussion

Our previous studies showed the effectiveness of NIR laser therapy in reducing CCI-induced pain in the rat[32]. A remarkable analgesic effect, whose peaked at about 30 min after laser treatment, was obtained with a protocol consisting in the irradiation of two points, the first one directly located on sciatic nerve ligation and the other one on lateral side of the calcaneus (paw joint). However, the analgesia decreased rapidly (about 2 hours later). In further studies, a more persistent analgesic effect was achieved when the irradiation of the two fixed points was followed by a scan on the whole leg, but even in this case the effect vanished 24 hours later[31].

Based on these evidences, in the present study a new protocol was designed and tested to obtain a long-lasting anti-hypersensitivity and protective effect through anti-inflammatory action and repair mechanisms. Results showed that laser treatment, performed by scans of the entire leg with the synchronized emission of a NIR, dual wavelength, high power source (MLS-MiS), was able to control pain and inhibit the progression of a persistent painful condition. The loose ligation of the sciatic nerve induces a damage characterized by painful sensations correlated with overt tissue alterations. As previously reported, CCI model elicits a pain syndrome characterized by mechanical and thermal hyperalgesia that begins about 3 days after nerve injury and reaches a plateau from 7 up to 30 days[33]. Laser treatment over these 3 weeks (10 sessions, every other day) counteracted the development of mechanical hyperalgesia already after two applications. The maximum anti-hyperalgesic effectiveness

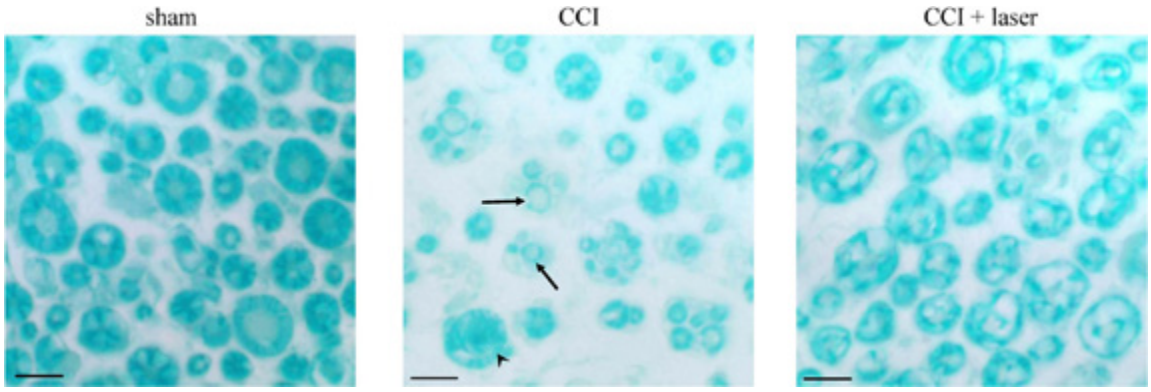

Figure 4. Luxol Fast Blue staining. Representative micrographs of sciatic nerve axons in a sham, CCI and CCI + laser groups showing a partial laser-dependent neuroprotection of myelin thickness. Original magnification 400 X. Scale bar = 20 μm.

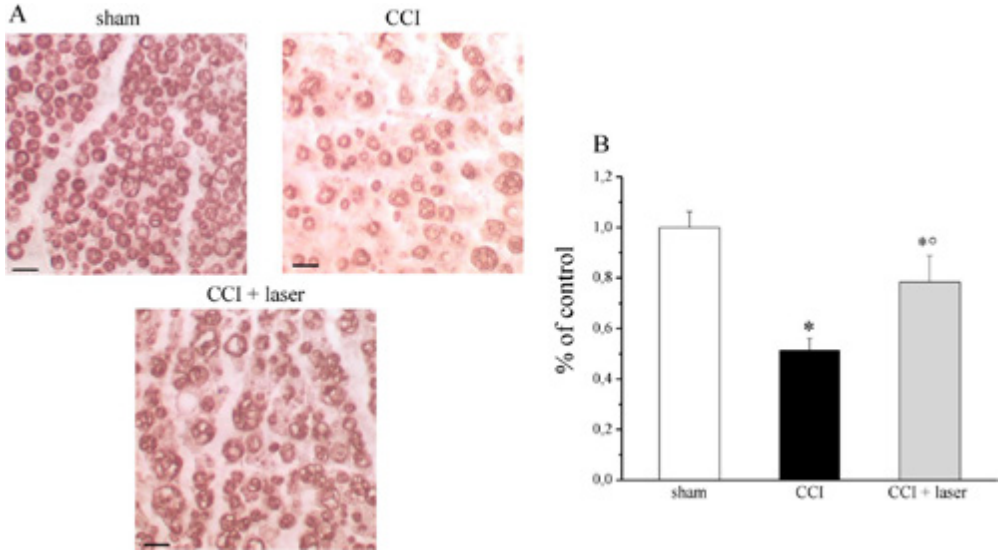

Figure 5. Myelin Basic Protein (MBP) expression. A, Protein expression of MBP was evaluated by immunohystochemistry in each experimental group. CCI group and CCI + laser group were compared to each other and with sham group. Original magnification 400X. Scale bar = 20 μm. B, AEC intensity was calculated by the integrated density of pixels for MBP. Control condition was arbitrarily set as 100% and results are expressed as mean ± S.E.M of 6 rats per group. Results are representative of at least three independent immunohistochemistry evaluations. *$P < 0.05$ vs sham group and °$P < 0.01$ vs CCI group. Each experimental point was performed in triplicate. Pictures are representative of fifteen field captured for each experimental point. *$P < 0.01$ vs sham group; °$P < 0.01$ vs CCI group.

was reached with 5 sessions, then the effect remained more or less stable until the end of the experiment. It is noteworthy that the measurements performed 30 min after laser irradiation did not show significant differences compared to the pre-treatment ones. While, over the first 5 sessions, an evident increase in the pain threshold was recorded between each before-irradiation measurement and the measurements performed before and after the previous laser session (48 h before). This means that the protocol used in this study does not induce an immediate analgesic effect, but rather a biological response rising more slowly but lasting longer over time.

The treatment protocol applied in this study was also able to reduce postural unbalance, a feature of neuropathy progression measured by hind limb weight bearing alterations. This measurement, in particular, may assess the somatosensory component of mononeuropathy highlighting spontaneous non evoked pain[34]. Laser treated animals showed a halving of postural unbalance measurement from the first treatment and this effect remained constant until the end of the laser sessions, as the Incapacitance test showed. Also in this case, the measurements performed 30 min after each laser irradiation did not show significant differences compared to the pre-treatment

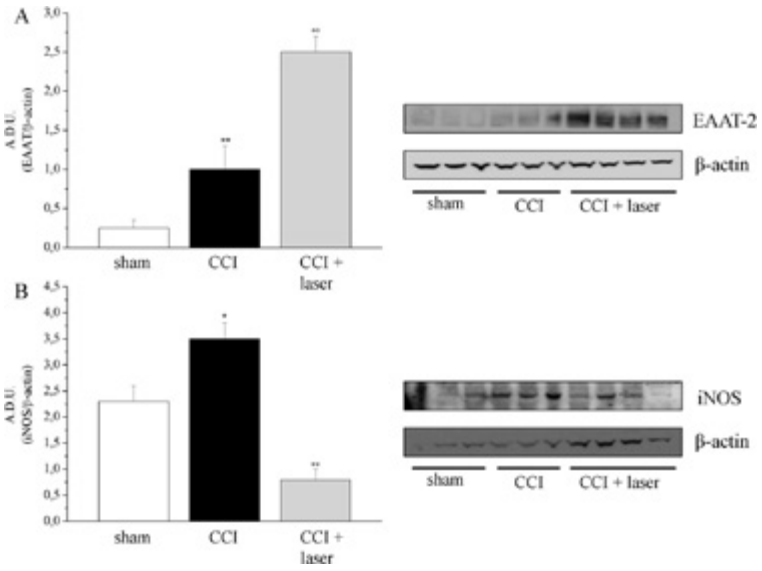

Figure 6. Western blot analysis of inflammatory markers. Spinal cords were isolated at the end of the experiment and proteins were run on SDS-PAGE. Proteins transferred on nitrocellulose membranes were then labelled with primary antibodies against EAAT-2 (panel A) and iNOS (panel B). β-actin normalization was performed f each sample. The graphs represent the means ± S.E.M of 6 rats per group. *P < 0.05 and **P < 0.01 vs sham group; °°P < 0.01 vs CCI group.

	Fiber diameter	Axonal diameter	Myelin thickness
sham	7.71 ± 0.32	3.17 ± 0.05	2.27 ± 0.16
CCI	6.33 ± 0.68**	2.72 ± 0.30**	1.81 ± 0.20**
CCI + laser	6.61 ± 0.49**	2.13 ± 0.18**	2.24 ± 0.18**°°

Table 3. Morphometric analysis of sciatic nerves. Morphometric analysis of sciatic nerves 29 days post-surgery showing the measurement of fiber and axonal diameter and of myelin thickness. Five µm sections stained with LFB were photographed at 100X magnification. The myelin thickness in photomicrographs taken from randomly selected fields was counted (6 rats/group) and analyzed using Origin 9.0 statistical software. The results showed a significant decrease in axonal and fiber diameter as well as in myelin thickness in CCI compared to sham group (**$P < 0.01$). Laser-treated group showed a significant increase in myelin thickness compared to CCI group (°°$P < 0.01$).

ones, confirming the paw pressure test results. The *ex vivo* analysis also highlighted a protective role of laser treatment in the central and peripheral nervous system.

In agreement with previous results[33,35,36], histology, immunohistochemistry and levels of inflammatory markers (evaluated by western blot) showed that CCI-induced morphometric alterations of the sciatic nerve that dramatically affect the proximal distal from the injury. Besides, CCI mediated nerve architecture derangement is accompanied by local inflammatory reaction response, which include oedema, infiltration of hematogenous immune cells and induction of various soluble factors like cytokines, chemokines and small signalling molecules as nitric oxide. These findings were further confirmed by the increased expression of iNOS detected in spinal cord samples. Laser treatment significantly prevented the reduction in myelin sheath thickness and hindered myelin degeneration, as highlighted by LFB staining and MBP immunohistochemistry. This result is in agreement with data reported by other authors, showing that NIR laser therapy was able to promote nerve fiber regeneration and improve the quality of myelin layers in a rabbit model of peripheral nerve injury[37]. Moreover, previous data obtained using sources with emission 808 nm and 904 nm, the same wavelengths used in the present study, demonstrated that these NIR radiations enhanced nerve repair after end-to-side neurorrhaphy of the median nerve in a rat model[38] and increased HNRNPK expression in cell culture[28]. HNRNPK is a member of the heterogeneous nuclear ribonucleoproteins (hnRNPs) subfamily known to be required for axonogenesis during development and several of its RNA targets are under strong post-transcriptional control during the regeneration process[39].

The anti-inflammatory effect elicited by laser application in the CCI model was clearly demonstrated by the significant reduction in iNOS expression in the spinal cord. Also this result is consistent with previous studies[28] that highlighted the increase of NLRP10 protein, an inflammasome inhibitor induced by laser radiation with 808 nm and 904 nm wavelengths.

In the central nervous system, the excitatory amino acid transporters (EAATs) remove glutamate from the synaptic cleft and extrasynaptic sites via glutamate reuptake into glial cells and neurons, allowing to keep its levels low and to terminate the synaptic transmission. Conditions that increase the levels of EAAT-2 expression, may avoid an excess of glutamate capable of triggering a series of biochemical cascades associated to excitotoxicity and neuronal damage[40]. The findings of this study show that repeated laser treatments were able to strongly increase EAAT-2 levels, corroborating the anti-inflammatory and beneficial effects of the therapy on nervous and glial cells.

In conclusion, the results of this study indicate that NIR laser therapy carried out with MLS-MiS laser source and suitable protocols is able to control pain and prevent alterations of the nervous system induced by nerve injury.

While our previous studies[31], reported a fast but not lasting analgesic effect was obtained with point by point irradiation, the protocol used in this study activated a slower but longer lasting biological response that counteracted hyperalgesia through three different mechanisms: (1) anti-inflammarory effect via inhibition of iNOS expression; (2) repair effect through preservation/restoration of myelin sheath; (3) protective effect on central nervous system via enhancement of EAAT-2 levels. The collected data present a preclinical evaluation for a future therapeutic application of laser in patients suffering from neuropathic pain induced by trauma. The protocols can be further studied in order to exploit both the rapid analgesic effect that can be obtained by trigger point irradiation and the more persistent protective effect, with direct action on the cause of pain.

Materials and Methods

Animals. In all the experiments described below, male Sprague–Dawley rats (Envigo, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used. Animals were housed in a Laboratory Animal Facility (CeSAL, Centro Stabulazione Animali da Laboratorio, University of Florence) and used one week after their arrival. Four rats were housed per cage (size $26 \times 41\ cm^2$), fed with standard laboratory diet and tap water ad libitum, kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines[41]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

CCI-induced peripheral mononeuropathy. Neuropathy was induced according to the procedure described by[7]. Briefly, rats were anaesthetized with 2% isoflurane. Under aseptic conditions, the right (ipsilateral) common sciatic nerve was exposed by blunt dissection at the level of the mid thigh. Proximal to the trifurcation, the nerve was carefully freed of the adhering tissue from the surrounding connective tissue, and 4 chromic catgut ligatures (4-0, Ethicon, Norderstedt, Germany) were tied loosely around the nerve with about 1-mm spacing between ligatures. After haemostasis was confirmed, the incision was closed in layers. The animals were allowed to recover from surgery and then housed one per cage with free access to water and standard laboratory chow. Another group of rats were subjected to sham surgery in which the sciatic nerve was only exposed but not ligated. Laser treatment started 7 days after surgery.

Laser treatment and study design. Treatment were performed with a Multiwave Locked System laser (MLS-MiS, ASA S.r.l., Vicenza, Italy), a class IV NIR laser with two synchronized sources (laser diodes): the first one is a pulsed laser diode emitting at 905 nm wavelength, with peak power from 140 W ± 20% to 1 kW ± 20% and pulse frequency varying in the range 1–2000 Hz; the second laser diode emitting at 808 nm wavelength can operate in continuous (max power 6 W ± 20%) or frequenced (repetition rate 1–2000 Hz, 50% duty cycle) mode. The two laser beams work simultaneously, synchronously and the propagation axes are coincident.

Seven days after the sciatic nerve ligation, animals were randomly distributed into three groups:

(a) **Sham** (n = 6), animals subjected to sham surgery in which the sciatic nerve was only exposed but not ligated.
(b) **CCI** (n = 6), animals subjected to the ligation of sciatic nerve, untreated with laser;
(c) **CCI + laser** (n = 6), animals subjected to the ligation of sciatic nerve, treated with laser. The treatment was performed 3 times a week over a 3 week period (days 1; 3; 6; 8; 10; 11; 13; 15; 17; 20; 22) for a total of 10 applications (Fig. 1) and consisted in a limb scan with the handpiece constantly moved over the treatment area. Irradiation was performed for 28 s with the following parameters: 30 Hz; 50% int (mean power 1840 mW); peak $power_{905}$ 1 kW ± 20%; 5,147 J/cm^2; 51,4 J.

Paw pressure test. The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy) according to the method described by[42]. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical mechanical probe. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. These limits assured a more precise determination of mechanical withdrawal threshold in experiments aimed to determine the effect of treatments. An arbitrary cut-off

value of 100 g was adopted. Laser treatment started 7 days after injury (performed on days 1; 3; 6; 8; 10; 11; 13; 15; 17; 20; 22) and paw pressure test was conducted on the same days immediately before and 30 min after laser treatment. The data were collected by an observer who was blinded to the protocol.

Incapacitance test. Weight bearing changes were measured using an incapacitance apparatus (Linton Instrumentation, UK) detecting changes in postural equilibrium after a hind limb injury[43]. Rats were trained to stand on their hind paws in a box with an inclined plane (65° from horizontal). The box was placed above the incapacitance apparatus. This allowed us to independently measure the weight that the animal applied on each hind limb. The value considered for each animal was the mean of 5 consecutive measurements. In the absence of hind limb injury, rats applied an equal weight on both hind limbs, indicating a postural equilibrium, whereas an unequal distribution of the weight on hind limbs indicated a monolateral decreased pain threshold. Data are expressed as the difference between the weight applied on the limb contralateral to the injury and the weight applied on the ipsilateral one[44]. This behavioural measurement was performed on days 1; 3; 6; 8; 10; 11; 13; 15; 17; 20; 22 immediately before and 30 min after laser treatment. The data were collected by an observer who was blinded to the protocol.

Tissue explants. On day 22, after the behavioural measurements, animals were sacrificed and the ipsilateral sciatic nerves were explanted. As previously reported, the portion containing the ligature was eliminated and a distance of 900 μm proximal to the ligation was chosen as optimal for evaluating the effect of laser treatment[9]. Contralateral nerves were also dissected, and equivalent portions were collected. After fixation in 4% buffered neutral formalin solution, the tissue block was embedded in paraffin, then cut in a microtome to 5 μm thickness and mounted on positively charged slides. The spinal cord of each animal was also collected and frozen in N_2 for western blot analysis.

Luxol fast blue staininig. To perform the Luxol Fast Blue (LFB) staining, sections were immersed overnight in 0.1% LFB solution at 56–60 °C. After washing, differentiation was initiated by immersion in 0.05% aqueous lithium carbonate for 15 s followed by multiple immersions in fresh 70% ethanol, until white matter could be distinguished and nuclei decolorized. After washing, sections were immersed in 0.8% periodic acid for 10 min and then rinsed in distilled water. Finally, sections were incubated with Schiff's reagent for 20 min and rinsed in distilled water for 15 min[45].

The sections were semiquantified by an arbitrary score starting from 1, mild infiltrate and oedema, up to 10, severe infiltrate and widespread oedema. The morphometric analysis was conducted as previously reported[33]. Sections were analyzed under light microscopy (100 × magnification). At least 6 randomly distributed 20X fields within the transversal section of sciatic nerve were captured for each section. Images were examined using an Olympus BX40 microscope (Olympus, Milan, Italy) and photographed using a digital camera Olympus DP50 (Olympus, Milan, Italy).

Myelin basic protein (MBP) immunohistochemistry. Slides were deparaffinized with xylene and rehydrated in ascending ethanol. Heat-induced epitope retrieval was performed for 3 min in sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). After extensive washing in TBS (PBS + 0.025% Triton X-100), endogenous peroxidase was hindered with 0.3% H_2O_2 in 0.3% methanol for 15 min. Sections were then blocked by incubation with Ultra V block (Thermoscientific, Milan, Italy) for 10 min. MBP was detected using a mouse anti-MBP antibody (Chemicon, Milan, Italy) diluted 1:100 at 4 °C overnight. After washing in TBS, sections were treated with an anti-mouse HRP-conjugated secondary antibody (1:1000, Invitrogen, Milan, USA) for 1 h at room temperature. Development with 3-amino-9-ethylcarbazole (AEC) chromogen (BioOptica, Milan, Italy) was performed for 10 min at room temperature following the manufacturer's instructions. After coverslipping, protein expression was determined by image analysis of the slides on a Zeiss Axioimager microscope (Carl Zeiss; Jena, Germany) at 40 × magnification.

Western blot of inflammatory markers in spinal cords. Protein extraction from spinal cords started with disruption and homogenization using the TissueLyser II (#85300 Qiagen). Samples were lysed on ice with CelLytic™ MT Cell Lysis Reagent supplemented with 2 mM Na_3VO_4 and 1x Protease inhibitor cocktail for mammalian cells (Sigma Aldrich). Tissue lysates were centrifuged at 16000 × g for 20 minutes at 4 °C and the supernatants were then collected. Protein concentration was determined using the BCA protein assay kit (#23227 ThermoFisher Scientific)[46]. Electrophoresis (50 μg of protein/sample) was carried out in 4–12% Bis-Tris Gels (Life Technologies, Carlsbad, CA, USA). Proteins were then blotted onto nitrocellulose membranes, incubated overnight with primary antibodies [anti-EAAT2 (ab41621; dilution 1:1000) and anti-iNOS (ab49999, dilution 1:1000) purchased from Abcam, (Cambridge, UK); anti-COX-2 (AM05213PU-N, dilution 1:1000) was from Origene (Rockville, MD, USA); anti-mPGES-1 (Item No. 160140, dilution 1:200) was from Cayman Chemical (Ann Arbor, MI, USA)] and then detected by enhanced chemiluminescence system (BioRad, Hercules, CA, USA). Results were normalized to those obtained by using an antibody against β-actin (purchased from Merck KGaA, Darmstadt, Germany) diluted 1:10000[47].

Statistical analysis. Behavioural measurements were executed on 6 rats per group (Sham, CCI, CCI + laser) performed in two different experimental sets. Measurements were taken in duplicate at least 1 min apart, the responses of both left and right paws were measured. Morphometric and immunohistochemical analyses were performed on 6 rats per group, evaluating four to five different sections of sciatic nerve per animal. Comparisons were carried out using Mann-Whitney nonparametric tests[48]. In all cases, the investigator was blind to the experimental status of each animal. Slides from control and experimental groups were labeled with numbers so that the person performing the image analysis was blinded as to the experimental group. In addition, all images were

captured and analyzed by an investigator other than the one who performed measures to avoid possible bias. Results were expressed as mean (S.E.M) with One-Way analysis of variance (ANOVA). A Bonferroni's significant difference procedure was used as a *post hoc* comparison. Data were analyzed using the "Origin 9.0" software (OriginLab, Northampton, MA, USA). Differences were considered significant at a $P < 0.05$.

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Author Contributions

The authors' contributions are as follows: L. Di Cesare Mannelli, C. Ghelardini and M. Monici designed the research, L. Micheli performed the *in vivo* experiments and wrote the manuscript, A Pacini and J.J.V. Branca performed the *ex vivo* analysis, L. Morbidelli and V. Ciccone performed the western blots and F. Cialdai and E. Lucarini performed the laser treatments. F. Cialdai also drew all the components of the Fig. 1 that represents the laser treatment protocol with time schedule and parameters used.

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Effect of NIR Laser Therapy by MLS-MiS Source on Fibroblast Activation by Inflammatory Cytokines in Relation to Wound Healing

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Abstract: The fine control of inflammation following injury avoids fibrotic scars or impaired wounds. Due to side effects by anti-inflammatory drugs, the research is continuously active to define alternative therapies. Among them, physical countermeasures such as photobiomodulation therapy (PBMT) are considered effective and safe. To study the cellular and molecular events associated with the anti-inflammatory activity of PBMT by a dual-wavelength NIR laser source, human dermal fibroblasts were exposed to a mix of inflammatory cytokines (IL-1β and TNF-α) followed by laser treatment once a day for three days. Inducible inflammatory key enzymatic pathways, as iNOS and COX-2/mPGES-1/PGE2, were upregulated by the cytokine mix while PBMT reverted their levels and activities. The same behavior was observed with the proangiogenic factor vascular endothelial growth factor (VEGF), involved in neovascularization of granulation tissue. From a molecular point of view, PBMT retained NF-kB cytoplasmatic localization. According to a change in cell morphology, differences in expression and distribution of fundamental cytoskeletal proteins were observed following treatments. Tubulin, F-actin, and α-SMA changed their organization upon cytokine stimulation, while PBMT reestablished the basal localization. Cytoskeletal rearrangements occurring after inflammatory stimuli were correlated with reorganization of membrane α5β1 and fibronectin network as well as with their upregulation, while PBMT induced significant downregulation. Similar changes were observed for collagen I and the gelatinolytic enzyme MMP-1. In conclusion, the present study demonstrates that the proposed NIR laser therapy is effective in controlling fibroblast activation induced by IL-1β and TNF-α, likely responsible for a deleterious effect of persistent inflammation.

Keywords: wound healing; NIR laser radiation; inflammation; fibroblasts; photobiomodulation

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1. Introduction

Any injury or infection triggers an inflammatory reaction via cytokines deriving from platelet degranulation and pathogen-associated molecular patterns. Moreover, in both cases, damaged cells release reactive oxygen species and non-specific factors which contribute to activate the inflammatory response in cells of the innate immune system, fibroblasts, and epithelial and endothelial cells [1]. The induction of the inflammatory response triggers a cascade of events mediated by recruitment, proliferation, and activation of several cell populations, primarily immune and stromal cells, as well as

further release of cytokines, vasoactive factors, and growth factors that all together contribute to the repair process [2,3].

Therefore, the correct progression of any acutely occurring inflammatory reaction is a key factor in the path leading to successful healing, which consists in repair/regeneration of damaged tissues and function recovery. However, the occurrence of alterations in the finely-tuned regulation of inflammation can cause pathologic conditions ranging from healing delay (e.g., chronic ulcers) to fibrosis. Moreover, conditions of tissue stress or altered function can induce an adaptive response, known as parainflammation or low grade chronic inflammation, which is an intermediate condition between basal homeostasis and acute inflammation, and is associated with serious diseases, including obesity, diabetes, atherosclerosis, asthma, and neurodegenerative diseases [4].

Inflammation is regulated by a plethora of cell populations, biochemical, and physical factors, but it is widely recognized that the cross-talk between macrophages and fibroblasts, and their ability to assume different phenotypes play a crucial role in determining not only the evolution of inflammation, but also the subsequent stages of the healing process.

During inflammation, macrophages shift from a pro-inflammatory phenotype (the so called M1), characterized by massive production of pro-inflammatory molecules, to an anti-inflammatory phenotype (the so called M2), which secretes suppressors of cytokine signaling, passing through intermediate phenotypes [3,5–7].

In response to pro-inflammatory mediators, resident fibroblasts or circulating fibrocytes become the protagonists of the stromal activation and transdifferentiate in myofibroblasts, their activated counterpart. Many pro-inflammatory mediators are implicated in fibroblast activation, migration, proliferation, and transdifferentiation, including the cytokines tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and the growth factors platelet derived growth factor (PDGF) and fibroblast growth factors (FGFs). Activated fibroblasts and other mesenchymal cells engage a crosstalk, which also reinforces the local immune response due to the induction of vasodilation throught production of nitric oxide (NO) and prostanoids, and stimulates angiogenesis via vascular endothelial growth factor (VEGF) production [8,9].

In a normal evolution of the process, the turning off of the inflammatory response, mediated by the shift of the macrophage phenotype from M1 to M2, opens the way to the remodeling phase, which is dominated by fibroblasts through the production of extracellular matrix (ECM) proteins and matrix metalloproteinases (MMPs) [10]. A well-timed resolution of inflammation is crucial for successful restoration of tissue architecture and function, while persistence of macrophage-fibroblast activation state, with excessive production of pro-inflammatory agents by fibroblasts and further recruitment of immune cells, leads to altered repair processes, from chronic wounds to fibrosis and scarring [11,12].

In summary, activated macrophages induce the stimulation of fibroblasts via production of transforming growth factor-β (TGF-β), TNF-α, IL-1, and other cytokines. In turn, activated fibroblasts can modulate the recruitment and behavior of immune cells via release of cytokines and vasoactive factors as NO and prostanoids. Activated fibroblasts, or myofibroblasts, regulate tissue remodeling by combining their ability to synthesize ECM proteins and that of assuming contractile properties [13,14]. Contractile activity of myofibroblasts increases ECM stiffness. In turn, ECM stiffness is, together with TGF-β1, among the most important factors in inducing myofibroblast differentiation and persistence. Therefore, inflammation dysregulation can generate a feed-forward loop with detrimental effects [15]. Therefore, the control of inflammation and fibroblast activation is crucial to obtain satisfactory morpho-functional recovery and avoid defective healing.

Whatever the cause of inflammation (wound, trauma, infection), at the tissue level it is characterized by redness, heat, oedema, pain, and loss of function.

In current clinical practice, a series of steroidal and nonsteroidal anti-inflammatory drugs can be used to control inflammation and the associated oedema and pain [16]. However, side effects, or even opposite effects on wound healing and other conditions

inducing inflammation, limit their use, especially considering long-term therapy, raising the need for alternative countermeasures [16]. Moreover, anti-inflammatory strategies focused on a specific target (e.g., TNF-α) did not produce the desired results [17]. Several physical therapies and devices aimed to favor the healing process through the control of inflammation and fibroblast behavior have been proposed, such as ultrasound, laser therapy, electrical stimulation, and vacuum-assisted closure [16,18,19]. Studies aimed at elucidating the effectiveness of these therapies in controlling inflammation and the deriving fibroblast activation might strengthen their use.

Laser therapy, currently called photobiomodulation therapy (PBMT), is one of the most widely applied to manage many different diseases characterized by acute or chronic inflammation. The benefits of PBMT in terms of anti-inflammatory [20], anti-pain [21–23], and anti-oedema [24] properties are widely documented in literature. Moreover, PBMT enhances cell energy metabolism and promotes anabolic and repair processes [25]. Further, PBMT has been shown to stimulate angiogenesis and collagen remodeling [16,26].

PBMT, being safe, non-invasive, and non-time-consuming (short-duration application) is also well accepted by patients.

Over the last years, new molecular insights into the action mechanisms of PBMT have been obtained. In particular, it has been demonstrated that in chronic inflammatory conditions, such as those related to periodontal diseases and osteoarthritis, PBMT is effective in reducing the expression of pro-inflammatory genes (TNF-α, IL-1β, IL-6, IL-8) through the downregulation of NF-κB signaling pathway via cAMP increase [27].

Another PBMT effect, which can be relevant in the evolution and outcome of the inflammatory response, is to induce a decrease in matrix metalloproteinases (MMPs) expression, as it has been recently demonstrated in an in vitro model of osteoarthritis [28]. MMPs are an important family of proteinases, able to degrade extracellular matrix components and covering a broad range of tasks in inflammation, acquired immunity, defense from injury and repair. MMPs are always present in acute and chronic, physiological and pathological inflammatory processes, and experimental evidence suggests that they can protect against or contribute to pathological evolution of inflammation [29,30].

Despite the abundant literature on the ability of PBMT to control inflammation, promote healing mechanisms, and counteract scarring, the effects that laser emissions commonly used in PBMT exert on fibroblasts activated by a strong and persistent inflammatory stimulation have not been clearly defined. In fact, for the most part, studies used in vitro models of fibroblasts in the basal state.

Therefore, the present study was aimed at investigating the effect of PBMT on activated fibroblasts. An in vitro model of fibroblasts, activated by exposure to inflammatory stimuli, was characterized for morphological features, canonical inflammatory and vasoactive cascades (inducible NO synthase and cyclooxygenase (COX)/prostaglandin synthase enzymes), and outcomes on angiogenesis and ECM remodeling. Then, the effectiveness and underlying molecular mechanisms of a high power, dual wavelength NIR laser source in reducing fibroblast inflammatory phenotype was investigated.

2. Materials and Methods

2.1. Cell Cultures

Normal human dermal fibroblasts (NHDF) were purchased from Lonza (Verviers, Belgium) and grown in Fibroblast Growth Basal Medium (FBS; Lonza, Basel, Switzerland) containing 10% Fetal Bovine Serum (FBS; Hyclone, Euroclone, Milan, Italy), 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Merck KGaA, Darmstadt, Germany). Cells were cultured at 37 °C with 5% CO_2 in Petri dishes and were split 1:3 twice a week until passage 10.

2.2. In Vitro Model of Inflammation

Cells (1×10^4) were seeded in 24-multiwell plates and allowed to adhere (when immunofluorescence analysis were planned, cells were seeded on 13 mm diameter glass coverslips placed inside the 24-multiwell plates). After 24 h, complete culture medium was replaced by fresh complete culture medium supplemented with a mix of IL-1β (10 ng/mL; #201-LB/CF R&D System, Minneapolis, MN, USA) and TNF-α (10 ng/mL; #201-LB/CF and #410MT, R&D System, Minneapolis, MN, USA). Cells were maintained with the cytokines mix for 48 h. Control samples were treated in the same way, omitting the cytokines mix.

2.3. Laser Treatment

At the end of the 48 h of stimulation with cytokines mix, the medium of all samples was replaced by a fresh complete culture medium. Then, samples which had been previously stimulated with the cytokines mix were divided into two groups: A "treated group", that received laser irradiation and an "untreated group" that was not laser irradiated. Laser treatment was performed with a Multiwave Locked System laser (MLS-MiS, ASA S.r.l., Vicenza, Italy) widely used in clinics. It is a class IV, NIR laser with two synchronized sources (laser diodes): The first one is a pulsed laser diode emitting at 905 nm wavelength, with peak power from 140 W ± 20% to 1 kW ± 20% and pulse frequency varying in the range 1–2000 Hz; the second laser diode emits at an 808 nm wavelength and can operate in continuous (max power 6 W ± 20%) or frequent (repetition rate 1–2000 Hz, 50% duty cycle) mode. The two laser beams work simultaneously and synchronously, and the propagation axes are coincident.

For laser exposure, only 6 wells of 24-well plates contained cells. The wells surrounding those with cells were filled with black cardboard to avoid light diffusion and reflection. The exposure was performed by placing the plate inside a holder, which allows the positioning of the laser handpiece at a 1.5 cm distance from the bottom of the wells, so that the spot of the two laser beams, impinging perpendicular to the sample surface, had the same diameter as a well (13 mm). Cells were irradiated for 10 sec with the following parameters: 10 Hz repetition rate; 50% int (mean power 1840 mW); peak power 1 kW ± 20%, fluence 5.19 J/cm^2. All treatments were performed under laminar flow hood at room temperature. The samples belonging to the untreated group were prepared and kept under the same conditions used for the exposed samples, except for laser irradiation.

2.4. Experiment Design

The following samples were prepared, analyzed, and compared:

(i) Samples stimulated with a mix of IL-1β and TNF-α for 48 h and then exposed to 3 laser treatments, repeated once a day, for 3 consecutive days under sterile conditions (CYKs + LASER Group);

(ii) samples stimulated with a mix of IL-1β and TNF-α for 48 h and not exposed to laser treatments CYKs Group);

(iii) samples not stimulated with a mix of IL-1β and TNF-α for 48 h and not exposed to laser treatments (CTRL Group).

For immunofluorescence analysis, an additional experimental group was included, namely cells exposed to laser treatment alone (LASER Group).

Six hours after the third laser treatment, all samples were prepared for the subsequent analysis described in the following paragraphs.

2.5. Immunofluorescence Analysis

Cells grown on glass coverslips and treated as previously described, were fixed for 5 min with ice cold acetone. Unspecific binding sites were blocked with PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Then, cells were incubated overnight at 4 °C with specific anti-NF-kB (1:50;

#sc-372, Santa Cruz, Dallas, TX, USA), anti-cyclooxygenase-2 (COX-2) (1:100; #TA313292, Origene, Rockville, MD, USA), anti-VEGF (1:50; #sc-57496, Santa Cruz), anti-α actin (1:100; #MAB1501X, Millipore, Billerica, MA, USA), anti α-smooth muscle actin (α-SMA) (1:100; #CBL171, Chemicon® by Thermo Fisher Scientific, Waltham, MA, USA), anti-tubulin (1:100; #05-829, Millipore, Billerica, MA, USA), anti-collagen I (1:100; #MAB3391, Millipore, Billerica, MA, USA), anti-fibronectin (FN) (1:100; #MAB1926-I, Millipore, Billerica, MA, USA), anti-MMP-1 (1:100; #MAB13439, Millipore, Billerica, MA, USA), and anti-α5β1 integrin (1:100; #MAB1999, Millipore, Billerica, MA, USA) primary antibodies properly diluted in PBS with 0.5% BSA. After washing three times with PBS-0.5% BSA, samples were then incubated for 1 h at 4 °C in the dark with: Alexa Fluor 555™ conjugated secondary antibodies [specifically: Anti-mouse IgG (#A-21422, Invitrogen™ by Thermo Fisher Scientific) for anti-NF-kB and anti-VEGF antibodies and anti-rabbit IgG (#A-21428, Invitrogen™ by Thermo Fisher Scientific) for anti-COX-2 antibody] and fluorescein isothiocyanate (FITC) conjugated specific secondary antibody [specifically: Anti-mouse IgG (#AP124F, Millipore) for anti α-SMA, anti-tubulin, anti-collagen I, anti-fibronectin, anti-MMP-1, anti-α5β1 integrin primary antibodies]. All secondary antibodies were diluted 1:200 in PBS with 0.5% BSA. Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. Again, samples were washed three times and then mounted on glass slides using Fluoromount™ aqueous mounting medium (Sigma-Aldrich St. Louis, MO, USA) [31]. In samples of incubated anti-NF-kB, anti-COX-2, and anti-VEGF, before mounting, nuclei were marked with DAPI (#D9542, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:5000 in PBS with 0.5% BSA for 30 min at room temperature. The fluorescent signal of samples stained with anti-NF-kB, anti-COX-2, and anti-VEGF antibodies was acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany). All other samples were evaluated by an epifluorescence microscope (Nikon, Florence, Italy) at 100x magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy). Based on the CCD images, a relative immunofluorescence quantification was carried out by image analysis routines (ImageJ 1.53 analysis software, National Institutes of Health, Bethesda, MD, USA) for samples stained with anti-collagen I and anti-α5β1 integrin antibodies. After appropriate thresholding to eliminate background signal and creation of a proper image mask, a pixel intensity histogram was acquired.

2.6. Western Blot

Cells derived from the different experimental conditions, were detached from 24 multi-well plates, collected in 15 mL tubes, and lysed with CelLytic™ MT Cell Lysis Reagent supplemented with 2 mM Na_3VO_4 and 1X Protease inhibitor cocktail for mammalian cells (Sigma-Aldrich). Cell lysates were centrifuged at 16000× *g* for 20 min at 4 °C, and the supernatants were then collected. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich). Electrophoresis with equal amounts of proteins (50 μg) was carried out in NuPAGE™ 4–12% Bis-Tris precast Gels (Thermo Fisher Scientific) as previously reported [32].

Proteins were transferred onto nitrocellulose membranes, blocked for 1 h in a PBS–0.05% Tween solution (Sigma-Aldrich) supplemented with 5% (wt/vol) of Blotting-Grade Blocker (Bio-Rad, Hercules, CA, USA). Membranes were then incubated overnight at 4 °C with the primary antibodies properly diluted in PBS–0.05% Tween solution supplemented with 1% (wt/vol) of Blotting-Grade Blocker: anti-inducible NO synthase (iNOS) (1:250; #sc-7271, Santa Cruz), anti-COX-2 (1:1000; #160106, Cayman Chemical, Ann Arbor, MI, USA), and anti-microsomal prostaglandin E synthase-1 (mPGES-1) (1:500; #160140, Cayman Chemical). Immunoblots were washed three times with PBS–0.05% Tween solution and then incubated for 1 h with the respective species-specific secondary antibody conjugated with horseradish peroxidase HRP (Promega, Madison, Wisconsin, US) diluted 1:2500 in PBS–0.05% Tween solution. The membranes were finally incubated

with SuperSignal™ West Pico PLUS chemiluminescent Substrate (Thermo Fisher Scientific), and the immunoreaction was revealed by ImageQuant LAS 4000 chemiluminescence system (GE Healthcare, Chicago, IL, USA). Results were normalized to those obtained by using an antibody against β-Actin (#A5441, Sigma-Aldrich) diluted 1:10,000 in PBS–0.05% Tween solution.

Immunoblots were analyzed by densitometry using Image J software, and the results, expressed as arbitrary density units (A.D.U.), were normalized to β-Actin.

2.7. Immunoassays for Prostaglandin E-2 and VEGF Quantification

Conditioned media were collected at the end of the experiment, frozen, and stored at −80 °C until use. Prostaglandin E-2 (PGE-2) and VEGF levels were measured using ELISA kit: Prostaglandin E_2 ELISA kit-Monoclonal (Cayman Chemical, Ann Arbor, Michigan, US) and VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively, following the manufacturer's instructions. Dosing of each sample was performed in double, and PGE-2 and VEGF levels were expressed as (pg/mL).

2.8. Statistics

Three different experiments were carried out in triplicate. Data are reported as means ±SD. Statistical significance was determined using two-sided Student's t test. A p value lower than 0.05 was considered statistically significant. For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide.

3. Results

3.1. Set up of an "In Vitro" Inflammatory Model in Fibroblasts Cultures

The human dermal fibroblasts NHDF have been treated with a mix of cytokines (IL-1β and TNF-α, each at 10 ng/mL) for 24 h and 48 h, then the occurrence of inflammatory features depending on the exposure time has been evaluated.

Microsomal PGE synthase-1 (mPGES-1), the pivotal inducible enzyme of the prostanoid inflammatory pathway, was evaluated by western blot after 24 h and 48 h of stimulation. A consistent rise in mPGES-1 was observed after both 24 h and 48 h, the increase being more evident at a longer time of exposure (Figure 1, upper panel, 0.8 ± 0.2 and 4.8 ± 0.9 fold increase of mPGES-1 expression in the presence of cytokines with respect to control, at 24 h and 48 h, respectively). The up-regulation of the mPGES-1 enzyme generated a significant increase in the final product, prostaglandin E2 (PGE-2), released by fibroblasts in the conditioned medium, documenting an activation of the enzymatic cascade (Figure 1, lower panel). Based on these results, the stimulation time of 48 h was chosen for further experiments.

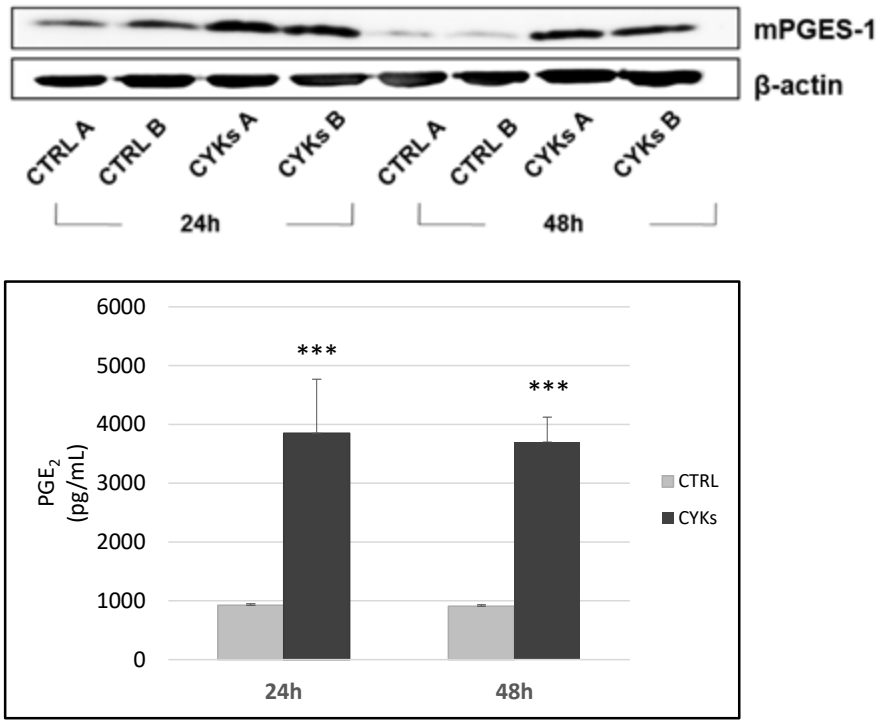

Figure 1. Development of an inflammation model on normal human dermal fibroblast (NHDF) cells. Fibroblasts were treated with IL-1β (10 ng/mL) + TNF-α (10 ng/mL) for 24 h and 48 h. Whole cell lysates were collected to assess mPGES-1 expression by Western blot (upper panel). Samples A and B represent intra-experimental duplicates. The measurement of PGE-2 performed by immunoenzymatic assay is reported (lower panel). At both times, there is an upregulation of the prostanoid system. Data represent means +/− SD (n = 3) *** $p < 0.001$ CYKs group vs. CTRL group.

3.2. Effect of Laser Treatment on Inflammatory Phenotype in Fibroblasts

3.2.1. Expression of Inflammatory Markers

In order to evaluate whether laser treatment could affect the inflammatory model described above, samples, after the stimulation with cytokine mix, were exposed to laser radiation according the following experimental protocol: NHDFs were treated with cytokine mix (IL-1β and TNF-α, each at 10 ng/mL) for 48 h; then, culture medium was replaced by fresh culture medium, and samples were divided into the following groups: (i) CYKs + LASER Group—samples previously stimulated with the cytokine mix and then exposed to laser treatment (3 treatments, repeated once a day, for 3 consecutive days); (ii) CYKs Group—samples previously stimulated with the cytokine mix and not exposed to laser treatment; (iii) CTRL Group-samples not stimulated with the cytokine mix and not exposed to laser treatment. Six hours after the third laser treatment (T = 126 h), all the samples were recovered and iNOS, COX-2 and mPGES-1 protein expression was evaluated by western blotting.

Following the exposure to inflammatory cytokines, a significant up-regulation of the inflammatory enzymes was observed (Figure 2). The group stimulated with cytokines and then treated with lasers showed a strong decrease in inflammatory enzyme expression compared to the group stimulated only with cytokines. For iNOS and COX-2, the decrease reached statistical significance (Figure 2).

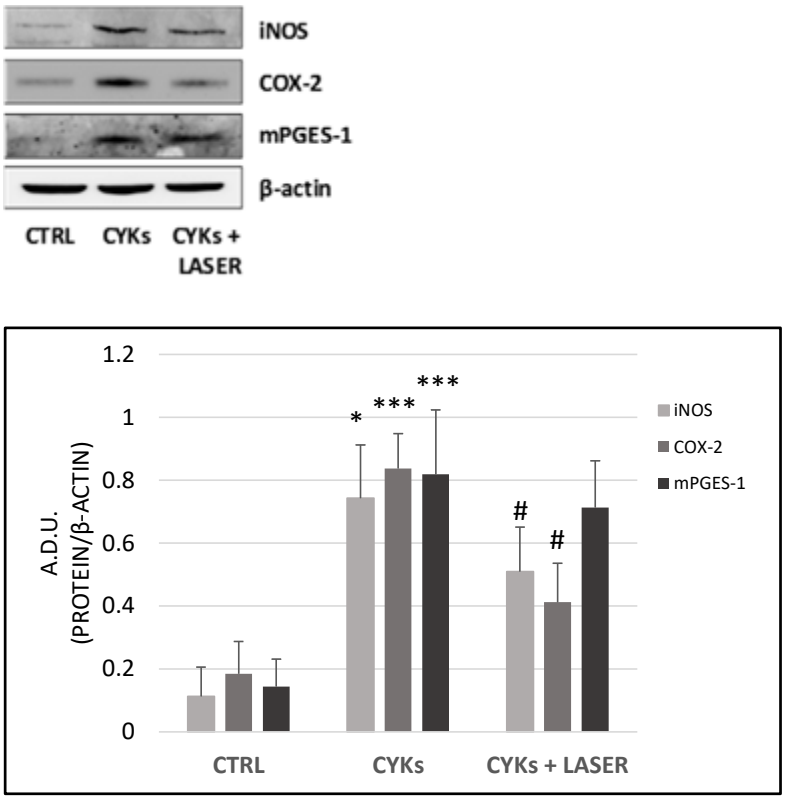

Figure 2. Effect of laser treatment on NDHF cells stimulated with pro-inflammatory cytokines. Fibroblasts were treated with IL-1β and TNF-α, each at 10 ng/mL for 48 h, then culture medium was replaced by fresh culture medium and samples divided into 3 groups: CYKs + Laser—samples stimulated with cytokine mix and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days); CYKs—samples stimulated with cytokine mix and not exposed to laser treatment; CTRL—samples not stimulated and not exposed to laser treatment. Six hours after the third laser treatment, whole cell lysates of all samples were collected, and Western blot was performed to assess protein abundance of iNOS, COX-2, and mPGES-1 (upper panel). Immunoblots were analyzed by densitometry and the results, expressed as arbitrary density units (A.D.U.), were normalized to β-Actin (lower panel). Data represent means +/− SD (n = 2) * $p < 0.05$ and *** $p < 0.001$ CYKs group vs. CTRL group, # $p < 0.05$ CYKs + Laser group vs. CYKs group.

To validate data obtained by Western blot, the main product of prostanoid enzymatic cascade, PGE-2, was measured in NHDF conditioned media recovered from the samples 6 h after the third laser treatment (T = 126 h). The cytokine mix-stimulated fibroblasts showed a significant increase in PGE-2 released in the medium in comparison with control samples (209 pg/mL in basal condition and 11000 pg/mL after 48 h of stimulation with the inflammatory mix). Although the resulting data were not significant, laser exposure reduced PGE-2 levels with a clear trend towards damping of the prostanoid pathway (Figure 3, upper panel).

Additionally, conditioned media were assessed for the release of the angiogenic factor VEGF, involved in neovascularization and granulation tissue formation. While inflammatory stimuli significantly increased VEGF levels, laser exposure strongly reduced VEGF availability in the medium, being the levels well below the basal, unstimulated condition (Figure 3, lower panel).

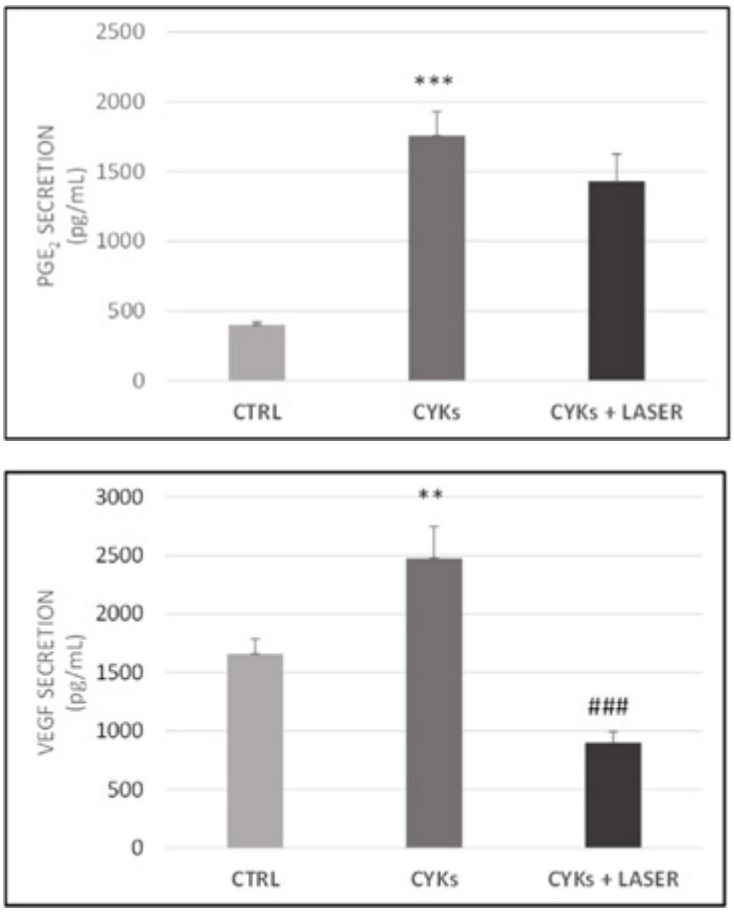

Figure 3. Modulation of PGE-2 and vascular endothelial growth factor (VEGF) release in NHDF cells exposed to pro-inflammatory cytokines and laser treatment. Fibroblasts were treated with IL-1β and TNF-α, each at 10 ng/mL for 48 h, then the culture medium was replaced by fresh culture medium and samples divided into 3 groups: CYKs + Laser—samples stimulated with cytokine mix and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days); CYKs—samples stimulated with cytokine mix and not exposed to laser treatment; CTRL—samples not stimulated and not exposed to laser treatment. Six hours after the third laser treatment, all samples were recovered, and PGE-2 (upper panel) and VEGF (lower panel) levels were evaluated in conditioned media using specific ELISA kits. Dosing of each condition was performed in double, and quantification is expressed as pg/mL. Data represent means +/− SD ($n = 2$) ** $p < 0.01$ and *** $p < 0.001$ CYKs group vs. CTRL group, ### $p < 0.001$ CYKs + Laser group vs. CYKs group.

Ultimately, the modulation of the inflammatory response at the cellular level was evaluated through confocal microscopy. Inflammation is a protective response characterized by a series of reactions, such as vasodilation and recruitment of immune cells to the site of injury. NF-κB is an inducible transcription factor, responsible for the activation of genes involved in this process, including COX-2 and VEGF [33]. The localization of the nuclear transcription factor NF-κB and the intensity of the fluorescent signal given by the expression of its downstream genes COX-2 and VEGF were analyzed in the samples described above. In control samples, the transcription factor seemed to remain outside the nucleus, since the fluorescent signal was mainly cytoplasmic (Figure 4a). NHDF stimulation with IL-1β and TNF-α induced a consistent increase in the expression of NF-κB, as evidenced by a higher fluorescence intensity. Furthermore, in many cells, a change in the localization was observed, with accumulation of the signal at the nuclear level (Figure 4b; white arrows). In samples stimulated with the cytokine mix and then treated with laser, a clear decrease in intensity of the signal linked to the transcription factor was observed, although some cells with NF-κB located in the nucleus

(Figure 4c; white arrows) were still present. In cells treated with laser alone, the presence of some NF-κB punctuation at nuclear level was observed (Figure 4d).

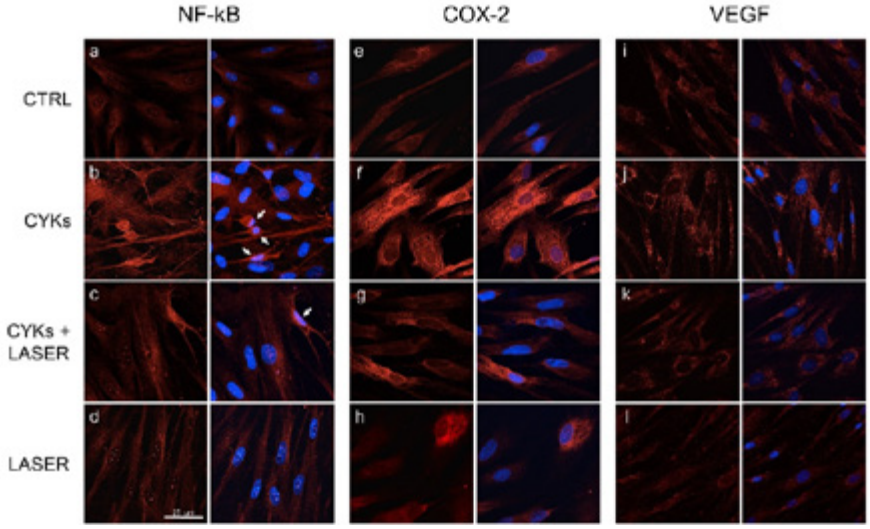

Figure 4. Laser treatment reduces inflammatory response in NHDF cells by limiting NF-κB translocation into the nucleus and down-regulating COX-2 and VEGF expression. Confocal analysis of NF-κB (panels (**a**–**d**)), COX-2 (panels (**e**–**h**)) and VEGF (panels (**i**–**l**)) expression and localization (magnification 63×) evaluated by immunofluorescence on NHDF in basal conditions (CTRL; panels (**a**,**e**,**i**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panels (**b**,**f**,**j**)), stimulated with IL-1β and TNF-α for 48 h, and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + LASER; panels (**c**,**g**,**k**)) and exposed to laser alone (LASER; panels (**d**,**h**,**l**)). For each series, the left panels show the protein of interest in red, while DAPI staining (blue) was merged on the right panels. White arrows indicate cells with nuclear localization of NF-kB. Bar = 25 μm.

A similar trend was described for COX-2. The enzyme expression resulted strongly enhanced by the cytokine mix (Figure 4f) in comparison with unstimulated and laser alone controls (Figure 4e,h), where the fluorescence signal was weak and located in the cytoplasm. In samples stimulated with the cytokine mix and then treated with laser, the signal was similar to that observed in control (Figure 4g). However, in the last condition, mixed cell populations were noticed, some still over-expressing the enzyme and others returned to control levels. Similarly, the VEGF signal also followed a modulation superimposable to that of COX-2, presumably dictated by the transcription factor NF-kB. VEGF labelling, not affected by laser alone (Figure 4k), increased by stimulation with cytokines mix (Figure 4j). Following laser treatment, the cytokine-induced VEGF intensity completely returned to the basal levels (Figure 4l), confirming the data obtained by protein dosage carried out on conditioned media, as previously illustrated (Figure 3, lower panel).

3.2.2. Morphology and Cytoskeleton Organization

The stimulation with the mix of IL-1β and TNF-α produced a marked change in cell morphology. The organization of the microtubules, which control cell architecture, changed as well. In the control samples, fibroblasts were generally star-shaped and spread on the substrate. The specific labeling for tubulin showed the well-known radial distribution of microtubules which branch off from a nucleation center (Figure 5a), usually anchored at the centrosome and the Golgi apparatus [34]. In the stimulated samples, the cells appeared spindle-shaped, elongated, with a dense, longitudinal microtubule network [34], where it was difficult to distinguish a nucleation center (Figure 5b). In the samples first stimulated and then treated with laser, fibroblasts regained a shape similar

to the controls, with a clearly distinguishable microtubule nucleation center and radially organized microtubules (Figure 5c).

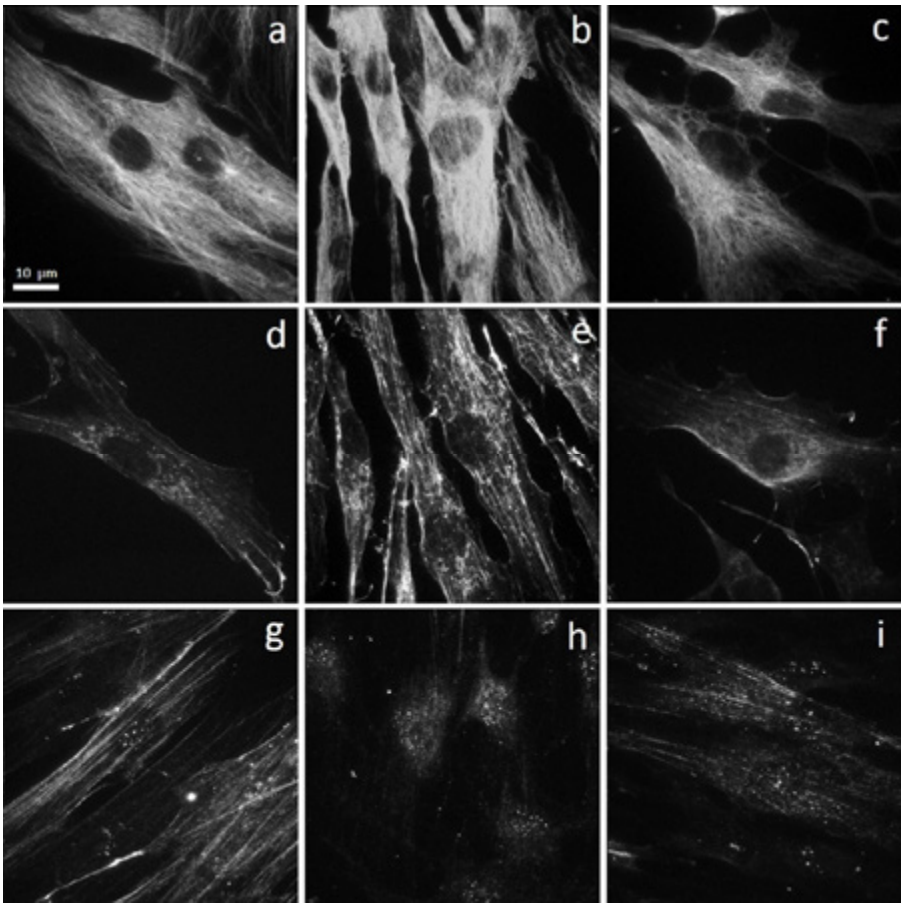

Figure 5. Effect of laser treatment on tubulin, α-actin, α-SMA expression, and distribution. Microscopy analysis of tubulin (**a**-**c**), α-actin (**d**–**f**), and α-SMA (**g**–**i**) expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panels (**a**,**d**,**g**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panels (**b**,**e**,**h**)), stimulated with IL-1β and TNF-α for 48 h, and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panels (**c**,**f**,**i**)). Bar = 10 μm.

As regards actin distribution and organization, control fibroblasts showed a perinuclear area rich of G-actin, a network of very thin microfilaments distributed in the cell cytoplasm, and a thin actin layer placed close to the plasma membrane (Figure 5d). In the stimulated fibroblasts, F-actin was predominant, with microfilaments arranged in parallel and thicker in comparison with those observed in control cells (Figure 5e). As already noted for tubulin, also in the case of actin, the stimulated cell samples which were then exposed to laser radiation recovered a condition similar to the control cells, with G-actin thickened in the perinuclear area, few very thin microfilaments and a thin actin layer close to the cell membrane (Figure 5f).

Alpha-smooth muscle actin (α-SMA) is the actin isoform that predominates within smooth-muscle cells. Its expression generally increases in the transition fibroblast-myofibroblast. In fact, myofibroblasts acquire a contractile phenotype, which is responsible for merging the wound edges in the healing process. Therefore, α-SMA is considered a marker of myofibroblast differentiation. In control samples, α-SMA staining revealed some stress fibers, which completely disappeared in fibroblasts stimulated with IL-1β and TNF-α, where the fluorescence signal coincided with the nucleus and was

detectable only in the nuclear area (Figure 5g,h). The samples exposed to laser radiation after the cytokine mix stimulation showed an intermediate situation. The signal in the nuclear area was still detectable, but fibers organized in parallel appeared (Figure 5i).

3.2.3. Extracellular Matrix Proteins and Membrane Integrin

Integrins are cell surface receptors which control various cellular functions. Integrin receptors connect the cell cytoskeleton with the ECM proteins, thus being involved in signaling changes of the extracellular microenvironment and leading to cellular responses. In particular, α5β1 integrin is a fibronectin receptor and has a well-defined role in cell adhesion, migration, and matrix formation, which are functions of crucial importance in physiological and pathological processes such as wound healing and fibrosis. In the control samples, α5β1 clusters were located at focal adhesion points mostly in the perinuclear area, along cellular protrusions, and at their ends, generally arranged parallel to the major axis of the cells (Figure 6a). In the samples stimulated with the cytokine mix, the expression of the integrin significantly increased (Figure 6b). In these samples, half of the cells still retained a morphology similar to the control (star-shaped and spread), but showed a higher density of integrin clusters with centripetal distribution in the perinuclear area. In the other half of the cells, characterized by spindle-shaped and elongated morphology, the α5β1 clusters became point-like, smaller, distributed in the perinuclear area, and at lateral intercellular surfaces forming cell–cell contact points (Figure 6b). After laser treatment, fibroblasts appeared similar to the controls, both for the signal intensity and distribution of α5β1 clusters (Figure 6c).

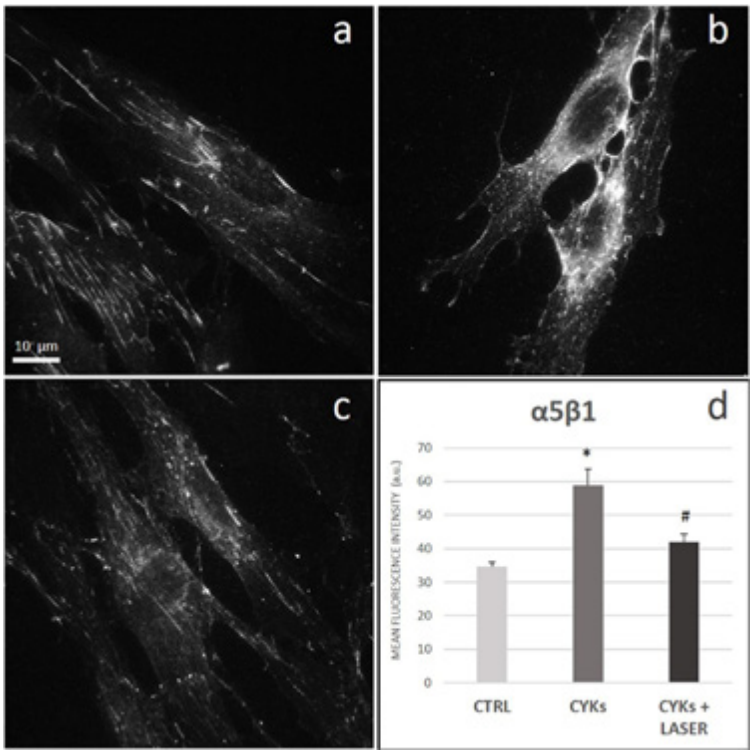

Figure 6. Effect of laser treatment on α5β1 expression and distribution. Microscopy analysis of α5β1 expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 µm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

Through its interaction with different cell types, cytokines, and other ECM molecules, and facilitating collagen fibrogenesis by scaffolding action, fibronectin plays a preeminent role in both wound healing and scarring [35,36]. Similarly to its receptor $\alpha 5\beta 1$, fibronectin significantly increased in fibroblast cultures stimulated with IL-1β and TNF-α, when compared to unstimulated controls, and formed a dense extracellular network of fibrils (Figure 7a,b). After laser treatment, fibronectin expression returned to the basal levels observed in control cells with evident reduction of extracellular fibrils (Figure 7c,d).

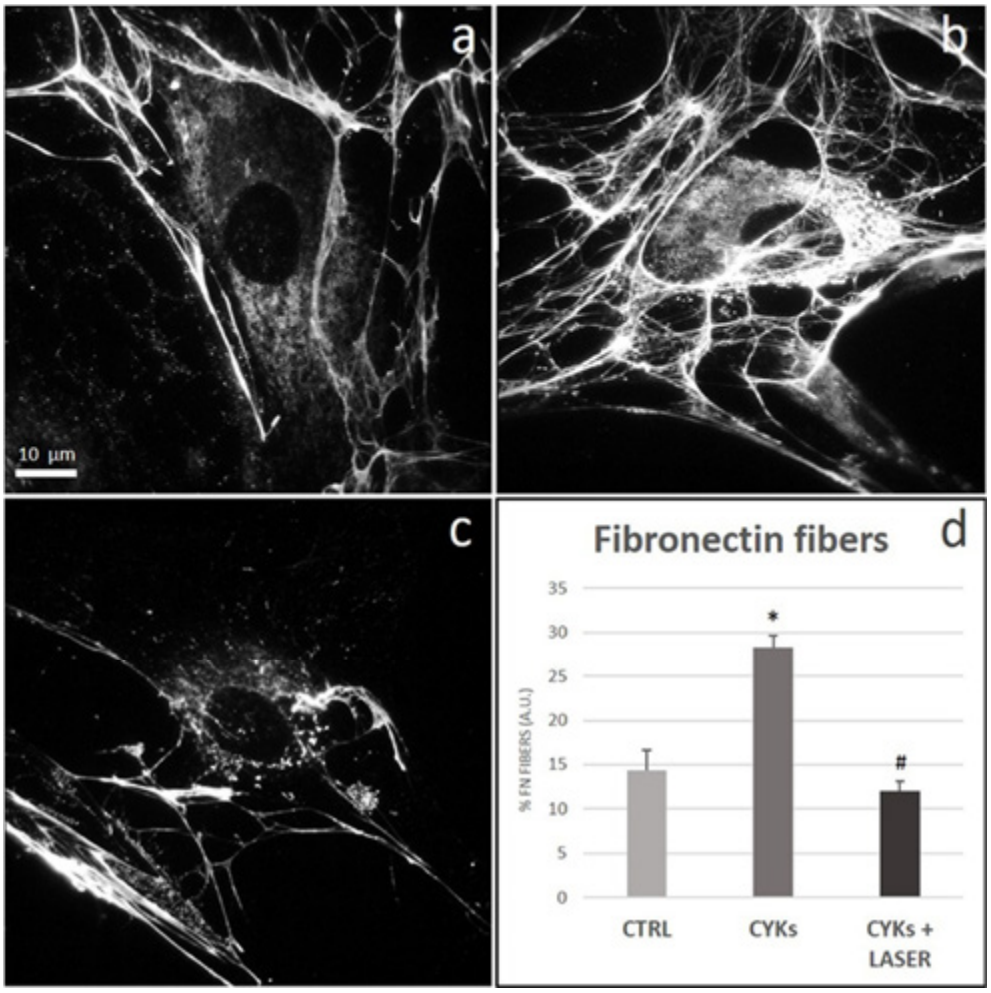

Figure 7. Effect of laser treatment on fibronectin expression and organization. Microscopy analysis of fibronectin expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 µm. The histogram reports the % of the surface area with fibers, acquired by ImageJ software after appropriate thresholding to only include the stained fibers (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

In addition, the synthesis of collagen I, one of the most abundant ECM components, was significantly enhanced by the exposure to the cytokine mix in comparison with control unstimulated cells (Figure 8a,b). Interestingly, stimulated fibroblasts showed an intracellular accumulation of collagen I, apparently in the endoplasmic reticulum and/or Golgi apparatus, while the protein was not released in the extracellular environment (Figure 8b). Cytokine-mix stimulated fibroblasts exposed to the laser treatment revealed

a collagen I signal similar to that observed in the control, both for distribution and fluorescence intensity (Figure 8c,d).

Matrix metalloproteinases (MMPs) are endopeptidases that can degrade the ECM proteins. They have important roles in fundamental physiological processes, such as embryonic development, morphogenesis, and tissue remodeling, and are involved in a number of diseases. MMPs are present in both acute and chronic wounds, where they regulate ECM degradation/deposition that is essential for wound healing. The excess protease activity can lead to chronic nonhealing wounds [37].

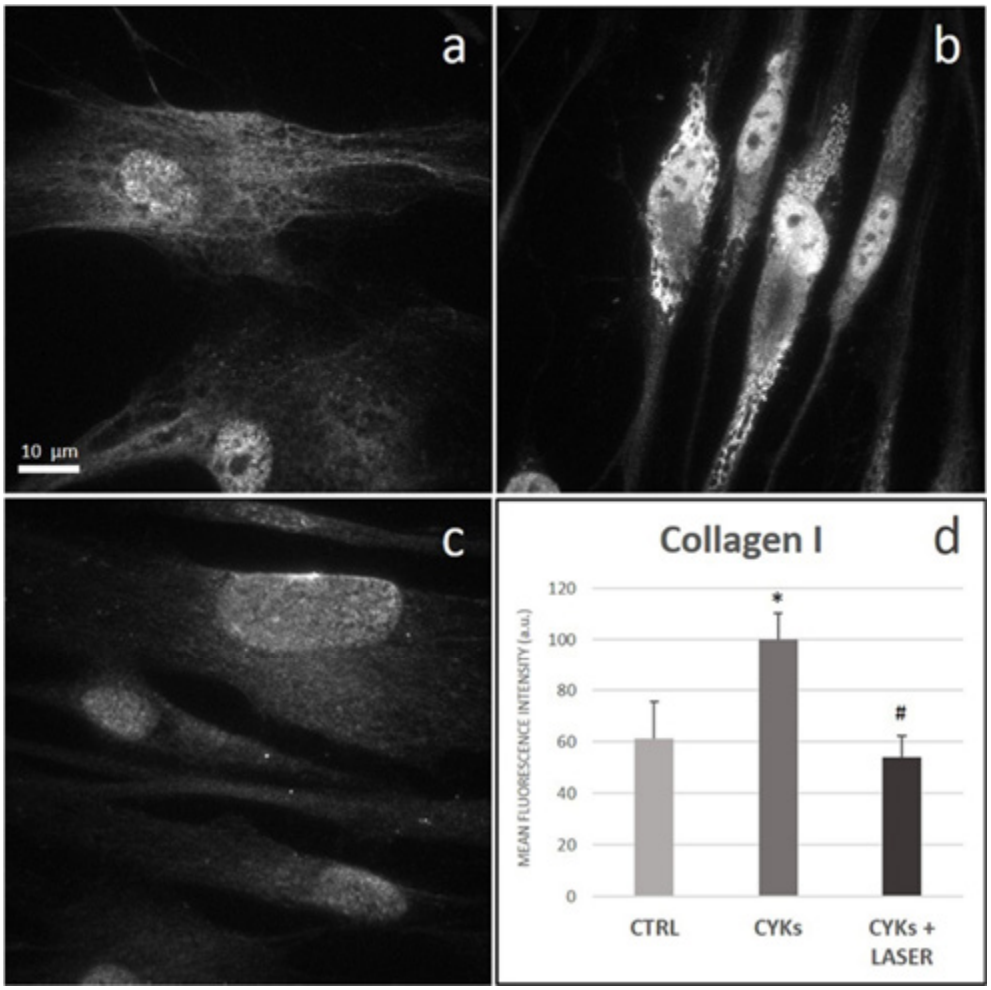

Figure 8. Effect of laser treatment on Collagen I expression and distribution. Microscopy analysis of Collagen I expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 µm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

Specifically, MMP-1 is able to degrade collagen types I, II, and III. Similarly to fibronectin and collagen, also MMP-1 significantly increased in fibroblasts stimulated with the cytokine mix (Figure 9b), compared to non-stimulated controls (Figure 9a). Laser treatment counteracted the effect of the cytokine mix and reported MMP-1 expression to a level comparable to that found in the basal state (Figure 9c).

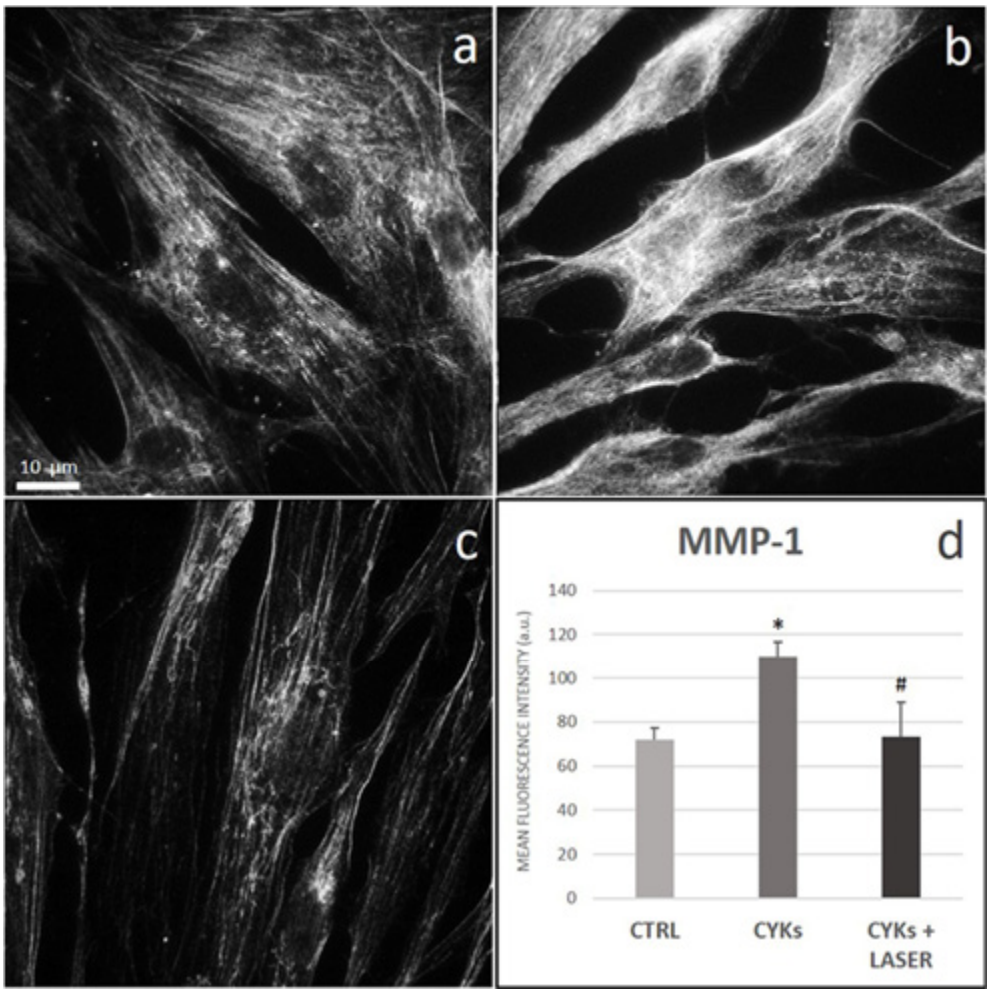

Figure 9. Effect of laser treatment on MMP-1 expression and distribution. Microscopy analysis of MMP-1 expression evaluated by immunofluorescence (magnification 100×) on NHDF in basal conditions (CTRL; panel (**a**)), stimulated with IL-1β and TNF-α for 48 h (CYKs; panel (**b**)), stimulated with IL-1β and TNF-α for 48 h and then exposed to laser treatments (3 treatments, repeated once a day, for 3 consecutive days) (CYKs + Laser; panel (**c**)). Bar = 10 µm. The histogram reports the mean pixel intensity, acquired by ImageJ software after appropriate thresholding and subsequent image masking (panel (**d**)). * $p < 0.05$ CYKs group vs. CTRL group; # $p < 0.05$ CYKs + Laser group vs. CYKs group ($n = 3$).

4. Discussion

The cytokines IL-1β and TNF-α have already been used at different concentrations, individually or in association, to stimulate an inflammatory response in various cell types, dermal fibroblasts included [28,38,39]. In this study, IL-1β and TNF-α have been used jointly to induce a pro-inflammatory phenotype in dermal fibroblasts, with the aim to investigate if PBMT delivered via a dual-wavelength NIR laser system (MLS-MiS) was effective in counteracting cell inflammatory response and modulating fibroblast functions involved in stromal activation, wound healing, and its alterations, which can lead to chronic ulcers or fibrosis. Preliminary experiments performed to define the protocol for preparing the in vitro model of inflammation in dermal fibroblast cultures showed that both 24 h and 48 h exposure to IL-1β and TNF-α produced a significant increase in the inducible enzyme mPGES-1 and in the release of its product PGE-2. The increase in mPGES-1 was higher after 48 h, while the increase in PGE-2 was similar at 24 h and 48 h. Therefore, a stimulation time of 48 h was chosen for the subsequent experiments in which

non-stimulated controls, samples stimulated with IL-1β and TNF-α, and samples exposed to laser radiation after the stimulation with the inflammatory cytokines were compared for their morphology, inflammatory profile, and expression of molecules involved in ECM remodeling. In stimulated samples, the inflammatory signals iNOS, COX-2, and mPGES-1 significantly increased in comparison with non-stimulated controls, in agreement with data reported in literature [40] and supporting the validity of the inflammatory model used. Samples stimulated and then treated with PBMT showed a significant decrease in iNOS and COX-2, compared to the stimulated but non-laser irradiated samples. The mPGES-1 level and that of the final product PGE-2 decreased, but not significantly, suggesting a multimodal action of PBMT, which could act at different cellular levels (gene transcription, protein expression, and localization), as demonstrated by the reported results.

Modulation of the three mediators mentioned above is closely related to their upstream activator NF-κB, an inducible transcription factor which is activated upon binding of pro-inflammatory cytokines, such as TNF-α, to their membrane receptors. In basal conditions, NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins. Following inflammatory stimuli, this protein moves to the nucleus, binds to specific elements on DNA, and recruits cofactors for the transcription of target genes *iNOS*, *COX-2*, and *mPGES-1* [41]. In the presence of inflammatory stimuli, the co-localization of the transcription factor NF-κB within the nucleus, observed by immunofluorescence, correlates with an increased expression of iNOS, COX-2, and mPGES-1 at the cytoplasmic level and a consequent increase of PGE-2 released in the extracellular medium. Following laser treatment, these values are significantly reduced, demonstrating the effectiveness of the laser source and treatment parameters used in counteracting the inflammatory response.

The anti-inflammatory properties of the NIR source used had already been highlighted by a proteomics study on laser-irradiated myoblasts, in which a marked increase in NLRP10, a strong inhibitor of the inflammasome, and in turn of IL-1β and interleukin-18 (IL-18) release, was observed [25]. These data are in agreement with previous studies showing the effectiveness of red and NIR radiation in reducing the inflammatory signals both in fibroblast cultures and at the wound level [42,43].

As previously mentioned, inflammation is a protective response characterized by a series of reactions modulated by the master regulator NF-kB, whose gene targets are involved both in the recruitment of immune cells to the site of injury and in vasodilation [33]. In this study, fibroblast production of VEGF at the cytoplasmic level and its secretion in the extracellular milieu were therefore analyzed. Compared to untreated controls, IL-1β and TNF-α stimulation of fibroblasts induced an increase in VEGF production and release in culture medium, that further confirms the validity of the model used for the present study. The proinflammatory cytokines-induced enhancement in VEGF levels is widely documented in vitro [44], and it occurs in vivo in chronic inflammatory diseases as well as in acute inflammatory response to infections and injuries. VEGF is produced by the most part of cell populations involved in wound healing, as platelets, immune cells (neutrophils and macrophages), fibroblasts, and endothelial cells, and reaches the maximum concentration during the proliferative phase. In the wound, VEGF promotes angiogenesis [45] and influences re-epithelialization and collagen deposition through stimulation of keratinocytes and fibroblasts [46]. However, if in a proper inflammatory response VEGF upregulation is needed to promote angiogenesis, excessive or persistent inflammation and VEGF production can lead to fibrosis and should be controlled. Laser treatment subsequent to IL-1β and TNF-α stimulation abolished the cytokine-mediated VEGF increase and brought VEGF levels back to values even lower than those seen in unstimulated controls. In literature, a modulation of VEGF expression following irradiation with red-NIR wavelengths has been described, the final effects depending on irradiation parameters and experimental models used [42,47,48]. The decrease observed in the present study, irradiating activated fibroblasts with the source and parameters described, further supports the strong anti-inflammatory action of the proposed laser treatment. At the same time, the results on cells exposed to laser alone, which did not

affect markers of fibroblast activation, substantiated the safety of laser irradiation on quiescent unstimulated cells.

IL-1β and TNF-α treatment induced also noticeable morphological changes with cytoskeletal rearrangements in the network of microtubules and actin microfilaments.

Microtubules form a scaffold which controls cell shape, intracellular transport, signaling, and organelle positioning. Microtubules are stiff and intrinsically polarized structures built of directionally aligned αβ-tubulin dimers. Their "minus" end is anchored at so called microtubule-organizing centers, whereas the "plus" ends can extend or shrink and interact with different intracellular structures [49]. Cells able to readily reorient their polarity axis, such as fibroblasts, generally present a radially organized microtubule array, whose changes are mutually related to cell polarity and can mechanically contribute to cell asymmetry by promoting cell elongation [34]. The results of the present study show that, following cell activation by cytokines, changes in microtubule density and orientation occurred and probably contributed to the observed cell elongation.

Following IL-1β and TNF-α stimulation, the actin filament network changed as well. Density and thickness of actin filaments increased, while their distribution underwent a rearrangement, giving rise to an array of filaments aligned parallel to the major cell axis. Considering that microtubules are connected to the layer of actin filaments close to the cell membrane through a complex of adaptor proteins often associated with focal adhesions, the changes in microtubule and actin filament networks are probably interrelated, and the rearrangement in α5β1 membrane integrin distribution observed in cytokine-stimulated cells further support this hypothesis. The inhibition of the inflammatory response due to laser treatment led to a partial recovery of the basal cytoskeleton organization in fibroblasts irradiated after cytokine stimulation. Cytoskeleton changes connected with effects produced by NIR laser irradiation have been previously described in different cell models [25,50] and depend on cell type, cell status, parameters, and sources used.

α-SMA is one of the six actin isoforms. Together with β- and γ-actin isoforms, α-SMA is expressed in some fibroblast/myofibroblast subpopulations in the basal state, where it has a cytoplasmic localization and participates in stress fiber formation. α-SMA is strongly induced by mechanical stress and TGF-β1 in activated myofibroblasts [51], therefore it is generally considered a marker of fibroblast-myofibroblast transdifferentiation. In the present study, dermal fibroblasts stimulated with IL-1β and TNF-α showed α-SMA expression seemingly concentrated in the nucleus, while cytoplasmic stress fibers, to some extent present in unstimulated cells, completely disappeared in the stimulated ones. These results are consistent with in-depth investigations on α-SMA distribution and roles carried out in the last two decades. It has been demonstrated that the apparent nuclear localization is due to deep invaginations of the nuclear membrane filled of α-SMA [52]. The role of the nuclear invaginations is currently quite completely unknown, but it has been hypothesized that these structures could be involved in cellular and nuclear mechanotransduction, nuclear transport, calcium signaling, cell differentiation [52–54]. Moreover, in agreement with our results, it has been found that TNF-α suppresses α-SMA expression and stress fiber formation in dermal fibroblasts and that persistent inflammation, mediated by TNF-α, might prevent normal matrix deposition and myofibroblast-dependent wound contraction mediated by TGF-β1 in physiological wound healing [55]. The inhibition of stress fiber formation would turn the cells into a phenotype more migratory and less able to generate tractional forces [51], with possible consequences and delay in the healing process.

Additionally, in the case of α-SMA, NIR laser treatment after IL-1β and TNF-α partially prevented the cytokine effect and some stress fibers reappeared inside the cells. α-SMA expression following red- or NIR-laser treatment has been widely studied being connected with the effectiveness of laser therapy in promoting wound healing and avoiding scarring. The results have been controversial, showing both down- and up-regulation of α-SMA expression [56–58]. This variability in results is possibly due to the

many different models (from cell cultures to animal models both normal and representing serious diseases, such as diabetes), laser sources, treatment protocols and parameters, and times at which analysis of α-SMA expression was performed. Interestingly, some studies in which the analysis of α-SMA expression was performed at different healing times after laser treatment showed that α-SMA expression changed in the different healing phases and resulted significantly different from controls only at specific time points [59]. The only unambiguous result is that laser irradiation is able to modulate α-SMA, but the modulation depends on many factors, among which the healing phase and corresponding cell phenotype (e.g., the phenotype of fibroblasts in the inflammatory phase is different from what they assume in the remodeling phase). This means that further studies are needed to develop treatment protocols suitable for the different patient's conditions and, in case of wounds, healing phase. However, the data of the present study indisputably demonstrate that, even at the cytoskeletal level, the source and the treatment parameters used are effective in counteracting the changes induced by cytokine stimulation, thus returning the cells to the basal state.

Compared to controls, the IL-1β and TNF-α stimulated fibroblasts showed increased fibronectin (FN) expression and assembly observed in the same samples. The increase in FN, a major ECM component, could be expected since the pro-inflammatory cytokines IL-1β and TNF-α, together with TGF-β, are considered potent fibrogenic initiators [60]. The increase in expression of α5β1 observed in the same samples is consistent with that of FN, considering that α5β1 is a membrane integrin able of binding FN [61].

A number of studies investigated the expression and role of α5β1 and its ligand FN in fibroblasts during inflammation and wound healing. In the healing process, α5β1-mediated fibroblast-FN interaction is crucial: α5β1 is involved in myofibroblast differentiation and granulation tissue formation by promoting FN assembly in a fibrillar structure. In the granulation tissue, a reduced ability to bind FN via integrin α5β1 might allow fibroblasts to migrate in the early FN-rich matrix and invade the wound [62]. On the other hand, some studies demonstrated that α5β1 integrin is able to confer strong cohesivity to 3D cellular aggregates linking adjacent cells together via FN, and that the FN with its dimeric structure is essential for this process [63]. Moreover, it has been suggested that α5β1-FN interaction contributes to clot retraction [63]. Therefore, α5β1 and FN play a crucial role in wound healing, and alterations in their expression can lead to healing impairment and fibrosis. These conditions can affect ECM remodeling by stimulating collagenase production and stimulating/inhibiting collagen/glycosaminoglycan biosynthesis depending on the target cells and experimental conditions.

The effects of the pro-inflammatory cytokines IL-1β and TNF-α on ECM remodeling and their role in fibrosis have been studied for many years with controversial results. In the present study, the cytokine-stimulated dermal fibroblasts showed increased expression of MMP-1 and collagen I, which have key roles in ECM degradation and building, respectively, thus modulating ECM turnover. In agreement with literature, the intracellular distribution of MMP-1 was associated with mitochondria [64] and, probably, the cytoskeleton. In fact, a relation between actin system dynamics and MMPs has been speculated because it has been observed that cytoskeleton changes often precede MMPs modulation and actin microfilament dynamics might be linked to the expression of MMP genes [65]. Regarding collagen I, contrary to what observed for FN, in stimulated fibroblasts it showed an intracellular localization and no extracellular fibrils were observed. If an increase in MMP-1 expression following IL-1β and/or TNF-α stimulation has been unanimously reported [39,66–68], the effects the two cytokines have on collagen synthesis remain uncertain. Many studies reported that both IL-1β and TNF-α inhibit collagen I synthesis [67,69–71], but other studies demonstrated that IL-1β and TNF-α increased collagen I synthesis in human renal fibroblasts [72] and in murine intestinal myofibroblasts [38], respectively. A proposed scenario is that, in some conditions, the antifibrotic effect of TNF-α is overwhelmed by its central role in driving inflammation [66].

Fibroblasts stimulated with IL-1β and TNF-α and then exposed to NIR laser radiation recovered features more similar to unstimulated controls as regards the expression and distribution of α5β1, FN, collagen I, and MMP-1. Therefore, laser treatment was also able to counteract the cytokine effects on α5β1 integrin and the proteins involved in ECM turnover and remodeling after injury. It is noteworthy that, in the case of FN, not only the expression returned to levels comparable with unstimulated controls, but in samples treated with laser radiation the fibrils showed a more ordered and parallel distribution. This effect of laser radiation on FN and collagen fibril organization has already been described [73] and could be connected with the laser radiation's ability to prevent fibrotic scars. The influence of red-NIR laser radiation on the expression of α5β1 integrin, FN, collagen, and MMP-1 has already been investigated in studies concerning laser application in the management of inflammatory response and wound healing. The results of these studies are controversial. A recent study on a model of diabetic wounded fibroblast cells showed that PBMT (660 nm wavelength) downregulated the expression of the genes *FN1*, *ITGA5*, and *ITGB1*, encoding for FN, α5, and β1 integrin subunits, respectively [74]. In a study on an immunosuppressed rat wounded model, PBMT by an 810 nm pulsed laser induced an increase in FN expression [75]. Enhanced FN expression was found also in human fibroblasts irradiated with a 940 nm diode laser [58]. A research on the effects of different protocols of PBMT in the healing of open wounds in rats showed that all the protocols used induced an increase in collagen deposition, but at different extent, depending on wavelength and fluence applied [43]. Using similar fluence but a different wavelength, a decrease in collagen production was found in wounded human skin fibroblasts [76]. In a rat model of wound healing, collagen deposition did not increase 3 days after laser treatment, but it increased significantly at day 7 after treatment [77]. Sakata et al. [28] found that, in chondrocytes stimulated with IL-1β, MMP-1 increased and then decreased after NIR irradiation applied post-stimulation, in complete agreement with what has been observed in the present study on fibroblasts activated by IL-1β and TNF-α.

From the outcomes of the studies mentioned above, it is evident that expression and function of α5β1 integrin, FN, collagen, and MMP-1 can be modulated through application of PBMT. However, results so uneven as those reported in the literature about PBMT effects demonstrate once again that it is very difficult to compare studies carried out using different experimental models, laser sources, and treatment parameters. Laser source and treatment protocol should be characterized for their biological effects before application for the management of specific pathological conditions.

In this paper, an in vitro model of fibroblast activation via stimulation with the pro-inflammatory cytokines IL-1β and TNF-α has been proposed and used to test the anti-inflammatory effect of a dual wavelength NIR laser source widely used in clinics to promote healing and reduce inflammation and pain. Like all in vitro models, a limit of the proposed model is to provide a very partial representation of what happens in vivo during inflammation and healing (a single cell population and two pro-inflammatory cytokines vs. many cell populations and a plethora of pro- and anti-inflammatory molecules). However, it can be considered representative of the early stage of the inflammation phase after an injury, when M1 macrophages produce great amounts of IL-1β and TNF-α. In the normal evolution of inflammation, macrophage phenotype is expected to shift from M1 to M2, with increased TGF-β production and a decrease in IL-1β and TNF-α levels [60]. Therefore, the proposed model can be considered also representative of altered evolution of inflammation with persistence of high levels of TNF-α, compared to TGF-β levels, due to the failure to switch from M1 to M2.

Using this model, the effectiveness of PBMT by a dual wavelength NIR laser source (MLS-MiS) in reducing inflammation has been tested, and the results obtained show that PBMT, administered through the laser source and protocol here described, is significantly effective in preventing the effects of IL-1β and TNF-α, thus modulating the cell inflammatory response and favoring cell return to the basal physiological state.

The anti-inflammatory effect of red-NIR laser radiation has been already reported in a number of studies but, to the best of our knowledge, it is the first time that it is evaluated and confirmed in an "in vitro" model of IL-1β and TNF-α activated dermal fibroblasts. Moreover, the significant anti-inflammatory activity of the laser emission tested in the present research is consistent with our previous studies carried out with the same laser source. In an in vitro model of myoblasts, it was found to increase the expression of NLRP10, a potent inhibitor of inflammasome activation and IL-1β and IL-18 production, as well as that of PP1, which regulates many important cell functions and favors cell recovery from stress to basal state [25]. In vivo, the same laser emission was able to reduce inflammatory infiltrate and accelerate the healing of ulcers in feline stomatitis [78] while, in a rat model of neuropathic pain induced by trauma, it significantly lowered inflammation and pain and preserved the myelin sheath [79]. It is well known that, when released by cells under pro-inflammatory stimuli, IL-1β and IL-18 induce the production of other pro-inflammatory cytokines, such as interferon-γ (INF γ), TNFα, IL-6, etc., thus triggering a cascade of events which further increase and perpetuate inflammation. Therefore, the ability of the proposed laser treatment to inhibit IL-1β and IL-18 release, through increased NLRP10 production, could explain its effectiveness in controlling fibroblast activation induced by IL-1β and TNF-α stimulation, thus damping excessive inflammatory response. Further studies could help to define treatment protocols specific for each different healing phase.

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Effect of MLS® Laser Therapy for the treatment of experimentally induced acute tendinopathy in sheep – a preliminary study.

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ABSTRACT

Tendon injuries are common in human athletes and sport horses. Unfortunately, traditional treatments are limited in their ability to completely heal injured tendons. Recent advances in low-level laser therapy (LLLT) have shown promising results. This study evaluated the effect of Multiwave Locked System (MLS®) laser therapy in collagenase-induced tendon lesions in sheep. Six animals were randomly assigned to two groups, with group 1 receiving ten MLS® Laser Therapy treatments at 5 J/cm^2 on the left hind limb and group 2 receiving the same number of treatment at 2.5 J/cm^2 on left hind limb. The right hind limb was considered a control for both groups. Clinical follow-up, ultrasonography and histological examinations were performed on the injured tendons. Clinical and histological evaluations demonstrated that using a therapeutic dose less than 5 J/cm^2 resulted in an anti-inflammatory effect. Moreover, the histological examinations showed a statistically significant reduction in cell number in both treated groups and a significant decrease in vascularization in the treated tendons in group 2. MLS® Laser Therapy appears to be an effective tool to improve collagen fiber organization in the deep digital flexor tendon.

INTRODUCTION

Overuse tendinitis and other tendon injuries are common among athletes [1,2] and represent a frequent cause of lameness in sport horses [3,4]. In the human medical field, Low Level Laser Therapy (LLLT) has been used to treat acute and chronic musculoskeletal pain and foster wound healing [5]. However, few studies have evaluated its effectiveness in treating patients with acute tendonitis and other tendinopathies [6].

As demonstrated in the literature, LLLT acts on two phases of the healing process[7]. First, it reduces PGE2 concentrations and inhibits cyclo-oxygenase [8,9,10,11]. Secondly, it modulates fibroblast metabolism and collagen deposition due to its anti-inflammatory effect. Histological changes observed in tendons receiving LLLT include increased collagen production [12], improved collagen bundle organization [13,14] and an increased number of small blood vessels [5,15].

Animal models are commonly utilized in tendon disorder research [16] and the collagenase-induced tendinitis model has been used to study acute inflammatory responses [8]. This model has been used in rats, sheep and horses, and mimics a traumatic tendon injury [17,18]. The sheep is recognised as a model for human and equine orthopaedic injuries, including tendinopathy, due to the similar connective tissue structure of the flexor tendons in these species [19,20,21]. Numerous authors have described the positive effects of LLLT in experimental trials in rats [9,16,22,23], mice [13,24] and rabbits [12]. However, to our knowledge, there are no studies investigating the effect of LLLT on experimentally induced tendinitis in sheep. This preliminary study was designed to investigate the effect of MLS® (Multiwave Locked System) laser therapy on an experimental model of collagenase-induced tendinitis in sheep in order to evaluate a specific treatment for human and animal athletes.

MATERIALS AND METHODS

This study was approved by the University Ethics Committee for Animal Experimentation (CEASA) and by the Italian Ministry of Health on 17 May 2010 (DM no. 97/2010-B). Six healthy adult female Bergamasca sheep weighing 50-60 Kg were included in the study. Prior to enrollment,

clinical and ultrasound examinations were performed to confirm tendon integrity.

A defect was produced in the deep digital flexor tendons (DDFT) of both hind limbs by collagenase injection as previously described [21,25]. Intravenous administration of 10 µg/kg of medetomidine (Sedator®, Ati srl Ozzano dell'Emilia, Italy), and 2 mg/kg of propofol (Rapinovet® Intervet Italia, Peschiera Borromeo, Italy) were used to anaesthetize the animals. After aseptic disinfection and placement in lateral recumbency, 500 IU of sterilized bacterial collagenase type 1A (C-9891; Sigma, Milan, Italy) in 0.13 ml of saline solution was injected bilaterally (left and right hind limbs) into the DDFT under ultrasound guidance. A 23-gauge needle was used to perform the injection. The needle was introduced 15 cm distal to the calcaneal bone and was inserted into the full thickness of the DDFT using a lateral approach with the hock joint flexed at 90°. A suture was applied close to the injection site to mark the precise location for treatment and tendon harvesting. Antibiotic therapy using amoxicillin-clavulanic acid (Synulox® Pfizer Italia, Rome, Italy) at a dose of 12.5 mg/kg SC was started and continued for 5 consecutive days. Buprenorphine (Temgesic® RB Pharmaceuticals, Slough, UK) at a dose of 0.01 mg/kg IM BID for 5 days was used to provide analgesia.

Seven days after collagenase injection, the 6 sheep were divided into two groups (group 1 and 2) and treated using MLS® Laser Therapy. The MLS® Laser Therapy was performed using an Mphi veterinary laser device (ASA, Arcugnano-VI, Italy), equipped with combined, synchronized and overlapping continuous and pulsed emissions from a single handpiece. Continuous emissions or continuous interrupted emissions were produced by an InGa(Al)As diode laser with the following parameters: wavelength of 808 nm, peak power of 1000 mW for continuous wave, mean power of 500 mW for continuous interrupted wave, spot area of 3.14 cm^2, spot diameter of 2 cm. Pulsed emissions were produced by an InGaAs/GaAs diode with the following parameters: wavelength of 905 nm, peak power of 25 W, mean power of 54 mW at 1500 Hz, pulsed wave, spot area of 3.14 cm^2, spot diameter of 2 cm. Following the protocol of Bjordal and Lopes-Martins (2013), the applications were performed daily for 5 days followed by 2 days of no treatment and then daily for 5 additional days [26]. All treatments were conducted by the same individual. Scan modality was based on the size and shape of the treatment area (Fig. 1). The equipment was calibrated before the start of every session using the Powermeter Ophir Nova II Display S/N 573995. In group 1, the left hind DDFT received MLS® laser treatment at a dose of 5 J/cm^2. In group 2, the left hind DDFT received MLS® laser treatment at a dose of 2.5 J/cm^2. The right hind DDFT was considered an internal control (without treatment) for both groups.

Fig. 1 MLS® Laser Therapy

Sheep in both groups were monitored daily by evaluating the circumference, swelling and heat of the limb at the point of injury. Pain on limb palpation and degree of lameness were also assessed using a previously developed scoring system ranging from a grade of 0 to 4 [27]. Tendon thickness and echogenicity of the wound area were evaluated ultrasonographically [17,28]. Ultrasound examinations, using a GE Medical System LOGIQ P5 machine and linear 6-10 MHz probe, were performed 7, 21 and 37 days after lesion creation.

At day 37 after tendon lesion creation (30 days after the first laser treatment), the animals were sedated and anesthetized as previously described. The animals were subsequently euthanized using an intravenous injection into the auricular vein of 10 ml of a combination of drugs approved for euthanasia (Tanax®, Intervet, Milan, Italy). After euthanasia, the tendons were surgically removed from the calcaneus to the end of the metatarsal region and the DDFT of both hind limbs harvested for histological analysis. Tendons were removed 5 cm proximally to 5 cm distally of the lesion previously marked by a cutaneous surgical stitch. Harvested DDFTs were cut into 1 cm pieces and the proximal–distal orientations were marked. Tissue samples for histology were fixed in 4% parformaldehyde (PFA) and embedded in paraffin. Sections were cut into 5 µm slices, mounted on microscope slides and stained using Harris hematoxylin and eosin (HE). Sections were analyzed for cell density, vascularization and tissue organization using specific markers to evaluate fibroblast and tissue/matrix organisation characteristics. A quantitative analysis was performed to compare differences in cell number between groups. Differences in vascularization were also evaluate by looking at the ratios of blood vessel areas. Three segments were processed from each tendon, with 5 slides taken from each segment and three microscopic fields examined per slide, resulting in a total of 540 fields evaluated.

Analyses were performed using STATISTICA 9 (StaSoft) software, and data were assessed for normality using a Shapiro–Wilk test. Differences among the experimental groups within each sampling were evaluated using a Kruskal–Wallis Test. In all analyses, a $p < 0.05$ value was considered significant.

RESULTS

After the collagenase 1A injection, an inflammatory reaction with a mild localized thickening of the DDFT was detected in all subjects. Lameness, ranging from grade 3 to 4, and pain (detected by palpation) remained evident for the first 3-5 days. A localized

increase in temperature around the point of injury was detected manually for the first 3 days. From day 7 of the MLS® Laser Therapy, an inflammatory reaction was observed in the treated limbs of group 1, with about 1 centimeter increase in wound circumference and an increase in the temperature of the metatarsus (Fig.2); whereas no worsening of lameness or pain was observed. This inflammatory response was not observed in the treated limbs of group 2.

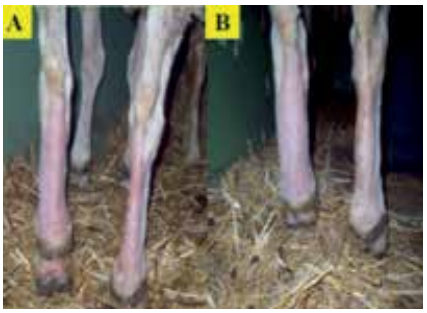

Fig. 2 Inflammatory reaction observed in the treated limbs of group 1 during MLS® Laser Therapy in left limb: A after collagenase induction, B 7 days after MLS® Laser Therapy

A progressive reduction in limb circumference was observed by the end of treatment for both groups. Limb circumference returned to a value close to the starting size only in group 2. In addition, local temperature, lameness and pain decreased in all subjects. The treated left DDFT showed a more rapid reduction in local inflammation compared to the right DDFT in all sheep. Ultrasound examinations detected the presence of a lesion in the DDFT 7 days after lesion creation and during the entire follow-up period. Better collagen-fiber alignment and more uniform filling of the lesions were observed in the treated limbs compared to the control limbs. During the follow-up period, group 1's treated limbs showed a marked thickening of the DDFT compared to the control limbs. Histological analysis revealed that there was disorganization of the extracellular matrix, increased vascularization and increased cell density in the DDFTs of the control limbs. In contrast, the sections obtained from tendons treated with MLS® Laser Therapy showed a more uniform and organized extracellular matrix, a lower number of cells and a better realignment of collagen fibers. The quantitative analysis revealed a significant decrease in fibroblasts in the treated legs compared to the control legs in group 1 (5 J/cm²). However, the ratio of the vessel areas did not differ between the control and treated tendons. In group 2 (2.5 J/cm²), the treated legs also had a decrease in fibroblast number compared to the control legs. A significant decrease in vascularity was observed in the treated tendons compared to the control tendons.

DISCUSSION

This is the first experimental study to evaluate the effect of LLLT on the tendon healing process in a sheep model. We evaluated the effects of two different doses of MLS® Laser Therapy in the acute phase of induced tendon lesions in order to determine a suitable therapeutic range for physiotherapy in human and veterinary medicine. The latest reviews on the effectiveness of LLLT in human medicine[5,6], highlighted the need to identify a specific treatment protocol for tendinopathy. Several authors [13,14,15,16,23,29,30,31] have reported the efficacy of LLLT in increasing the calcaneal tendon's mechanical properties as well as increasing the alignment of collagen fibers and angiogenesis, with doses between 3 and 5 J/cm². In the present animal model study, we initially decided to use 5 J/cm², which is the average value reported in the literature for treatment of acute tendinitis in human medicine. A 50% reduction in radiant fluence was elected for the second group (2.5 J/cm²) because the clinical symtomps and ultrasonographical data demostrated an increase of inflammatory response in group 1 during and after MLS® treatment. The tendon circumference in the first treatment group did not return to normal after a month of follow-up. In contrast, in the second group, the tendons' external morphology returned to the physiological state by the end of the trial. In particular, the clinical manifestations of two sheep worsened slightly in the initial treatment phase, with an increase in circumference at the injection site, a local rise in temperature and a slow remission of symptoms. The aggravation of the inflammatory condition, observed during the applications of MLS® Laser Therapy in group 1, appears to indicate that a dose of 5 J/cm² was excessive for treatment of acute tendinitis with this type of laser emission. Instead, the dose of 2.5 J/cm² (group 2) appears to be suitable to produce an anti-inflammatory and biostimulating effect on tendon healing. Results from histological examinations indicated that both treatments induced a statistically significant decrease in cell number, although the values only returned to normal in the second group. Moreover, the MLS® dose of 2.5 J/cm² (group 2) caused a significant decrease in blood vessel area and better improvement of collagen fiber organization in deep digital flexor tendon compared to group 1 and the control group.

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AUTHOR DISCLOSURE STATEMENT

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Laser therapy in the management of neuropathic pain: preliminary experience on 43 patients

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ABSTRACT
The aim of this case series is to report on the effect of MiS, a new laser therapy device which uses two synchronized emissions with 905 nm and 808 nm wavelengths, pulsed and continuous, respectively, with high peak power, in the management of neuropathic pain. A total of 43 patients (mean age: 53 years, from 23 to 85 years) presenting neuropathic pain associated to different anatomical areas, such as cervical zone, spine, foot/ankle, hand/wrist, shoulder, elbow, hip and knee were treated with laser therapy by MiS. Pain (VAS score) and functionality (therapist evaluation) were evaluated at the end of treatment. The severity of pain decreased over time and was lower at the end of treatment. MiS laser therapy demonstrated to be safe and effective in patients affected by neuropathic pain and represents a valuable tool for the management of these patients.

INTRODUCTION
Pain is described as a complex, subjective experience, involving the transduction of harmful environmental stimuli together with the cognitive and emotional processing by the brain [1,2]. Neuropathic pain is a form of chronic pain resulting from any kind of damage to the central or peripheral nervous system without nociception [3,5]. Neuropathic pain is a painful condition that may comprise different types of pathologies: such as postherpetic neuralgia, painful diabetic polyneuropathy, post-surgery neuropathic pain, multiple sclerosis, spinal cord injury, stroke and cancer. Patients with neuropathic pain often have spontaneous pain, allodynia, and hyperalgesia.
The estimation of the incidence and prevalence of neuropathic pain is difficult because of the lack of simple diagnostic criteria for large epidemiological surveys in the general population [6]. A portion of these patients is specifically affected by peripheral neuropathy and seek medical treatment to alleviate the pain and improve the function associated to conditions that are localised at several body levels: spine, being lumbar-sciatic pain a very common problem, cervical area, elbow, wrist and hand, knee, ankle and foot, hip. For instance, sciatica is a form of radicular pain, and is described as a disease of the peripheral nervous system. It is a very common condition and the main cause of absences from work, with great economic impact on society [7]. The trend in terms of life expectations getting longer suggests that more and more people will be experiencing this type of pain in their life. Chronic neuropathic pain is characterised by complexity of neuropathic symptoms, poor outcomes and difficult treatment decisions.
On the biological side, nerve inflammation plays an important role in the development and progression of neuropathic pain. For instance, recent studies have indicated that hypoxia-inducible factor 1a (HIF-1a) is crucial in inflammation [8], while other previous studies have identified the relationship between proinflammatory cytokines, and neuropathic pain development [9-12].
Therapeutic options are in many cases related to conservative treatment, consisting of modifying the pain-precipitating activity, biomechanical correction with physiotherapy or the use of antidepressants, analgesics and/or steroids [13,14]. Specifically, painkillers are the main drugs to treat pain, although these have shown only 30% effectiveness in patients with neuropathic pain [15-17]. Unfortunately, these drugs have undesirable side effects and, currently, there is a worldwide trend in opioid reduction for acute and chronic pain management [18-20]. Physical methods are an interesting alternative to the pharmacological treatment because of the absence of side effects.
Recent studies have reported the use of laser therapy in patients with peripheral somatosensory neuropathy and neuropathic pain [21,22]. Specifically, clinical studies on the effects of laser therapy on injured nerves reported an increase in nerve function [21]. Moreover, laser therapy demonstrated to be effective for promoting axonal growth in injured nerves in animal models [23-26].
Positive effects of MLS® therapy in promoting repair processes of peripheral nerves, acting on the recovery of the lesioned function and the muscle mass and inducing faster myelinization of the regenerated nerve fibers, have been reported by Gigo-Benato et al. [27].
In vitro studies were carried out to characterize the effect of MLS® pulse and have shown that MLS® treatment induces an increase of NLRP 10, a protein with anti-inflammatory action.

NLPR 10 inhibits the activity of caspase 1 and PYCARD protein complex, which promote the maturation of the inflammatory cytokines interleukin-1 β (IL-1β) and interleukin 18 (IL-18). Therefore, ultimately, NLPR 10 inhibits the production of pro-inflammatory interleukins IL-1β and IL-18, reducing inflammation [28]. The decrease in inflammation leads to a normalization of vascular function and thus to a decrease of the edema. Obviously, the decrease in inflammation and edema results in a decrease of pain symptoms, that are frequently present in patients.

MiS is a medical device for laser therapy which combines the synchronized pulse of traditional MLS® therapy with the high peak power typical of high intensity laser therapy. These specific characteristics allow MiS to act on pain and its causes, leading to significant and persistent improvement of pain symptomatology and concomitant recovery of functionality.

This case series collects the case reports of two physiotherapy centers that have treated 43 patients for peripheral neuropathy using MiS, a new laser therapy, reporting changes in pain and in function, and safety associated to the use of the device.

MATERIALS AND METHODS

This is a case series collecting patients from routine practice in two Italian physiotherapy centers: Fisiolab (Vicenza- Italy) and Rehability Center (Padova- Italy). Forty-three patients of both sexes affected by several conditions related to peripheral neuropathies have been included in the series.

During the treatment, patients and therapists wore safety glasses to prevent eye damage.

Diagnosis and instrumental evaluation (i.e. X-Ray, Ultrasounds, CT, MRI), when available, were recorded.

Additionally, patients were evaluated by the specialist performing the treatment before therapy start.

The patients that were included in this series received MiS (ASA Srl, Italy) treatment focused on the peripheral neuropathy as stand-alone treatment or as a part of their treatment programme.

MiS is a class IV NIR laser with two synchronised sources, one consists in 6 pulsed 905 nm laser diodes, the second is a continuous/frequency-modulate 808 nm laser diode. Maximum average power is 6W± 20%, while maximum peak power is 1kW. MiS has 2 interchangeable handpieces (with diameters of 2 and 5 cm).

The total number of sessions and the time of each session were adjusted based on each patient response to the treatment and ranged from 2 to 13 sessions, with a duration ranging from 6 to 20 minutes (according to body location). The used frequency was 30 Hz for all the body areas, while intensity was adapted to the anatomical site as follows: 80% for shoulder and hip, 70% for spine, 60% for elbow, wrist/hand, knee, ankle/foot and 50% for head and cervical area. Dosage was adjusted based on size of the area to be treated, patient and pathology characteristics and condition stage.

Trigger points, when present, were treated in all patients with the following parameters: Frequency: 10 Hz, time: 23 s, Intensity: 25%. In the trigger point phase, the hand piece was perpendicular to the treated points.

Pain evaluation was performed before and after each laser session using a Visual Analogue Scale (VAS) scale. It is a scale comprising 10 grades, with 10 representing 'unbearable pain' and 0 representing 'no pain'. It is a pain scale commonly used in the medical field, and it was shown to be a reliable and valid measure of pain [29,30]. Safety has been specifically assessed and the therapists recorded any side effect and/or rebound effect happened during the treatment. Functional evaluation and global assessment were reported by the specialist for each patient.

RESULTS

Demographic and clinical characteristics of all patients at baseline were recorded. Patients demographic characteristics are reported in Table I.

For 34 patients, peripheral neuropathy treatment was the focus of the overall therapy cycle, while 9 patients received other MiS treatments beside the peripheral neuropathy protocol (i.e. specific for edema, muscle pain and contracture) in their therapeutic path.

VAS pre and post treatment, along with change in VAS expressed as a percentage of the initial value are reported in Table II, divided by treated anatomic areas. As expected when dealing with neuropathic pain, average pain at baseline was moderate to severe (mean was >7 for all groups). Overall, VAS pre-treatment mean was 7,8 and VAS post-treatment mean was 1,6, corresponding to a decrease in pain of 79,5%. Pain completely disappeared in patients treated for elbow, hip and shoulder problems. Considering all the groups, improvement was at least 60% respect to baseline, meaning that initial pain score was reduced of above 60% at the end of the treatment cycle.

It has to be noted that some patients were not seeking medical treatment for pain, but for symptoms related to nerve irritation, as for example paraesthesia, dysesthesia, hyporeflexia, etc. In these cases, the treatment with MiS

Table I - Demographics

Sex	M=55,8% F=44,2%
Active (sport activity)	YES=46,5% % NO = 44,2% NA=9,3%
Age	Mean= 53 yrs (23 to 85)

Table II - VAS pre and post treatment divided by anatomical distribution of the treated areas

Area	Patient #	VAS Pre (mean)	VAS post (mean)	δVAS%
Spine	17	8,8	2,2	75%
Cervical area	3	8,3	3	63,9%
Elbow	4	9	0	100%
Knee	4	8	1,5	81,3%
Ankle/foot	3	7	2	71,4%
Hip (mainly pudendal nerve)	9	7	0	100%
Shoulder	1	9	0	100%
Wrist/Hand	2	7,5	2,5	66,7%
TOTAL	**43**	**7,8**	**1,6**	**79,5%**

gave excellent results and the therapists have reported strong improvements in sensitivity and dysesthesia reduction.

In general, looking at VAS value trend, it was possible to appreciate pain decrease during time, rather than intra-session. Some patients, reported fluctuation in VAS score between the sessions during the treatment cycle. This could be related to a prompt increase of physically demanding activities by the patients after perceiving benefit from the initial laser therapy sessions.

In general, laser treatment provided a positive impact on pain and function on the majority of the patients, only for 2 of them no significant improvement after the laser therapy cycle was reported.

DISCUSSION

Neuropathic pain can substantially impair quality of life as it often associates with other problems, such as loss of function, anxiety, depression, disturbed sleep and impaired cognition and physical therapies have been suggested as potential alternative for treatment [6]. The results of this case series show that patients treated with MiS for peripheral neuropathy had an improvement in terms of pain symptoms measured with VAS, even when starting from high VAS values, typical of neuropathic pain. The improvement was gradual and was normally seen after some sessions rather than at the end of each laser treatment, suggesting that MiS is able to induce biological responses whose effects depend on the evolution of the underlying biological processes over time, which could be interesting to address in future basic and clinical studies. MiS inherits the wavelengths (808 nm and 905 nm), the characteristic synchronized modulation of continuous and pulsed emissions, and the scientific evidence of the action mechanisms from MLS® laser therapy. Experimental and clinical research demonstrated that MLS® pulse exerts a positive effect in the treatment of many musculoskeletal diseases [31-34]. This effect is related to anti-inflammatory, anti-edema and tissue healing actions [28,35]. Besides relying on MLS® pulse features, MiS is characterised by a very high peak power in the order of kW. The modulation in short pulses allows to control the peak power avoiding damaging thermal effects.

In the literature, Kobiela Ketz et al [36] suggested that the reduction of hypersensitivity mediated by laser treatment in a model of neuropathic pain induced by spinal nerve injury could be exerted by modulating macrophages and microglia components. Preliminary *in vivo* investigation related to laser therapy use in neuropathic pain relief highlighted therapeutic effects that might be used for clinical application in neuropathic cases [37].

In the specific field of neuropathic pain, preclinical experiments carried out on animal models demonstrated that the treatment with MiS promotes the recovery of the myelin sheat in nerve fibres that have been damaged in the lesion area, as confirmed by histological and immunohistochemical evaluations [38]. These data support the concept that laser therapy by MiS could be a suitable tool in the management of neuropathic pain.

No rebound effect has been observed, thus confirming the safety of the device in this cases series, which included individuals with different characteristics, pathologies and stage of conditions.

Patients gave a positive feedback on the treatment feeling, especially when the 5 cm handpiece was used on large areas, as its shape allowed a sort of massage over the patient's skin, making the treatment well accepted and contributing to build compliance to session attendance.

CONCLUSION

This case series reports on the use of MiS in the management of 43 cases of neuropathic pain localised in different anatomical areas. Based on the results reported, the new MiS laser therapy demonstrated to be safe and effective in patients affected by neuropathic pain. Therefore, laser therapy by Mis may represent a valuable and well-accepted tool for the management of peripheral neuropathies.

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ORIGINAL ARTICLE

Photobiomodulation therapy (PBMT) on acute pain and inflammation in patients who underwent total hip arthroplasty—a randomized, triple-blind, placebo-controlled clinical trial

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Abstract

When conservative treatments fail, hip osteoarthritis (OA), a chronic degenerative disease characterized by cartilage wear, progressive joint deformity, and loss of function, can result in the need for a total hip arthroplasty (THA). Surgical procedures induced tissue trauma and incite an immune response. Photobiomodulation therapy (PBMt) using low-level laser therapy (LLLT) and/or light-emitting diode therapy (LEDT) has proven effective in tissue repair by modulating the inflammatory process and promoting pain relief. Therefore, the aim of this study was to analyze the immediate effect of PBMt on inflammation and pain of patients undergoing total hip arthroplasty. The study consisted of 18 post-surgical hip arthroplasty patients divided into two groups ($n = 9$ each) placebo and active PBMt who received one of the treatments in a period from 8 to 12 h following THA surgery. PBMt (active or placebo) was applied using a device consisting of nine diodes (one super-pulsed laser of 905 nm, four infrared LEDs of 875 nm, and four red LEDs 640 nm, 40.3 J per point) applied to 5 points along the incision. Visual analog scale (VAS) and blood samples for analysis of the levels of the cytokines TNF-α, IL-6, and IL-8 were recorded before and after PBMt application. The values for the visual analog scale as well as those in the analysis of TNF-α and IL-8 serum levels decreased in the active PBMt group compared to placebo-control group ($p < 0.05$). No decrease was observed for IL-6 levels. We conclude that PBMt is effective in decreasing pain intensity and post-surgery inflammation in patients receiving total hip arthroplasty.

Keywords Phototherapy · Low-level laser therapy · Light-emitting diodes · Total hip arthroplasty (THA)

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by the wear of articular cartilage, marginal osteophyte formation, ligament, synovial and meniscal changes, and damages of the subchondral bone [1, 2]. During early stages of the disease, the degenerative process is slow but progresses over time. In advanced stages of OA, abnormal remodeling of cartilage and formation of osteophytes irreversibly destroy the affected joint [3]. Activities of daily living (ADLs) involving load bearing at the hip joint become compromised due to pain, and prognosis is often poor [4] directly interfering with quality of life of patients [1–4].

Conservative treatment is often no longer effective in later stage of OA, and total hip arthroplasty (THA) is an alternative often used in these cases [5] to relieve symptoms [6, 7].

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Although known as a surgical extreme procedure, post-surgical quality of life (QoL) improves and many patients often return to work [6, 7]. Even knowing that THA surgery can be successful, there are issues that need to be addressed related to postoperative pain management. Inadequate postoperative pain management is a worldwide problem, and the need to improve its management is well documented [5]. However, the tissue trauma leads to an inflammatory reaction and immune response with release of some mediators such as cytokines [8–11]; therefore, the surgical procedure can cause significant postoperative pain [12]. Postoperative pain is associated with increased hospital length of stay, delayed ambulation, and long-term functional impairment [13]. A more focused effort is needed to develop postoperative pain management, particularly during the first few days after surgery [14].

Postoperative pain management following THA is a major concern for both patients and their caregivers particularly during the first few days after surgery [14]. The rise of inflammation dominates the initial phase of repair and a postoperative pain management will likely include the use of NSAIDs for analgesia. Published studies show that most NSAIDs have an adverse effect on osteoblast growth by cell cycle arrest and apoptosis induction [15]. Also, the potential risk of heart attacks and strokes has been known for years, and it applies to even short-term use of the medication for people with or without heart disease [16].

Photobiomodulation therapy (PBMt), using low-level laser therapy (LLLT) and light-emitting diode therapy (LEDT), has been shown to be an effective in pain reduction, modulation of inflammation, and promoting repair of tissue [3, 17–20]. Additional studies have demonstrated positive effects of PBMt in cell proliferation, microcirculation, vascular neoformation, collagen production from fibroblasts, and bone repair [21–23]. PBMt is virtually without side effects and has minimal contraindications for use. Comparisons with non-steroidal anti-inflammatory drugs (NSAIDs) in animal studies found optimal doses of PBMt and NSAIDs to be equally effective in treatment of different musculoskeletal disorders [24–35]. PBMt offers a better risk-benefit profile compared to NSAIDs and is a safe, non-pharmacological adjunct therapy in the management of acute pain.

However, there is a lack of information regarding the use of non-pharmacological complementary therapies that offer less risk of side effects, since the use of non-steroidal anti-inflammatory drugs (NSAIDs) are commonly associated with these effects [36, 37]. In addition, although NSAIDs are widely prescribed, they have been shown to have limited efficacy in pain relief [36, 37]. In this perspective, the aim of this study was to evaluate the effect of PBMt that combines multiple light sources, power outputs, and wavelengths on acute pain and serum levels of inflammatory markers in patients following postoperative hip arthroplasty.

Material and methods

Study design and ethics statement

A randomized, triple-blinded (patients, therapists, and outcome assessors), placebo-controlled trial was performed. The present study was submitted and approved by the research ethics committee (process number 066490). All patients voluntarily agreed to participate and signed the informed consent statement. The study was conducted at, between July and August of 2015.

Characterization of sample

The sample size calculation was performed based on a pilot study performed by our research group. For sample size calculation, we considered the β value of 20% and α of 5%. In pilot study used as reference for sample size calculation, it was observed that PBMt led to decreased pain (our primary outcome) using visual analog scale (VAS) to 53.90 mm (± 11.50) immediately after PBMt irradiation compared to baseline (68.80 ± 13.30). Thus, the calculation resulted in a sample of 9 volunteers per group, 18 volunteers in total.

Eighteen post-surgical THA patients participated in the study. Each patient underwent the same surgical procedure and technique performed by the same surgeon. During surgery, the head and part of the neck of the femur were resected; the acetabulum was prepared to receive high-density polyethylene that fits into a metal hemi-sphere to replace the femoral head which was then connected to a rod inserted in the medullary canal of the femur. The fixing of the femoral head may or may not be cemented. The surgeon used an acrylic plastic polymethylmethacrylate, and the rod was fixed under pressure [38]. The consolidated standard of reporting trials (CONSORT) flowchart summarizing experimental procedures and subjects are shown in Fig. 1.

Inclusion criteria and exclusion criteria

Inclusion criteria:

- Patients that were in immediate postoperative period from 8 to 12 h after total hip arthroplasty, due to diagnosis of OA. The diagnosis of OA was conducted by an orthopedic surgeon, based on the previous history of OA in these patients. In addition, the surgeon evaluated radiological images in the anteroposterior profile of the hip of these patients. The surgeon used Dejour's classification [39] to describe the severity of hip OA;
- Both genders.

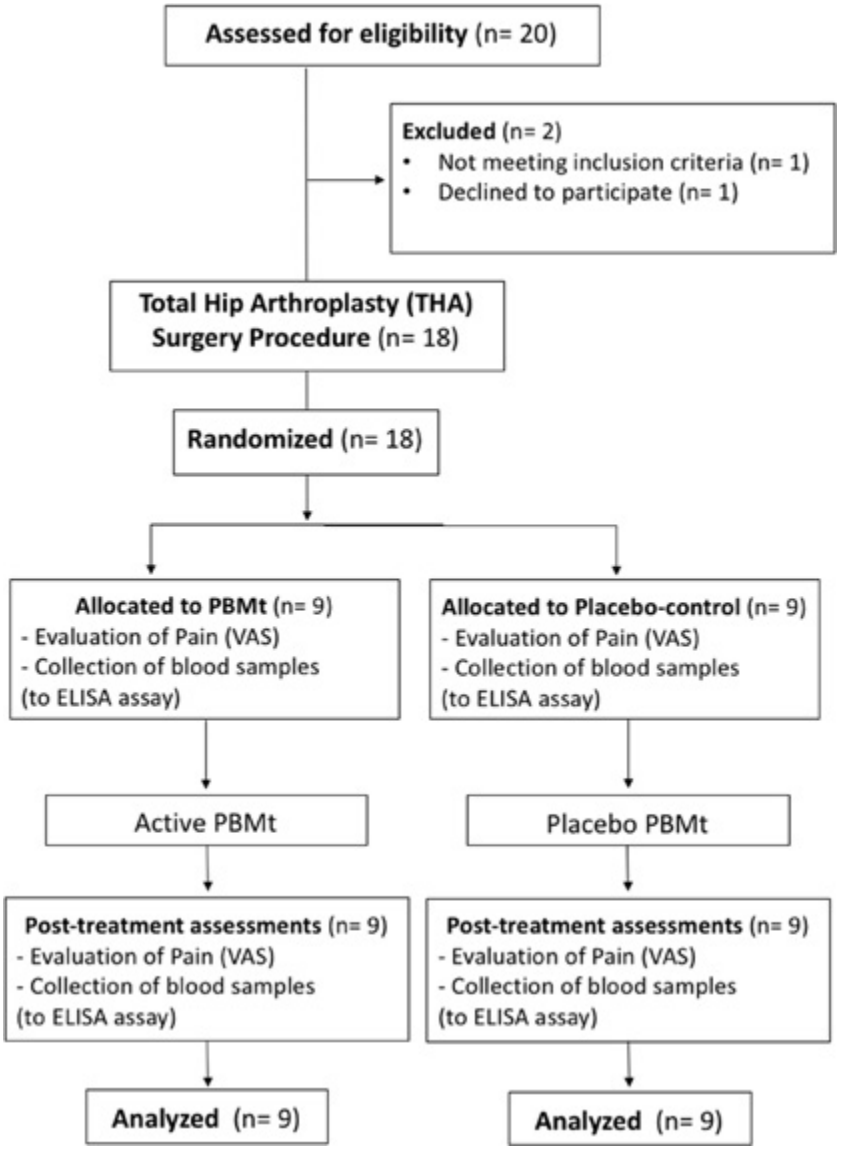

Fig. 1 CONSORT flowchart

Exclusion criteria:

- Any hip surgery that was not due to OA;
- Any hip surgery other than total arthroplasty;
- Neurological and cognitive problems, as dementia, mental retardation, communicative deficit, or any other condition that would make it impossible to understand the study procedures;
- Postoperative complications such as deep vein thrombosis and infections.

Randomization

Prior to initiation of treatment, the 18 patients were randomized into two experimental groups (nine patients per group). Randomization was carried out by a simple drawing of lots (A or B) performed by a participating researcher not involved with the recruitment or evaluation of patients. This same researcher was responsible for programming the PBMt device according to the result of the randomization and was instructed to not disclose the identity of the devices to anyone involved in the study. The PBMt device used displayed the same setting and emitted the same sounds regardless of the programmed dose and mode (active PBMt or placebo PBMt). Patient and therapist were blinded throughout the treatment. Randomization labels were created using a randomization table at a central office. Concealed allocation was achieved through the use of sequentially numbered, sealed, and opaque envelopes.

Interventions

A single application of PBMt (active or placebo) was performed within postoperative period from 8 to 12 h, immediately

after the baseline evaluation. The active and placebo PBMt were performed using the same device and the irradiated sites were the same in both therapies. To ensure blinding for patients and therapists, the device emitted the same sounds and the same information on the display regardless of the programmed mode (active or placebo). The device in the placebo mode and in the active mode had the brightness of the red light source, but in the placebo mode, the emitted light had only 0.5 mW of power output for each red diode, and both super-pulsed laser, infrared LEDs, and magnetic field were turned off. This amount of power used in placebo mode is negligible but ensures the brightness of the red light without therapeutic effects. In this way, it is impossible to discern between the two PBMt modes (placebo and active). The device was previously coded as active or placebo modes, and only one researcher not involved in the randomization, treatment, and evaluation was aware of these codes. The intervention specifications were as follows:

1. *PBMt group:* PBMt was applied using a cordless, portable PainAway/PainCure™ device (manufactured by Multi Radiance Medical, Solon-OH, USA) at five sites/points over the full extent of the surgical scar, with a distance of 2 cm between sites (Fig. 2). The cluster style emitter contains 9 diodes comprising 1 super-pulsed laser diode (905 nm, 2.7 mW average power, 8.5 W peak power, and 0.81 J dose for each diode), 4 red LEDs (640 nm, 15 mW average power, and 4.5 J dose for each diode), and 4 infrared LEDs (875 nm, 17.5 mW average power, and 5.25 J dose for each diode). The total irradiation time per site was 300 s and the total energy delivered was 39.8 J. The choice of these parameters was based on a previous study using the same technology [40]. The optical power of the device was checked before irradiation (in each patient) by a researcher that was not involved in data collection and analysis. For such, it was employed a Thorlabs® thermal power meter (Model S322C, Thorlabs®, Newton-NJ, USA). The full description of PBMt parameters is provided in Table 1.
2. *Placebo-control group:* The placebo PBMt was delivered using the same device that active PBMt but without any emission of therapeutic dose (placebo mode). Patients received a total negligible dose of 0.6 J per point/site in placebo mode. Furthermore, the sources of infrared light and superpulsed are off and the electromagnetic field is inactive.

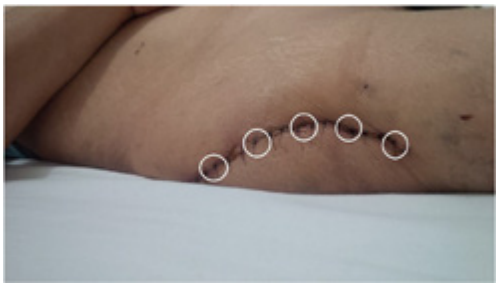

Fig. 2 Sites of PBMt and placebo irradiation (white circles) on the surgical scar

Outcomes

The outcomes were pain intensity and levels of cytokines (interleukin [IL]-6, IL-8, and tumor necrosis factor alpha [TNF-α]) obtained at baseline (pre-treatment) and immediately (within 10 min) after irradiation with PBMt (post-treatment). These outcomes were collected by an assessor who was not aware of patient allocation to their treatment groups.

The primary outcome of the study was pain intensity measured by visual analog scale (VAS). Visual analog scale (VAS) evaluates pain intensity levels perceived by the patient, with assistance of an assessor, on a scale ranging from 0 to 100 mm, with 0 being "no pain" and 100 being "the worst possible pain".

The secondary outcome of the study was the cytokine levels measured by *enzyme-linked immunosorbent assay (ELISA) method. For such*, blood samples were collected by a qualified nurse blinded to group allocation and were obtained from the antecubital vein. One hour after collection, each sample was centrifuged at 3000 rpm for 20 min. Pipettes were used to transfer the serum to Eppendorf® tubes, which were stored at − 80 °C until analysis. The levels of IL-6, IL-8, and TNF-α in the blood samples were determined by enzyme-linked immunosorbent assays, using a commercial kit and following the manufacturer's instructions (BD Biosciences®, USA). Spectrophotometric readings were performed in a SpectraMax® Plus 384 Absorbance Plate Reader (Sunnyvale, CA, USA) with 450-nm wavelength and correction to 570 nm. The results were expressed in pg/ml.

Statistical analysis

The statistical analysis was conducted following the principles of intention-to-treat analysis [41]. Initially, the data was tabulated and evaluated for normality using the Shapiro-Wilk test. As a normal distribution was verified, unpaired, two-tailed, Student's t test was used to detect difference among groups. The significance level was set at $p < 0.05$. The data in the graphs are presented as mean and standard error of the mean (±SEM). The researcher that completed the statistical analysis was blinded to randomization and allocation of patients in experimental groups.

Table 1 Parameters for PBMt

Number of lasers	One super-pulsed infrared
Wavelength (nm)	905 (± 1)
Frequency (Hz)	3000
Peak power (W)	8.5
Average optical output (mW)	2.7
Power density (mW/cm^2)	9.66
Energy density (J/cm^2)	2.9
Dose (J)	0.81
Spot size of laser (cm^2)	0.4
Number of red LEDs	4 Red
Wavelength of red LEDs (nm)	640 (± 10)
Frequency (Hz)	2
Average optical output (mW)—each	15
Power density (mW/cm^2)—each	16.66
Energy density (J/cm^2)—each	5
Dose (J)—each	4.5
Spot size of red LED (cm^2)—each	0.9
Number of infrared LEDs	4 Infrared
Wavelength of infrared LEDs (nm)	875 (± 10)
Frequency (Hz)	16
Average optical output (mW)—each	17.5
Power density (mW/cm^2)—each	19.44
Energy density (J/cm^2)—each	5.83
Dose (J)—each	5.25
Spot size of LED (cm^2)—each	0.9
Magnetic field (mT)	35
Irradiation time per site (s)	300
Total energy delivered (J)	39.8
Aperture of device (cm^2)	4
Application mode	Cluster probe held stationary with slight contact with patient skin with a 90-degree angle.

Results

Eighteen acute postoperative arthroplasty patients were recruited for this study and completed all procedures with no dropouts. The average age in PBMt group was 69 (± 5.6), height of 165.00 cm (± 11.00), and body weight of 70 (± 9.56), 55.5% were male and 44.4% female. In the placebo-control group, average age was 67 (± 6.4), height of 169.00 cm (± 5.00), and body weight of 79 kg (± 11.00), 33.3% were male and 66.6% female. There was no difference between experimental groups for demographics' characteristics ($p > 0.05$).

Figure 3 demonstrates that PBMt significantly decreased ($p < 0.05$) pain compared to placebo-control. At baseline (before treatment), placebo-control group showed 56.70 mm (± 10.00) at VAS scale, while PBMt group showed 65.60 (± 12.40), without difference between groups. Immediately following treatment, PBMt group decreases pain intensity when compared to placebo-control group ($p < 0,05$), as shown in Fig. 3.

Fig. 3 Change in pain assessed using 100 mm VAS. Values are mean and error bars are SEM. Asterisk indicates significant difference between placebo/control and PBMt groups ($p < 0.05$)

As shown in Fig. 4, the levels of IL-6 showed no statistically significant difference between the placebo-control (pre-treatment 361.67 ± 29.89 pg/ml and post-treatment 352.88 ± 18.32 pg/ml) and PBMt groups (pre-treatment 350.30 ± 33.38 pg/ml and post-treatment 338.19 ± 28.60 pg/ml) at none time points tested.

The levels of IL-8 in placebo-control and PBMt groups were 191.29 pg/ml (± 15.52) and 190.28 pg/ml (± 15.99), respectively, showing homogeneity between groups at baseline evaluation. At post-treatment evaluation, there was a statistically significant decrease in IL-8 in favor of PBMt group compared to placebo-control group ($p < 0,05$), as showed in Fig. 5.

The levels of TNF-α in placebo-control and PBMt groups were 467.73 pg/ml (± 24.47) and 469.88 pg/ml (± 26.50), respectively, showing again homogeneity between groups at baseline evaluation. At post-treatment evaluation, there was a statistically significant decrease in TNF-α in favor of PBMt group compared to placebo-control group ($p < 0,05$), as shown in Fig. 6.

Discussion

The results of this study support the hypothesis that photobiomodulation therapy applied to the surgical incision in the postoperative period reduces acute pain and inflammation in patients after hip arthroplasty. Previous studies using laser therapy and the visual analog scale as a means of evaluation also reported less pain in the post-surgical period, corroborating the results of this study [42–44].

Photobiomodulation therapy has been identified as an effective, safe, and non-invasive modality able to modulate the inflammatory process [17, 18, 20, 36]. The current study adds to this collective body of evidence. It is

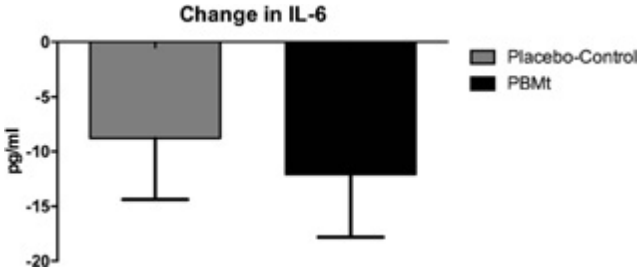

Fig. 4 Change in IL-6 levels measured by ELISA immunoenzymatic assay. Values are mean and error bars are SEM

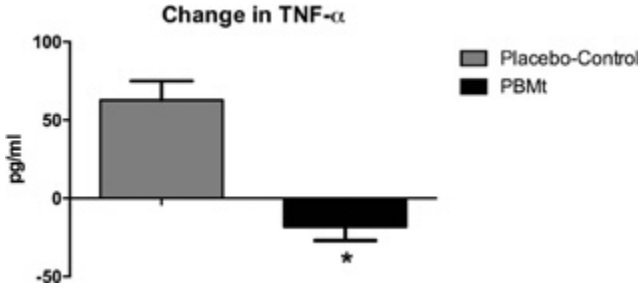

Fig. 6 Change in TNF-α levels measured by ELISA immunoenzymatic assay. Values are mean and error bars are SEM. Asterisk indicates significant difference between placebo-control and PBMt groups ($p < 0.05$)

known that surgical injuries in the hip joint induce immune response mediated by inflammatory cytokines [8–11].

There was modulation of the inflammatory process in the arthroplasty postoperative period in the group treated with the active phototherapy. These results are in accordance with previous studies in the literature in which authors demonstrated decreased in pain scores and inflammatory markers in patients treated with laser therapy over the surgical incision in tibial fractures postoperative [45]. Decrease in pain and reduced administration of analgesic drugs was also observed in patients treated with laser therapy in distal radius fracture in the immediate postoperative period [46].

In order to observe the action of PBMt on serum levels of proinflammatory citokynes IL-6, IL-8, and TNF- α, mediators released during acute inflammation, we performed an analysis using the immunoenzymatic test. In this study, we chose not to perform blood tests before the surgery, since there is a prior consensus in the scientific literature regarding the increase of cytokines and their kinetic behavior, which occur after post-surgical hip arthroplasty, highlighting the acute inflammatory character [9, 11, 47].

Our results show reduced IL-6 levels in the group treated with effective PBMt, although this reduction was not statistically significant. The results are consistent with previous studies that investigated the effects of PBMt in decreased IL-6 release [17, 21]. This reduction in IL-6 release may not be significant because of the short time interval chosen between the end of surgery and the application (8 to 12 h after surgery). Several authors report that an increase in peak concentrations occurs on the first day and remains until the third day post-surgery [10, 11].

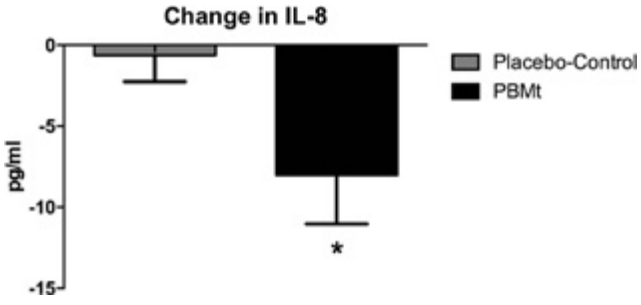

Fig. 5 Change in IL-8 levels measured by ELISA immunoenzymatic assay. Values are mean and error bars are SEM. Asterisk indicates significant difference between placebo-control and PBMt groups ($p < 0.05$)

Regarding the release of IL-8, our results show a statistically significant decrease in serum levels of this cytokine in the group treated with effective PBMt. This allows us to infer that PBMt attenuates the levels of this cytokine. A previous study showed increased IL-8 at the end of surgery with peaks in 6 h and decrease on the first day, indicating its chemotactic character and short duration [11]. The findings of this study also indicate a statistically significant decrease in serum levels of proinflammatory cytokine TNF-α. Since in the early stages of inflammation, an increased presence of TNF-α stimulates IL-6 and IL-8 synthesis attributing the acute inflammatory process in surgical trauma [8, 10, 11], modulation of this cytokine minimizes this process. Thus, once the action of these mediators is controlled, the patient has a less aggressive inflammatory process due to the surgical procedure [11]. Acute inflammation can lead to functional impairment to the patient. Therefore, modulation, not elimination, of the inflammatory process and its subsequent signs and symptoms is paramount to the success of the tissue repair and quality.

Our results provide us with evidence that a single application of PBMt added to postoperative treatment attenuates inflammation and significantly reduces acute inflammatory pain. These results point to a possible decrease in the administration of analgesic drugs, which besides having high cost for health systems and have adverse or side effects [36, 37]. Improving pain control may decrease length of stay, decrease costs, enhance functional recovery, and improve long-term functional outcomes [13].

The results also provide a better understanding of the role of PBMt with the parameters used on the modulation of inflammatory mediators, as well as in the decrease of pain, being of great value, since it may guide future therapeutic interventions. However, it is important to highlight that the parameters

used in the present study were chosen according to previous study [40] and according to instructions provided by the manufacturer. Further studies are important to substantiate the findings described in this study and to investigate if the parameters used are the most adequate for pain control in patients submitted to THA.

This study has some limitations such as the lack of assessment of cytokines prior to surgery in order to have more data to ensure the homogeneity of groups, which happened due to our full access to patients only after the surgery, making impossible to analyze the inflammatory markers before the surgery. However, it is important to highlight that the analysis of these mediators after the surgery (before treatments) showed homogeneity between the groups. Finally, it is important to report that magnetic therapy may have an effect of reducing pain, and this aspect warrants further investigation.

Conclusion

In this study, PBMt (phototherapy) with the parameters used as the immediate postoperative treatment of hip arthroplasty provided pain decrease and decreased serum levels of proinflammatory cytokines (IL-6, IL-8, and TNF-α). PBMt is a safe treatment and without reported side effects, and can be suggested as a possible therapeutic modality in the immediate hip arthroplasty postoperative period.

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Compliance with ethical standards

The present study was submitted and approved by the research ethics committee (process number 066490). All patients voluntarily agreed to participate and signed the informed consent statement.

Competing interests Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH, USA), a phototherapy/photobiomodulation device manufacturer. Douglas Scott Johnson is an employee and a shareholder of Multi Radiance Medical (Solon-OH, USA). The remaining authors declare that they have no conflict of interests.

Ethical aspects All experimental procedures were submitted and approved by the Research Ethics Committee of Nove de Julho University (process number 066490). All patients signed an informed consent form prior to enrollment.

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Published online 2014 June 21. **Research Article**

The Effect of Low-Level Laser on Postoperative Pain After Tibial Fracture Surgery: A Double-Blind Controlled Randomized Clinical Trial

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Background: Postoperative pain is a common complication that can lead to serious morbidities and delayed recovery.
Objectives: The aim of this study was to investigate the effect of low-level laser therapy on acute pain after tibial fracture surgery.
Patients and Methods: In this randomized clinical trial, 54 patients who were candidate for tibial fracture surgery were allocated randomly to two groups, namely, control and laser therapy. Both groups had the same type of surgery and technique of spinal anesthesia. Patients in laser group were treated with the combination of two lasers (GaALAs, 808 nm; and GaALInP, 650 nm) at the end of the surgery while control group received laser in turn-off mode with the same duration as laser group. Patients were evaluated for pain intensity according to the visual analogue scale (VAS) and the amount of analgesic use during 24 hours after surgery.
Results: Laser group experienced less pain intensity in comparison with control group at second, fourth, eighth, 12^{th}, and 24^{th} hours after surgery (P Value < 0.05). In addition, the amount of consumed opioid in laser group was significantly less than the control group (51.62 ± 29.52 and 89.28 ± 35.54 mg, respectively; P Value, 0.008).
Conclusions: Low Level Laser Therapy is a proper method to reduce postoperative pain because it is painless, safe and noninvasive and is easily accepted by patients.

Keywords: Low Level Laser Therapy; Postoperative Pain; Tibial Fracture Surgery

1. Background

One of the undesirable complications of surgery is postoperative pain that may result in serious morbidities such as agitation, hypertension, mood changing, tachycardia (1, 2) and delay in wound healing, which can be more dangerous in patients with the underlying diabetes mellitus, hypertension, or coronary heart diseases as it may lead to fatal complications such as myocardial infarction (3). There is a high variability among patients in tolerance to pain and analgesic requirement (4, 5). The studies show that about 80% of patients experience a mild to severe pain after surgery (6). There is inadequate postoperative analgesia in the half of all surgeries, can lead to chronic postoperative pain (7). Several methods are available to control and reduce postoperative pain such as administering opioids or nonsteroidal anti-inflammatory drugs (NSAIDs) and patient-controlled analgesia (PCA). It is established that the use of systemic opioids alone is not sufficient to relieve postoperative pain. In most cases, inadequate dosage is prescribed to reduce the side effects of these drugs like respiratory depression and therefore, the medication cannot control pain completely (8, 9). Analgesic nephropathy, skin reactions, and peptic ulcers are common side effects of nonsteroidal anti-inflammatory drugs (10). Recent advances present new techniques for prevention and reduction of postoperative pain. One of the most important technologies of this century is the use of low-level laser (LLL) at the site of surgery (11).

Low-level laser therapy (LLLT) was pioneered at Russia and Hungry and then at Europe in early 1960s. It is a branch of laser treatments that has been indicated for pain killing and wound healing. LLLT uses irradiation with laser light of low intensity and its effects are not due to producing heat. These nonthermal effects are thought to be mediated by a photochemical reaction that alters cell membrane permeability, leading to increased mRNA synthesis and cell proliferation. FDA has started differ-

Implication for health policy makers/practice/research/medical education:
Postoperative pain is a common complication that can lead to serious morbidities and delayed recovery. The aim of this study was to investigate the effect of low-level laser therapy on acute pain after tibial fracture surgery

ent investigations on LLLT for 15 years and has approved the use of LLLT for pain relief in carpal tunnel syndrome since 2002 (11, 12). It is also used to treat damages in sport injuries and musculoskeletal disorders. In addition, it is applicable to reduce neck pain and the size of keloid scarring after surgery (13-17). Many studies found that LLL stimulates respiratory cycle in mitochondria and increases adenosine triphosphate molecules (14) that reduce swelling and pain (16). In another study, applying LLL directly over painful points was useful in treatment of stress fracture of tibia (18). The LLL is effective in relieving pain of knee osteoarthritis, breast augmentation surgery, and cryosurgical treatment of oral leukoplakia (15, 17).

2. Objectives

Pain following orthopedic surgeries are considered severe pain (19, 20); hence, the aim of this study was to investigate the effect of LLLT on acute pain after tibial fracture surgery.

3. Patients and Methods

This double-blind, controlled, randomized clinical trial was conducted in 2012-2013 in Imam Khomeini Hospital, Ahvaz, Iran. The study was approved by the Ethical Committee of Jundishapur University of Medical Sciences (ETH-654) and all subjects signed an informed consent.

Sample size was calculated at 27 in each arm of the study by setting the power at 80% and the values for $Z_{1-\alpha/2}$, $Z_{1-\beta}$, P_1, and P_2 at 1.96, 0.84, 0.68, and 0.32, respectively, based on a previous observational study (21). A total of 54 patients aged between 18 and 60 years who were candidate for tibial fracture surgery in American Society of Anesthesiologists (ASA) classes I and II were allocated randomly to two equal groups of control and laser. All subjects were matched based on their age, weight, and height. Patients who were pregnant, those with malignant tumors, benign tumors with malignant potential, hypersensitivity to light, e.g. systemic lupus erythematosus, coagulopathies, high intracranial pressure, history of chronic pain, those on long-term opioids or other painkillers during the preceding month, or those who did not agree to undergo spinal anesthesia were excluded from the study.

Monitoring equipment including electrocardiograph, pulse oximeter and sphygmomanometer were employed for all patients; they received 10-mL/kg intravenous lactated Ringers' solution and then spinal anesthesia was induced by the anesthesiologist.

Spinal anesthesia was induced by intrathecal administration of 10-mg 0.5% bupivacaine (Astrazeneca Co., Germany) with 25-gauge needle in the sitting position and with the midline technique.

If the systolic blood pressure dropped by 20% or more, 10-mg ephedrine would be injected intravenously. Upon achieving successful anesthesia, pull-tight elasticated tourniquet was clamped and operation was started. The surgical procedures were similar in both groups and included open reamed interlocking intramedullary nailing, which is the preferred approach for treatment of tibial shaft fractures (22).

After the surgery and before the final bandage in surgery room, patients in laser group were treated with a combination of two lasers (Canadian Optic and Laser Center, Canada): (1) GaALAs hand held probe (PLP-IR) with wavelength of 808 nm and 300-mW output power in continuous mode (dose, 6 J/cm^2; area, 1 cm^2; and time, 20 s/point); and (2) GaALInP hand held probe (PLP-R) with wavelength of 650 nm and 100-mW output power in continuous mode, (dose, 3 J/cm^2; area, 1 cm^2; and time, 30 s/point).

Each tibial fracture was radiated from four sides in contact technique with the combination of IR and R laser in dose of 9 J/cm^2 (medial, lateral, anterior, and posterior sides of fracture region and popliteal fossa). For radiation on popliteal fossa, the legs were elevated by 60° angels.

In addition, trigger points on muscles and surgical wounds (6-8 points) were radiated with 4 J/cm^2 by the same combination of IR and R lasers (ten seconds of each laser; 3 J/point IR plus 1 J/point R laser).

For placebo laser treatment in control group, all those sites were treated with the lasers in turn-off mode with the same duration.

One of authors who was blind to the group allocation and did not participate in the laser therapy procedures, filled out the questionnaires. The amount of total analgesic and pain intensity at second, fourth, eighth, 12th, and 24th hours after the surgery were investigated in both groups. Pain intensity was quantified by visual analogue scale (VAS) in which zero and ten represented analgesia and worst possible perception of pain, respectively. If VAS was three or more, 0.3 mg/kg of pethidine was injected intravenously.

3.1. Statistical Analysis

The data are presented as mean ± standard deviation (SD). We performed Shapiro-Wilk test and Levene's test for normality of the data distribution and equality of variances. Independent samples t test, repeated measure test, and Bonferroni post hoc test were used to analyze the data. P Value of less than 0.05 was considered as statistically significant. All the statistical analyses were done by SPSS software version 16 (SPSS Inc, Chicago, IL, USA).

4. Results

Demographic characteristics of participants are presented in Table 1. Two groups were similar in terms of age, weight, height, and body mass index. There was no significant difference between groups regarding the duration of surgery (57.34 ± 3.2 and 56.29 ± 3.4 minutes in control and laser groups, respectively; P = 0.71) and anesthesia duration (84.14 ± 5.21 and 85.02 ± 4.98 minutes in control and laser groups, respectively; P = 0.69).

Table 1. Demographic Characteristics of the Participants [a,b]

Groups	Age, y	Weight, kg	Height, cm	BMI, kg/m^2
Control Group	24.61 ± 2.76	71.22 ± 11.34	169 ± 6	72.16 ± 12.71
LLLT Group	25.05 ± 2.68	72.27 ± 10.80	171 ± 5	70.09 ± 13.23
P value	0.628	0.777	0.791	0.706

[a] Abbreviations: LLLT, low-level laser therapy; and BMI, body mass index.
[b] Data are presented as mean ± SD.

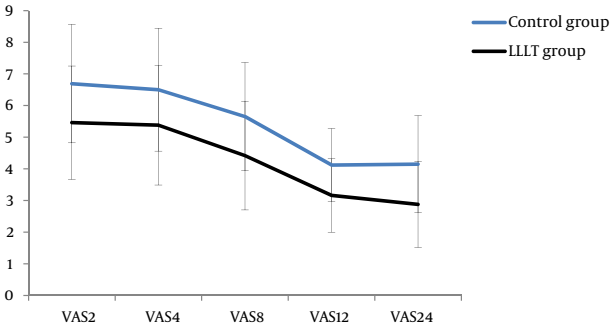

Figure 1. Pain Intensity at Different Hours in lllt and Control Groups

Table 2. Postoperative Pain Intensity [a, b]

Groups	VAS at Different Time Points After Surgery				
	2nd h	4th h	8th h	12th h	24th h
Control Group	6.69 ± 1.87	6.50 ± 1.94	5.65 ± 1.71	4.12 ± 1.15	4.15 ± 1.53
LLLT Group	5.46 ± 1.79	5.38 ± 1.89	4.42 ± 1.72	3.16 ± 1.16	2.88 ± 1.36
P value	0.019	0.041	0.013	0.006	0.012

[a] Abbreviations: LLLT, low-level laser therapy; and VAS, visual analogue scale.
[b] Data are presented as mean ± SD.

Based on VAS, mean scores of pain intensity after operation in different periods are presented in Table 2. Pain reduced considerably at second, fourth, eighth, 12th, and 24th hours after surgery in laser group in comparison with the control group. Although there were no significant differences in pain intensity between the second and fourth, the fourth and eighth, the eighth and 12th, as well as the 12th and 24th hours in each group ($P > 0.999$, $P = 0.110$, $P = 0.681$, and $P > 0.999$ in control group; $P > 0.999$, $P = 0.099$, $P = 0.097$, and $P > 0.999$ in laser group, respectively), there were significant differences between the second and eighth, the second and 12th, the second and 24th, the fourth and 12th, the fourth and 24th, as well as the eighth and 24th in each group ($P < 0.001$, $P = 0.010$, $P < 0.001$, $P = 0.009$, $P < 0.001$, and $P = 0.002$ in control group; $P < 0.001$, $P = 0.002$, $P < 0.001$, $P = 0.002$, $P < 0.001$, and $P < 0.001$ in lase group, respectively; Figure 1).

The mean of total amount of analgesic (pethidine) used in laser group was significantly less than control group. The mean of total amount of analgesic was 51.62 ± 29.52 and 89.28 ± 35.54 mg in laser and control groups, respectively ($P = 0.008$).

5. Discussion

Pain as a stressor, stimulates the physiological and psychological responses. Its outcomes have a direct effect on the postoperative complications, recovery time, and patient's satisfaction with the health system. The aim of this study was to investigate the effect of LLL with the wavelengths 650 and 808 nm on pain after tibial fracture surgery. The results of this study showed that pain reduction was significant at the second, fourth, eighth, 12th, and 24th hours after surgery (P Value ≤ 0.05). Similarly, Moore et al. showed that low level gallium-aluminum-arsenide laser for four to six minutes at the end of the cholecystectomy had no significant effect on pain reduction at the first and the fourth hours after surgery; however, the effect was significant at the eighth, 12th, 24th, and 48th hours after surgery (21). Hegedus et al. reported that the use of LLL (wavelength, 830 nm; continuous wave; and power, 50 mW) in patients with knee osteoarthritis resulted in pain reduction and improvement in joint movement (15). Jackson et al. found that laser irradiation with wavelength of 630 to 640 nm at the beginning and at the end of breast augmentation surgery reduced the postoperative pain (23). Moreover, Ribeiro et al. reported that AsGaAl laser (660 nm) could decrease the pain as well as postoperative recurrence rate in patients with oral leukoplakia (17).

The results of our study showed the mean total amount of analgesic use in laser group was significantly lower than the control group ($P < 0.05$). This finding is consistent with the findings of other researchers who reported that LLLT could decrease pain during and after the surgery and had a positive effect on wound healing and edema (12). LLLT is used in muscular fatigue (24), wound healing, and pain reduction in dental procedures in patients with and without diabetes (25-27). The researches showed that LLL could cause analgesia by reducing prostaglandin E2 (28, 29), raising endorphin level, and increasing urinary excretion of serotonin, the pain receptors stimuli. LLLT also has a negative effect on pain neurotransmitters and prevents accumulation of acetylcholine, a pain stimulus in the receptors (30).

The results of this study showed that the combination of laser therapy and analgesic medications had better effect during the 24 hours of recovery after the surgery. Laser radiation at wavelengths of 650 and 808 nm (R and IR laser) can decrease postoperative pain and analgesic use in postoperative period. LLLT does not have side effects like respiratory depression, skin reaction, and analgesic nephropathy that are seen with other methods. It is recommended to perform more studies concerning the applications of LLLT in anesthesia field as it is a noninvasive, safe, and acceptable analgesic method in patients in recovery or surgery room.

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Authors' Contributions

Study concept and design: Sholeh Nesioonpour and Soheila Mokmeli. Analysis and interpretation of data: Sarah Hojjati and Salman Vojdani. Manuscript preparation: Salman Vojdani, Ahmadreza Mohtadi, Reza Akhondzadeh, Kaveh Behaeen, and Shahnam Moosavi. Collection of data: Salman Vojdani and Sarah Hojjati. Critical revision: Sholeh Nesioonpour.

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Efficacy of class IV diode laser on pain and dysfunction in patients with knee osteoarthritis: a randomized placebo-control trial

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Objectives
The aim of this study was to investigate the effect of class IV diode laser on knee pain and functions in patients with knee osteoarthritis.

Patients and methods
Fifty patients with a mean±SD) age of 55.68±8.88 years, height of 173.84±4.946 cm, weight of 83.86±5.28 kg, and BMI of 27.78±1.89 kg/cm^2 were randomly assigned equally into two groups (25 patients in each group). Group I received a multiwave locked system laser plus exercises and group II received placebo laser plus exercises three times weekly for 4 weeks. Exercise program was applied for both groups three times weekly for 4 weeks. The exercises included range of motion, stretching, isometric, and isotonic resisted exercises to the quadriceps and hamstring muscles. Pain was evaluated using a visual analog scale and knee function by using the Western Ontario and McMaster Universities Index of Osteoarthritis (WOMAC). Statistical analyses were performed to compare differences between baseline and post-treatment results for both groups.

Results
Visual analog scale and WOMAC were significantly decreased in both groups after 4 weeks of treatment, with a more significant decrease gained in group I ($P>0.0001$).

Conclusion
Class IV diode laser combined with exercise was more effective than exercise alone in the treatment of patients with knee osteoarthritis. Multiwave locked system laser combined with exercise effectively decreased pain and WOMAC as compared with the placebo laser plus exercises group.

Keywords:
class IV laser, knee function, knee osteoarthritis, multiwave locked system, pain

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1110-6611

Introduction

Osteoarthritis (OA) is a group of conditions that lead to joint signs and symptoms, which are associated with defective integrity of articular cartilage that can affect the normal daily activities in the elderly with higher social and financial influences on either patients or society [1]. It is characterized by joint inflammation, synovitis, and articular cartilage degeneration with many clinical manifestations such as pain, reduced range of movement, crepitus, tenderness, impairment of muscular performance, and functional capability [2].

The most commonly involved joint is the knee with impaired life quality and associated morbidity [3]. Knee OA commonly affects the elderly, and especially women [4]. One-third of the people aged 65 years and older have knee OA, which is evident by radiography. The incidence of the OA tends to increase with aging, with increasing pain and disability of the lower limb, which can seriously affect one's life activities [3]. Although OA is commonly seen as a progressive pain, and chronic disorder with functional limitation, early therapeutic approach can minimize its symptoms [5].

The treatment includes several interventions, both pharmacological and nonpharmacological, according to the degree of joint destruction [6]. Treatment of OA aimed to relieve pain, increase the limited range of motion (ROM), and promote cartilage regeneration. Weight reduction and exercise alone or combined with patient education, electrical stimulation, magnetic field, ultrasound, and low-level laser therapy (LLLT) could relieve pain and improve function [7,8]. Although these modalities provide only symptomatic relief and cannot modify the degenerated cartilage structure, its side effect is low and comparable to the other NSAIDs [9]. Laser

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therapy was reported to have an analgesic effect that can relieve both acute and chronic musculoskeletal pain [10]. Moreover, it is commonly used in the treatment of degenerative knees OA in animals and humans. Studies on animals reported that LLLT decelerates the arthritic process by altering level of prostaglandin, increasing the levels of proteins, and improving the repair of degenerating cartilages by increasing the number of chondrocytes and the thickness of the articular cartilage [11,12].

Researchers investigated the clinical effect of laser in the treatment of knee OA. Some authors reported a positive effect on pain relief [13], whereas the other authors disagreed with this result [14,15]. These controversial results may be because of the differences in parameters (wavelength, dose, time, area, technique) used in treatments by different studies [16]. Thus, it is important to choose optimum parameters to achieve therapeutic response in patients with knee OA. In the previous studies, LLLT was used in the treatment of knee OA in specific points [17,18], with an average of 6 J/point. Scanning of the related area such as the quadriceps, hamstring, and the calf muscles was not easy. Because of the large area of laser radiation and low-power laser generator, the time of application will be too much or even not applicable. Now, the presence of new types of class IV lasers, which provide a high laser power (<0.5 W), help to deliver an adequate level of fluency (energy density) that is sufficient to cover a large area of treatment and to stimulate the physiological responses [19]. The safety of new high-power laser, which approved by Food and Drug Administration, helps to scan the related area in addition to stimulating specific points on the joint line.

The multiwave locked system (MLS) is a novel treatment used for a variety of diseases causing pain and inflammation. The MLS laser is a synchronized laser, which has continuous emission of 808 nm with pulsed emission of 905-nm diode lasers. In comparison with the LLLT, the synchronization of two laser wavelengths provides a high-power class IV laser (808 nm, with a maximum power 1 W, and 905 nm, with a maximum power of 25 W). The advantage of this combination is postulated to have a better penetrability and the possibility of increasing the emitted energy [20]. From the available literature, there was no study that investigated the effect of this combination on the deep articulating pain as in knee OA. Therefore, the objective of this study was to investigate the effect of class IV diode laser on knee pain and functions in patients with knee OA.

Patients and methods

Patients

A single randomized blinded placebo-controlled trial was approved by University's Ethics Research Committee. A rheumatologist who performed a baseline evaluation for all patients was blinded to the study purpose or design. Patients were subjected to imaging before the decision of accepting them in the trial. On the basis of radiographic finding, patients with grade less than or equal to 3 in the Kellgren and Lawrence grading of OA were included in the study. The estimated sample size was performed by GPower 3.1 program (Universitat Kiel, Germany) for windows with α errors of 0.05, power (1-β error) of 0.80, effect size of 0.85, and using Wilcoxon–Mann–Whitney test for two groups in the analysis of data to detect changes in pain level. The effect size was based on the previous studies [21,22]. The estimated sample size was 48 patients. The number of samples was increased to 50 for possible dropout. A total of 50 male patients participated in the study. The patients were recruited from physical therapy and rehabilitation department of Al-Nour Hospital, Mecca, Saudi Arabia. A written consent was collected from the patients for participation in the study and to publish their results.

The inclusion criteria were as follows: (a) anterior or posterior knee pain for at least 3 months, (b) limitation in knee ROM and posterior knee muscle tightness, (c) BMI less than or equal to 30 kg/m^2, and (d) at least a score of 25 on the Western Ontario and McMaster Universities Index of Osteoarthritis (WOMAC) as self-reported disability questionnaire.

Patients were excluded if they had previous surgery, rheumatoid arthritis, fractures, more than 20° genu valgum or genu varum, previous intra-articular injection of corticosteroid or hyaluronic acid, or any cardiovascular, respiratory, or other musculoskeletal problems that interfere with patient participation in exercise. Patients were assigned randomly into two groups of 25 patients in each group. Group I received MLS plus exercises and group II received placebo laser plus exercises. Randomization was performed by online graphPad program (GraphPad Software, San Diego, California, USA) after assigning a specific number for every patient. Patients did not know which group they were assigned to and which treatment they would be given. The therapists were blinded to the group assignment as well, and therefore neither patients nor the therapist knew who was in which group.

Assessment

Patient's age, weight, height, and BMI were recorded. Pain level was measured by visual analog scale (VAS) and knee function using WOMAC.

Assessment of pain

The VAS was used for evaluation of pain for all patients. It is a line divided into 10 equal sections, with 10 representing 'unbearable pain' and 0 representing 'no pain'. Each participant was asked to indicate the level of his pain by marking on this scale. A ruler was used to measure the distance in centimeters from 0 to the marked point. It is an ordinal scale commonly used by researches, and it was shown to be a reliable and valid measure of pain [23]. Measurement was performed at baseline and 4 weeks after treatment.

Assessment of knee function

The lower limb and knee joint functions were evaluated by using WOMAC. The WOMAC scale could evaluate pain, stiffness, and lower limb and knee function. WOMAC is considered as a reliable and valid measure for evaluation of patients with hip and knee OA [24]. Five items of the WOMAC scale were used to measure pain, two items for stiffness, and 17 items for physical function. Each item was graded on a five-point scale of 0–4, where 0, no pain/limitation; 1, mild pain/limitation; 2, moderate pain/limitation; 3, severe pain/limitation; and 4, extreme pain/limitation [21]. After confirmation of its validity and reliability, an Arabic version of WOMAC was used in patients with knee OA [25].

Treatment

Multiwave locked system laser therapy

Mphi laser device (ASA, Arcugnano, Italy) was used in this study. It provides synchronized and overlapping continuous and pulsed emissions of Ga–Al–Ar laser emitted in a single handpiece. Mphi has continuous emission of wavelength 808 nm with peak power of 1000 mW, mean power of 500 mW, spot diameter of 2 cm, and spot area of 3.14 cm^2. Pulsed emission has a wavelength of 905 nm, peak power of 25 W, mean power of 54mWat, with a frequency of 1500 Hz, with the same diameter and spot area.

While the patient assumed a supine lying position, the affected knee was slightly flexed and supported with a pillow underneath. Laser was applied into two subphases: scan and trigger point subphases. In the scan phase, both the anterior and posterior knee surfaces were scanned, with an average area of 100 cm^2, time of application of 6 min and 17 s per session, and a total energy of 214 150 J. In trigger point treatment, laser probe was perpendicular and in contact to four points on the anterior knee surface around the patella and two points on the posterior knee surface on the medial and lateral hamstring insertion. The energy delivered was 2.14 J/cm^2, 6.175 J on each point in an average time of 16 s [21]. The total time of laser session was about 9 min. MLS laser was applied to all patients in group I three sessions/week for 4 consecutive weeks. Calibration of laser equipment was done by the manufacturing company. For placebo laser, patients in group II attended the physical therapy department and received sham laser with the same equipment, time, area, and points of application before applying the exercise program three sessions/week for 4 consecutive weeks.

Exercises

Exercise program was applied thrice a week for 4 weeks for all patients in both treatment groups. The program included (a) 5-min ROM exercise to lower limb joints in pain-free range from nonweight-bearing position, (b) 10 min stretching exercise to the hamstring and calf muscles, and (c) isometric strengthening exercise to the quadriceps muscle using sand bags while the patient raised his leg straight for 10 times for three sets with 5-min rest in between each set. The patient performed an isotonic resisted exercise, three sets of 10 times each, to the quadriceps muscle using Multigym device with a variable resistance according to patient tolerance. Hamstring strengthening exercise, three sets of 10 times each, was performed from prone lying position using sandbags [18,21]. The total time of exercise was about 45 min, plus the rest periods between different exercise modes according to patient tolerance. The patient was instructed to repeat the program of exercises at home and a handout prescription of exercises was given for all patients. Hot packs were allowed in case of muscle soreness after exercise.

Data analysis

Unpaired *t*-test was used to compare the mean values of patient's age, weight, height, and BMI for both treatment groups. Changes in VAS and WOMAC between groups were analyzed by Mann–Whitney *U*-test. Each group's results were analyzed by Wilcoxon's signed-rank test to compare between baseline and after 4 weeks. The level of statistical significance was set as P less than 0.05.

Results

The aim of the study was to investigate the effect of class IV diode laser on knee pain and functions in

patients with knee OA. Fifty male patients participated in the study. Their mean age was 55.68±8.88, weight was 83.86±5.28, height was 173.84±4.946, and BMI was 27.78±1.89. They were randomly assigned into two groups of 25 patients in either group I or group II. Unpaired *t*-test was used to compare the demographic data of patients including age, weight, height, and BMI, and revealed nonsignificant changes in their means between the two treatment groups, as shown in Table 1.

Wilcoxon's matched-pairs signed-ranks test was used to compare between baseline and post-treatment results and revealed significant decreases in VAS and WOMAC in both group I and group II (Table 2). Mann–Whitney test was used to compare the baseline and post-treatment scores of VAS and WOMAC and showed nonsignificant differences in baseline results between both groups, as shown in Table 2. Significant decreases were observed in post-treatment results in either VAS or WOMAC, with a significant decrease gained in group I more than group II, as shown in Table 2.

Discussion

The aim of the study was to investigate the effect of MLS on knee pain and functions in patients with knee OA. MLS combined with exercise was effective more than placebo laser plus exercises in the treatment of patients with knee OA. MLS combined with exercise was more effective in decreasing VAS score and WOMAC subscales as compared with the placebo laser plus exercises group.

The result of the present study was consistent with those of the previous studies, which support the effect of LLLT in the treatment of arthritis. Researchers have found favorable analgesic, anti-inflammatory, and biostimulating effects of laser. Diode laser significantly reduces the chronic pain as in rheumatoid arthritis, chronic arthritis, and knee injuries [14,21,22,26,27].

In knee OA, the main aims of treatment are to relieve pain, to improve lower limb function, and to alleviate joint destruction by changing the inflammatory process. Laser diode can reduce pain indirectly by increasing the microcirculation [28], and increasing oxygenation to tissues with reduction of knee swelling [29] and the intensity of inflammation. Laser reduces the inflammatory process by altering prostaglandin synthesis, decreasing interleukin 1, enhancing lymphocyte response, and decreasing C-reactive protein and neopterin levels [30,31]. Laser therapy can reduce pain at the tissue level by altering the release of chemical mediators such as histamine and bradykinin, which are released from injured tissues [32], and decreasing the release of substance P, which decreases the threshold of pain [33]. These effects lead to an increase in the knee functional performance, and an improvement in the ambulation duration and quality of life [21]. Laser has a biostimulating effect as it influences the cellular metabolism through stimulation of cytochrome oxidase enzyme, which enhances the oxidative phosphorylation and increases ATP production, which in turn regulates other cellular processes leading to normalization of biological functions at the cellular level [34].

The result of the present study was contradictory to the finding of Gur *et al.* [14] and Tascioglu *et al.* [15], who found no significant effect of laser on pain in patients with knee OA. Although they used a semiconductor diode laser, the energy density delivered to patients (1.5, 2 or 3 J/point) was different from the present study (6 J/point). Laser was delivered at low power output of 11.2 or 50 mW and with different wavelengths (904 or 830 nm), which showed that the power output and the wavelength are important factors that the laser effect was dependent on [16]. In addition, it may provide evidence of the importance of the combination of wavelengths (808–905 nm) that was used in the current study. MLS is considered a high-power laser with two synchronized wavelengths (808 and 905 nm) resulting in deeper tissue penetration [19]. This combination may be able to reach a deeper area such as the knee joint and is responsible for pain relief and improving knee and lower limb function.

As the concentration of chromophores in skin and subcutaneous tissue increased, the absorption of laser

Table 1 Demographic characteristics of patients in both treatment groups

	Group I (mean±SD)	Group II (mean±SD)	*t*-Value	*P*-value
Age (years)	56.08±9.83	55.28±8.00	0.315	0.093[c]
Weight (kg)	82.64±3.78	85.08±6.29	−1.662	0.164[c]
Height (cm)	174.84±5.489	172.84±4.20	1.445	0.151[c]
BMI (kg/m^2)	27.06±1.43	28.49±2.06	−2.835	0.452[c]

[c]Nonsignificant changes.

Table 2 Changes in visual analog scale and Western Ontario and McMaster Universities Arthritis Index in both treatment groups

	VAS		*P*-value	WOMAC pain		*P*-value	WOMAC stiffness		*P*-value	WOMAC function		*P*-value
	Pretreatment	Post-treatment		Pretreatment	Post-treatment		Pretreatment	Post-treatment		Pretreatment	Post-treatment	
Group I (25 patients)	25.52	13.90	0.0001[a]	26.72	15.08	0.0001[a]	24.30	20.60	0.0001[a]	26.16	13.88	0.0001[a]
Group II (25 patients)	25.48	37.10	0.0001[a]	24.28	35.92	0.0001[a]	26.70	30.40	0.0001[a]	24.84	37.12	0.0001[a]
P-value	0.991[c]	0.0001[b]		0.543[c]	0.0001 [b]		0.544[c]	0.009[b]		0.746[c]	0.0001[b]	

Pretreatment and post-treatment values were expressed as mean rank. VAS, visual analog scale; WOMAC, Western Ontario and McMaster Universities Arthritis Index. [a]Significant differences between baseline and post-treatment using Wilcoxon's matched-pairs signed-rank test, $P \leq 0.05$. [b]Significant differences between two treatment groups using Mann–Whitney test, $P > 0.05$. [c]Nonsignificant differences.

is increased. When the wavelengths increased up to 1000 nm, the penetration reaches deeper tissues and can reduce pain and inflammation in the deeper area such as the knee joint [19,35]. The optimum dose of the therapeutic laser is dependent on three factors: power output, wavelength, and time of application. Class IV laser with a longer wavelength (up to l000 nm) over a longer period of time produces a higher therapeutic dosage, which is delivered to the tissue and can stimulate the tissues effectively [36].

The result of the present study indicates that exercise therapy alone or combined with MLS laser is clinically able to decrease pain and improve function. Exercise, when applied actively, was shown to be safe, economical, and effective in the treatment of patients with knee OA. Home-based isometric exercise of the knee extensor and flexor muscles has shown to have a beneficial effect on the long-term increase in muscle strength [37]. Stretching exercises to hamstring muscle when combined with isotonic muscle strengthening provide a useful treatment combination for middle-aged adult patients with knee OA [38]. Combined use of exercise with MLS laser has shown to have clinical significance in providing a potent effect in reducing pain and improving lower limb and knee function [18]. The reduction of pain and posterior knee muscles spasm and tightness in addition to the anti-inflammatory effect of laser may help decrease the inflammatory process. Moreover, MLS may recover the exercise muscles through improving muscle conditions, enhancing skeletal muscle contractile function, and postexercise recovery, which is considered as the cause for improving knee and lower limb function, as reflected in the improvement in WOMAC score.

Conclusion

Class IV diode laser combined with exercise was more effective than exercise alone in the treatment of patients with knee OA. MLS laser combined with exercise effectively decreased pain and WOMAC subscales as compared with exercise alone. MLS laser is an effective physical therapy modality that may provide better outcomes for patients with knee OA, especially when used in combination with exercise. Further studies may be recommended to investigate the effect of MLS on quadriceps muscle strength and recovery, as well as the changes in the inflammatory process inside the knee joint in patients with knee OA. In addition, the effect of MLS in the treatment of other types of painful and arthritic joints such as rheumatoid arthritis should be considered in future studies.

Limitation

All the recruited patients were male patients. All patients were instructed to perform a home exercise program and the exercise compliance was obtained from family members. Although the family members or the participants themselves reported any deficiency in the exercise prescription at home, we considered this a limiting factor in the present study.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

The Effect of Low-Level Laser in Knee Osteoarthritis: A Double-Blind, Randomized, Placebo-Controlled Trial

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Abstract

Introduction: Low-level laser therapy (LLLT) is thought to have an analgesic effect as well as a biomodulatory effect on microcirculation. This study was designed to examine the pain-relieving effect of LLLT and possible microcirculatory changes measured by thermography in patients with knee osteoarthritis (KOA). ***Materials and Methods:*** Patients with mild or moderate KOA were randomized to receive either LLLT or placebo LLLT. Treatments were delivered twice a week over a period of 4 wk with a diode laser (wavelength 830 nm, continuous wave, power 50 mW) in skin contact at a dose of 6 J/point. The placebo control group was treated with an ineffective probe (power 0.5 mW) of the same appearance. Before examinations and immediately, 2 wk, and 2 mo after completing the therapy, thermography was performed (bilateral comparative thermograph by AGA infrared camera); joint flexion, circumference, and pressure sensitivity were measured; and the visual analogue scale was recorded. ***Results:*** In the group treated with active LLLT, a significant improvement was found in pain (before treatment [BT]: 5.75; 2 mo after treatment : 1.18); circumference (BT: 40.45; AT: 39.86); pressure sensitivity (BT: 2.33; AT: 0.77); and flexion (BT: 105.83; AT: 122.94). In the placebo group, changes in joint flexion and pain were not significant. Thermographic measurements showed at least a 0.5°C increase in temperature—and thus an improvement in circulation compared to the initial values. In the placebo group, these changes did not occur. ***Conclusion:*** Our results show that LLLT reduces pain in KOA and improves microcirculation in the irradiated area.

Introduction

Since Endre Mester began his pioneering investigations, numerous clinical and basic research studies have demonstrated the physiological effects and medical applicability of low-level laser therapy (LLLT). Its application was initiated based on previous work that demonstrated properties of low-level laser that exert a positive influence on fibroblast[1] and osteoblast[2] proliferation, collagen synthesis,[3] and bone regeneration.[4] In vivo examinations have also shown that LLLT significantly stimulates the activity of alcalic phosphatase and calcium accumulation.[5] In addition to cartilage damage and bone metabolism, pathological alterations are also known to exhibit reduced circulation in the vessels of the joint parallel to the degenerative changes. Numerous authors have reported increased microvascularization as a histological effect of the laser beam.[6,7] While examining revascularization—a phase of wound healing—Mester found a significant increase in the number of vascularized areas in laser-treated patients.[8] In light of the domestic and international literature, the aim of this study is to gather evidence of the analgesic effect of low-level laser as well as its possible effect in increasing microcirculation. In order to obtain objective data, thermographic measurements were taken, and follow-up examinations were performed to control for the permanency of the effects obtained.

Patients and Methods

Both female and male patients with mild to moderate knee osteoarthritis (KOA) were recruited to the study. Reasons for exclusion included considerable deformity of the varus or valgus, ankylosis, intense synovitis, or gonitis observed during physical examination; erosive or destructive alterations detected by radiograph (Kellgren-Lawrence stage 4); and the usual contraindications for laser therapy (Table 1).

Thirty-five patients were selected for the examinations, but only 27 patients (22 women and 5 men) completed the

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Table 1. Participation in the Study

Inclusion criteria	*Exclusion criteria*
Men or women between the ages of 30 and 65 with: 1) Knee pain intensity above 40 mm on VAS 2) Mild to moderate knee osteoarthritis confirmed by x-ray	1) Lab results abnormal (inflammatory and infectious disease, malignant tumor). 2) Arterial circulatory blockage in lower limbs. 3) Observed during physical examination: – considerable deformity of the varus and valgus – ankylosis – intense synovitis – gonitis 4) Detected in radiograph: – erosive, destructive alteration 5) Usual contraindications for laser therapy

study, 18 of whom were in the active LLLT group and 9 in the placebo LLLT group. Eight patients from the placebo group who left the experiment provided no reasons for doing so, nor did they return to the institute. The demographic data on the patients included in the study are summarized in Table 2.

During the study patients received no steroids, antidepressants, or sedatives. A detailed case history and physical status were recorded. Various examinations were conducted prior to treatment in order to rule out other diseases and to attain patient homogeneity (Table 3). Those who underwent treatment were given full disclosure and signed an agreement form on participation in the study.

Permission was granted for this study by the Institute's Research Ethics Committee. The patients received no other therapies or pain medication.

Treatments were administered on the same days twice a week over a period of 4 wk with an OPTIKOP KLS GaAlAs diode laser (power 50 mW, continuous wave, wavelength 830 nm) or with a placebo probe (power 0.5 mW) of the same appearance and display. The probes were numbered 1 (active) and 2 (placebo). Randomization was ensured by having patients randomly choose sealed envelopes from a bowl containing an equal number of slips with either number 1 or 2, which corresponded to one of the laser probe numbers. Neither the patients nor the operator knew which was the active or placebo LLLT probe. Treatment was administered in skin contact only over the joint which caused the most explicit complaints. The dose delivered was 6 J/point.

In one session, a patient was given a total dose of 48 J/cm^2. The size of the point in the focus of the laser light was nearly 0.5 mm^2; that is to say, the power density was approximately 50 $mW/0.5\,mm^2$, i.e., 10 W/cm^2. The laser has European certificate no. CE 0120. The device is self-checked in accordance with European Standards (CE) and requires no special staff. Treatment was administered over the femoral and tibial condyles in every case since enthesis is often responsible for the complaints mentioned by the patients. Laser irradiation was aimed at the synovia and cartilage in the joint line. The points that were irradiated were the medial and lateral epicondyle of the tibia and femur, the medial and lateral knee joint gap, and the medial edge of the tendon of the biceps femoris muscle and semitendinosus muscle in the popliteal ditch (Fig. 1).

Valgization was carefully performed on the knee joint when the medial knee joint gap was being treated, and varization was carried out when treatment was administered to the lateral knee joint gap. The knee joint was flexed on treating the popliteal ditch. In order to judge the efficacy of the treatment, subjective (pain on a 10-cm scale), semi-objective (pressure sensitivity on the Ritchie index scale), and objective (flexion in degrees, circumference in centimeters, and thermography with temperature [°C]) parameters were measured (Table 4). Thermographic measurements were used to observe microcirculatory changes during the treatment period, and a computer system enabled us to digitize the image.[9,10]

Considering that thermographic measurement is very sensitive but that its specificity is low, an attempt was made to set appropriate standard examination conditions. The examination room was therefore kept at a constant temperature (21–23°C) and free from drafts at a humidity of 70–80%. Prior to measurement, patients rested for 15 min and then the affected part of the body was washed with alcohol.

Table 2. Demographic Data

Distribution of patients			*Kellgren-Lawrence stage*				
	Men	*Women*		*Men*	*Women*	*Comorbidities*	
						Diabetes mellitus	2
No. of patients	5	22	I	2	3	Hypertonia	9
						Hypothyreosis	1
Age of patients	37–44	32–65	II	10	9	Ulcus ventriculi	1
Average age	41.00	51.40				Hyperlipidemia	1
Overall average	49.48		III	—	3	Arteriosclerosis universalis	2
						Myoma uteri	2

Table 3. Examinations Before Treatment

Dexascan	
X-ray (comparative image of bilateral knee joints)	
Doppler (arteries of the lower limbs: a. femoralis; a. poplitea)	
Laboratory	
Blood	WBC, RBC, HBG, HTC, sedimentation rate, CBC, BUN, Se creatinin, glucose, Se bilirubin , K, Na, Se ALP, SGOT, SGPT, gamma-GT, Se Ca, Se P, ELFO, Se protein, RF, Se urea, Se cholesterol, Se triglyceride
Urine	protein, pus, glucose, UBG, pH, ketone, bilirubin, blood, specific gravity, sediment

Patients were told to avoid coffee, alcohol, and cigarettes prior to measurement since these can influence circulatory conditions. In every case, medial and lateral comparative measurements were performed from anterior–posterior and posterior–anterior angles.

A basic (or zero) examination was performed prior to treatment; all other measurements were carried out weekly after the second treatment at the same time each week. In order to control for the permanency of the effect obtained, control measurements were performed 2 wk and 2 mo after completing the therapy.

Results

The graph shows changes in the four parameters examined, plotted against time, for treatment with active and

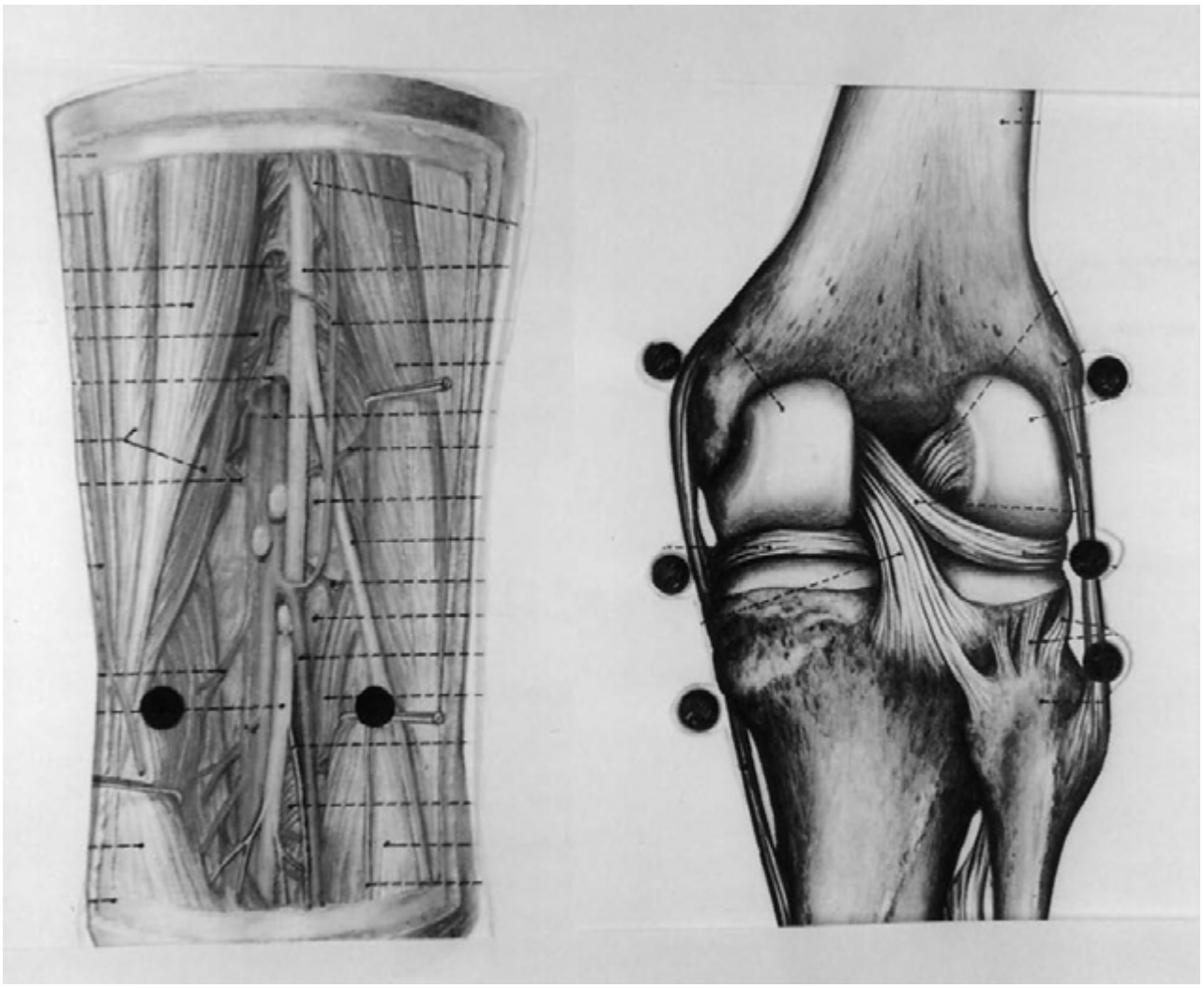

FIG. 1. Irradiated points.

Table 4. Outcome Measures

Pressure sensitivity (Ritchie index)	
0	Not sensitive
1	Pressure sensitive
2	Pressure sensitive, which patients also demonstrate through facial expressions
3	Pressure sensitive, which patients also demonstrate through facial expressions and by retraction of limb)
Pain (10 cm visual analogue scale)	
Flexion (Domján-Bálint mobimet: degree)	
Circumference (cm)	
Thermography (°C)	

placebo LLLT probes. Certain examination times were compared to the initial data; a comparison was also made between the two groups for the time of examination. For statistical analysis, *t*-tests were used for within-group differences and ANOVA for between-group comparison over time.

Joint flexion was 105.83° before treatment (BT) in the active laser group (Fig. 2a), and 122.27° immediately after the last treatment session (AT); 124.33° 2 wk AT; and 122.94° 2 mo AT. For treatment with the placebo probe (Fig. 2b), joint flexion was 107.22° BT, 115.22° AT, 116.11° 2 wk AT, and 112.11° 2 mo AT. For the active LLLT group, a significant

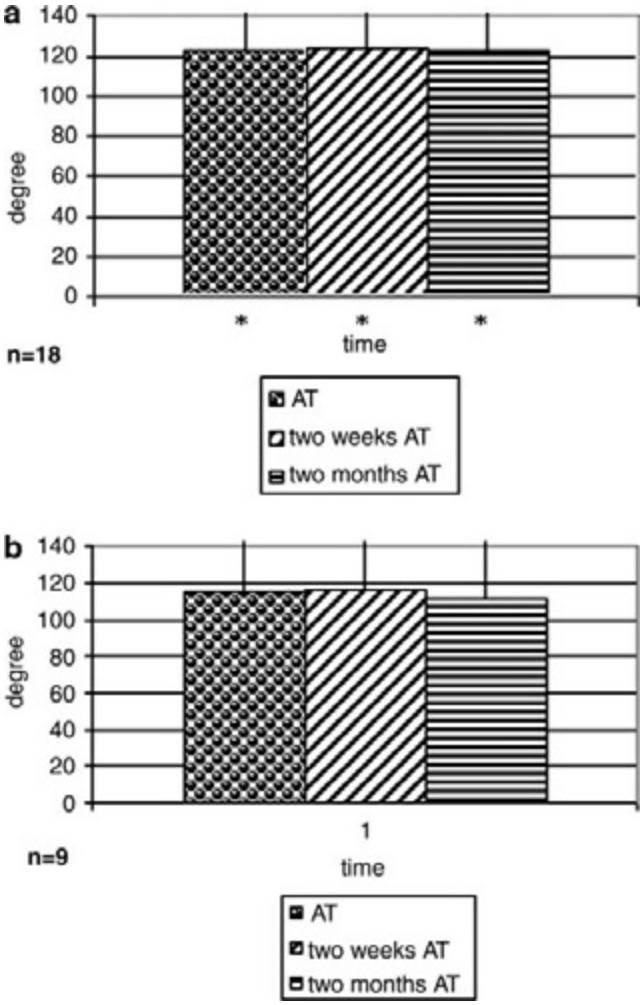

FIG. 2. (**a**) The effect of laser treatment on joint flexion. Treatment resulted in significant improvement in joint flexion at all times examined. (**b**) The effect of placebo laser treatment on joint flexion. We observed no significant change from treatment at any of the times examined. AT, after treatment.

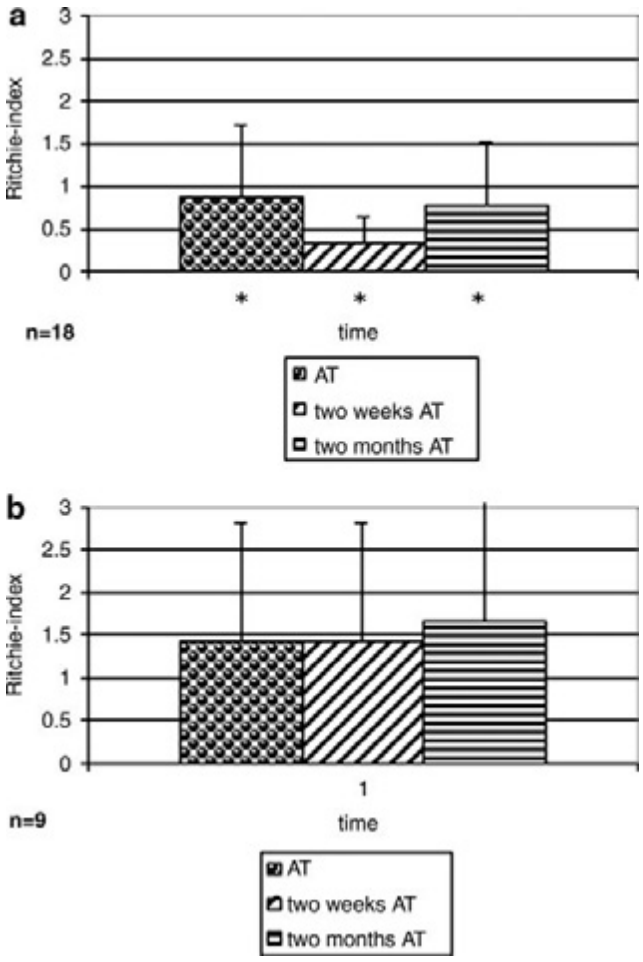

FIG. 3. (**a**) The effect of laser treatment on pressure sensitivity of the joint. Treatment resulted in significant improvement in joint flexion at all the times examined. (**b**) The effect of placebo laser treatment on pressure sensitivity of the joint. We observed no significant change from treatment at any of the times examined.

change could be detected compared to the initial value at every time examined. This trend could not be observed for the placebo group ($p < 0.05$).

Pressure sensitivity of the joint for treatment with the active probe (Fig. 3a) was 2.33 BT, 0.83 immediately AT, 0.33 2 wk AT, and 0.77 2 mo AT as measured using the Ritchie index. For treatment with the placebo probe (Fig. 3b), pressure sensitivity was 2.11 BT, 1.44 directly AT, 1.44 2 wk AT, and 1.66 2 mo AT. There was only a significant change at all the times examined for the active LLLT group compared to the initial value, whereas none was detected for the placebo LLLT group ($p < 0.05$).

Pain in the joint for treatment with the active probe (Fig. 4a) was 5.75 BT, 1.71 immediately AT, 1.05 2 wk AT, and 1.18 2 mo AT on a 10-cm scale. For treatment with the placebo LLLT probe (Fig. 4b), pain was 5.62 BT, 4.13 immediately AT, 4.07 2 wk AT, and 4.12 2 mo AT. A significant change could be detected at all times examined for the active LLLT group compared to the initial value, whereas this trend could not be observed for the placebo LLLT group ($p < 0.05$).

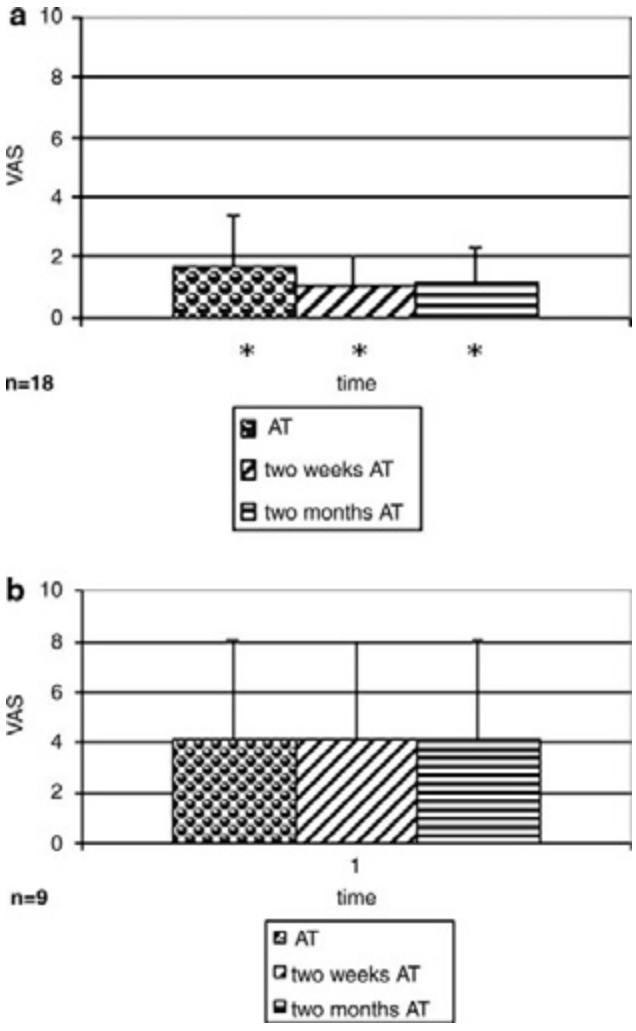

FIG. 4. (**a**) The effect of laser treatment on pain in the joint. Treatment resulted in significant improvement in joint flexion at all the times examined. (**b**) The effect of placebo laser treatment on pain in the joint. We observed no significant change from treatment at any of the times examined.

The circumference of the joint was 40.45 cm BT for treatment with the active probe, 39.61 cm immediately AT, 39.58 cm 2 wk AT, and 39.86 cm 2 mo AT. For the group treated with the placebo LLLT probe, circumference was 40.44 cm BT, 39.86 cm immediately AT, 39.87 cm 2 wk AT, and 40.05 cm 2 mo AT. With regard to the examined parameters, no significant changes appeared for the effective or placebo group under the effect of the treatment ($p \geq 0.05$).

Increased metabolism and a richer blood supply to tissues beneath the surface represented important factors in the thermographic results. Where tissues have a higher metabolism and there is a richer blood supply beneath the surface skin, more infrared rays are emitted. The opposite also holds true.

During the treatment period, weekly thermograms showed increasing temperature in previously cold areas and an extension of the warmer area (Fig. 5a and 5b). There was no increase in skin temperature in the placebo LLLT group (Fig. 6a and 6b).

At follow-up measurements 2 mo after probe (Fig. 7a and 7b) therapy, the thermographic changes remained elevated by at least a 0.5°C in patients who experienced pain relief. An increased temperature was even observed in the nontreated control side in all patients who were treated with the active LLLT.

Discussion

Our measurement results provide evidence that treatment with the active LLLT probe resulted in significant improvement for all evaluated parameters. In the placebo LLLT group, we found nonsignificant changes in joint flexion and pain. In the active LLLT group, we found significant improvement with regard to joint flexion, pain, and pressure sensitivity in the active group in comparison with the placebo group at the times examined. The positive effects obtained from active LLLT still persisted 2 mo after treatment. The lack of effect on knee circumference was expected and has not been demonstrated with other therapies. In the placebo LLLT group, three patients gave an account of an explicit reduction in their complaints, which is in line with placebo improvement in studies of other KOA therapies.

It is a weakness of the study that we did not use other validated tools for measurement of KOA pain and disability such as the WOMAC questionnaire or the Lequesne index. However, there is a high correlation between pain scores and these tools, and there is little reason to believe that incorporation of these tools would have altered our results.

Over the years more than 100 double-blind, placebo-controlled studies have been published on the effects of LLLT. These articles also showed the favorable anti-inflammatory effect of LLLT.[11–13] Based on the objective, semi-objective, and subjective measurements after laser and placebo treatments in patients with seropositive rheumatoid arthritis, Barabás came to the conclusion that laser treatment exerts a positive influence on the clinical signs and laboratory parameters of this disease.[14] Ohshiro also demonstrated a positive effect on microcirculation and verified changes by thermography in parallel with the reduction of pain.[15]

In studies where the temperature of the skin was measured, it was reported to have risen in the irradiated site.[15–18]

Mester noticed an increase in the migration index of T lymphocytes after laser irradiation. He observed that this

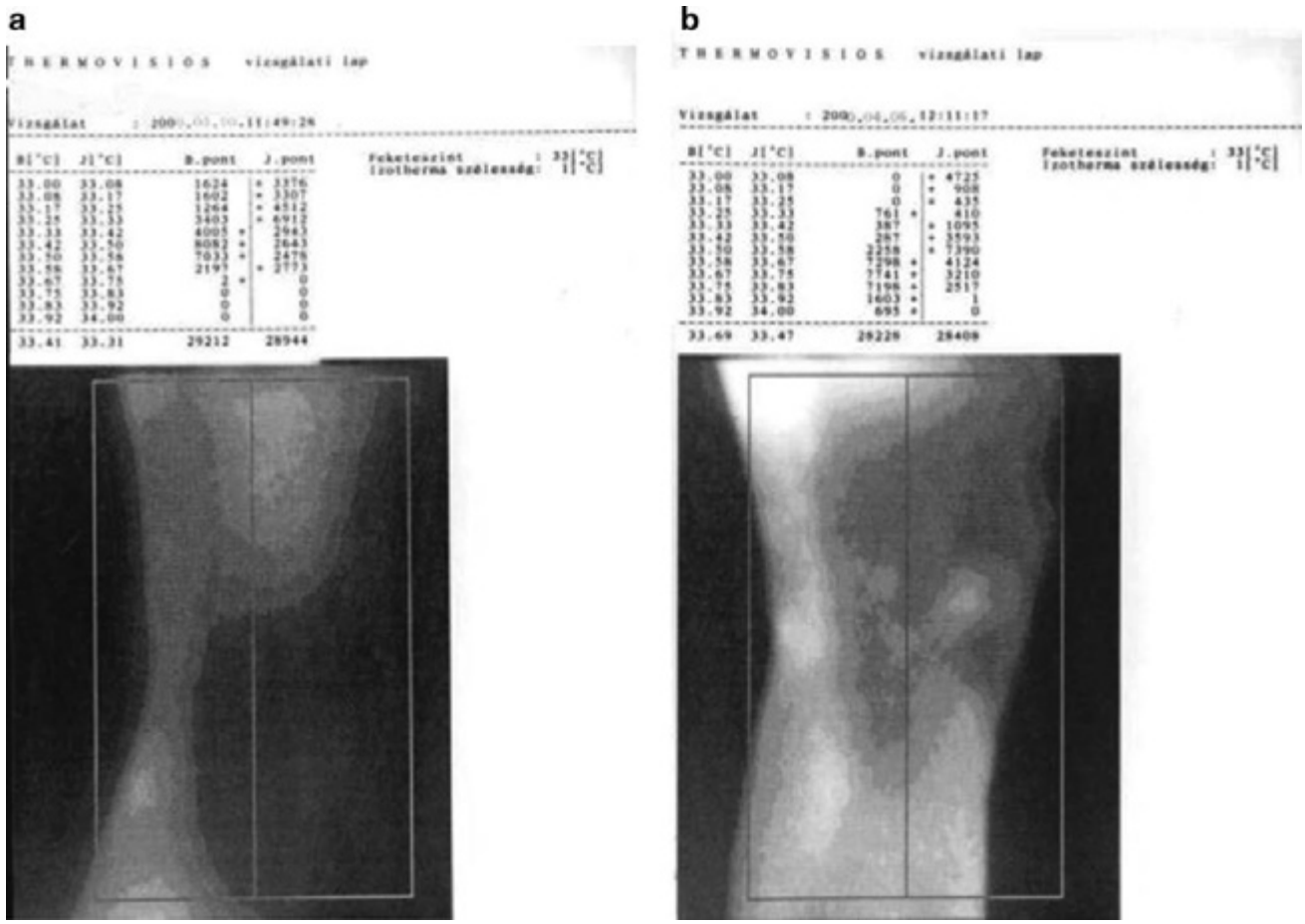

FIG. 5. (**a**) Lateral image of a right knee before eight active low-level laser therapy (LLLT) treatments. White and grey colors represent higher temperatures, greyer and black colors represent colder temperatures. (**b**) Lateral image of a right knee after eight active LLLT treatments.

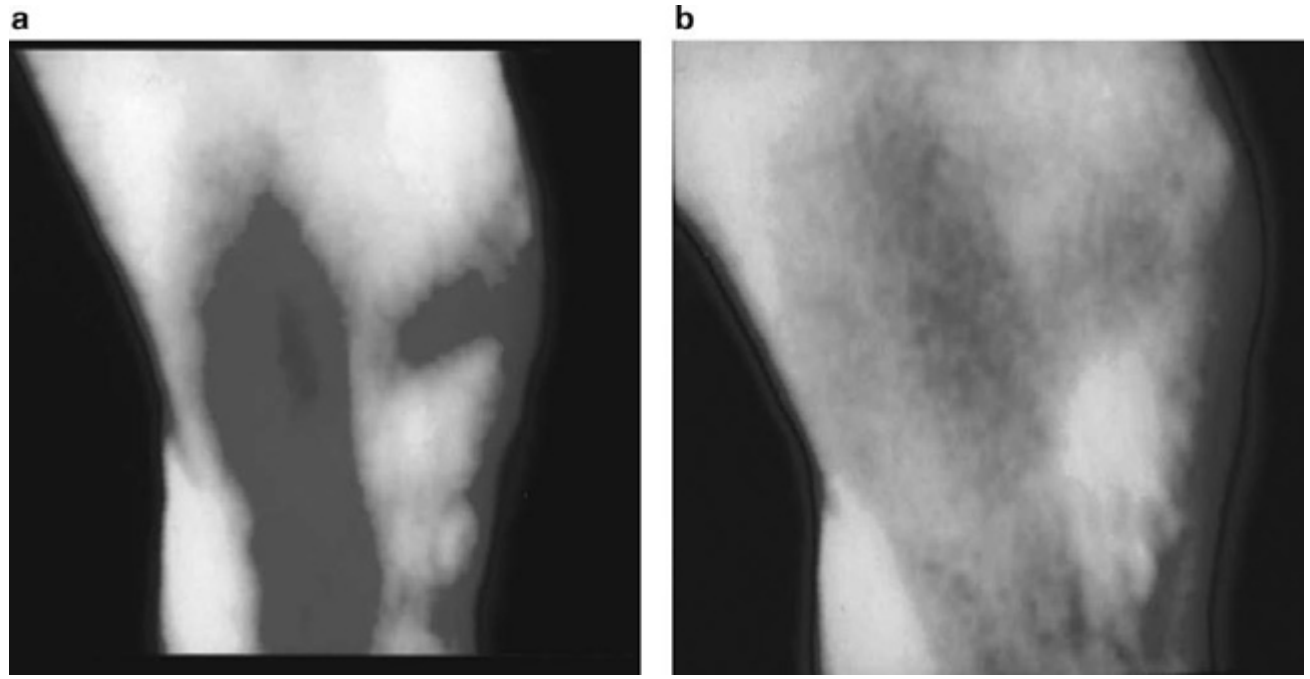

FIG. 6. (**a**) Medial thermogram of the left knee before eight placebo LLLT treatments. (**b**) Medial thermogram of the left knee after eight placebo LLLT treatments.

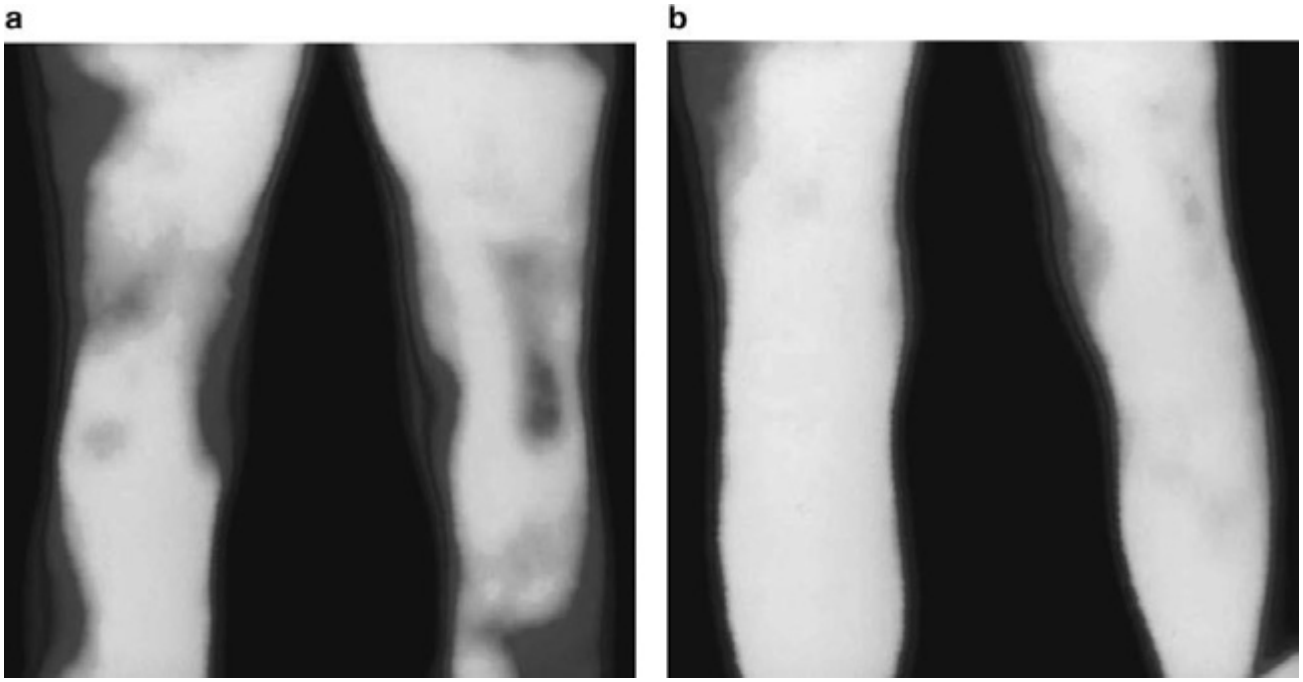

FIG. 7. (**a**) Image from posterior–anterior angle before eight treatments of the right knee joint. (**b**) Image from posterior–anterior angle after eight treatments of the right knee joint.

change can be transmitted by pouring the medium of treated cells on nontreated lymphocytes. In patients with bilateral leg ulcer that failed to respond to conservative treatment, while treating the wound of one limb he also noticed slower but unambiguous wound healing on the other side.[8] Other authors have reported effects proximal and distal from the irradiated area.[19–21]

With qualitative evaluation of the results obtained, we noticed an increase in temperature, suggesting circulatory changes at a good distance from the treated points and on the untreated side. On the other hand, we did not find this clear change in the control group.

In summary, low-level laser represents an effective treatment for short-term improvement in patients suffering from painful KOA.

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Disclosure Statement

No competing financial interests exist.

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Efficacy of low-level laser therapy on pain and disability in knee osteoarthritis: systematic review and meta-analysis of randomised placebo-controlled trials

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ABSTRACT

Objectives Low-level laser therapy (LLLT) is not recommended in major knee osteoarthritis (KOA) treatment guidelines. We investigated whether a LLLT dose–response relationship exists in KOA.

Design Systematic review and meta-analysis.

Data sources Eligible articles were identified through PubMed, Embase, Cumulative Index to Nursing and Allied Health Literature, Physiotherapy Evidence Database and Cochrane Central Register of Controlled Trials on 18 February 2019, reference lists, a book, citations and experts in the field.

Eligibility criteria for selecting studies We solely included randomised placebo-controlled trials involving participants with KOA according to the American College of Rheumatology and/or Kellgren/Lawrence criteria, in which LLLT was applied to participants' knee(s). There were no language restrictions.

Data extraction and synthesis The included trials were synthesised with random effects meta-analyses and subgrouped by dose using the World Association for Laser Therapy treatment recommendations. Cochrane's risk-of-bias tool was used.

Results 22 trials (n=1063) were meta-analysed. Risk of bias was insignificant. Overall, pain was significantly reduced by LLLT compared with placebo at the end of therapy (14.23 mm Visual Analogue Scale (VAS; 95% CI 7.31 to 21.14)) and during follow-ups 1–12 weeks later (15.92 mm VAS (95% CI 6.47 to 25.37)). The subgroup analysis revealed that pain was significantly reduced by the recommended LLLT doses compared with placebo at the end of therapy (18.71 mm (95% CI 9.42 to 27.99)) and during follow-ups 2–12 weeks after the end of therapy (23.23 mm VAS (95% CI 10.60 to 35.86)). The pain reduction from the recommended LLLT doses peaked during follow-ups 2–4 weeks after the end of therapy (31.87 mm VAS significantly beyond placebo (95% CI 18.18 to 45.56)). Disability was also statistically significantly reduced by LLLT. No adverse events were reported.

Conclusion LLLT reduces pain and disability in KOA at 4–8 J with 785–860 nm wavelength and at 1–3 J with 904 nm wavelength per treatment spot.

PROSPERO registration number CRD42016035587.

Strengths and limitations of this study

- The review was conducted in conformance with a detailed a priori published protocol, which included, for example, laser dose subgroup criteria.
- No language restrictions were applied; four (18%) of the included trials were reported in non-English language.
- A series of meta-analyses were conducted to estimate the effect of low-level laser therapy on pain over time.
- Three persons each independently extracted the outcome data from the included trial articles to ensure high reproducibility of the meta-analyses.
- The review lacks quality-of-life analyses, a detailed disability time-effect analysis and direct comparisons between low-level laser therapy and other interventions.

INTRODUCTION

Approximately 13% of women and 10% of men in the population aged ≥60 years suffer from knee osteoarthritis (KOA) in the USA.[1] KOA is a degenerative inflammatory disease affecting the entire joint and is characterised by progressive loss of cartilage and associated with pain, disability and reduced quality of life (QoL).[1] Increased inflammatory activity is associated with higher pain intensity and more rapid KOA disease progression.[1 2]

Some of the conservative intervention options for KOA are exercise therapy, non-steroidal anti-inflammatory drugs (NSAIDs) and anti-inflammatory low-level laser therapy (LLLT). There is evidence that exercise therapy reduces pain and disability and improves QoL in persons with KOA.[3 4] NSAIDs are recommended in most KOA clinical treatment guidelines and is probably the most frequently prescribed therapy category for osteoarthritis, despite intake of these

drugs is associated with negative side effects,[5] which is problematic, especially since the disease requires long-term treatment. Furthermore, a recently published network meta-analysis indicates that the pain relieving effect of NSAIDs in KOA beyond placebo is small to moderate (depending on drug type).[6] Likewise, in the first systematic review on this topic, the pain relieving effect of NSAIDs was estimated to be only 10.1 mm on the 0–100 mm Visual Analogue Scale (VAS) better than placebo.[7]

LLLT is a non-invasive treatment modality,[8 9] which has been reported to induce anti-inflammatory effects.[9–14] LLLT was compared with NSAID in rats with KOA by Tomazoni *et al* in a laboratory; NSAID (10 mg diclofenac/knee/session) and LLLT (830 nm wavelength, 6 J/knee/session) reduced similar levels of inflammatory cells and metalloproteinase (MP-3 and MP-13). In addition, LLLT reduced the expression of proinflammatory cytokines (interleukin-1β (IL-1β) and IL-6 and tumour necrosis factor α), myeloperoxidase and prostaglandin E_2 significantly more than NSAID did.[10 11]

LLLT has been applied to rabbits with KOA three times per week for 8 weeks in a placebo-controlled experiment by Wang *et al*.[12] At the end of treatment week 6, they found that LLLT had significantly reduced pain and synovitis and the production of IL-1β, inducible nitric oxide synthase and MP-3 and slowed down loss of metallopeptidase inhibitor 1. Two weeks later, LLLT had significantly reduced MP-1 and MP-13 and slowed down loss of collagen II, aggrecan and transforming growth factor beta, and the previous changes were sustained.[12] These findings indicate that the effects of LLLT increase over time.

Pallotta *et al*[14] conducted a study on LLLT in rats with acute knee inflammation, which demonstrated that even though LLLT (810 nm) significantly enhanced cyclooxygenase (COX-1 and COX-2) expression it significantly reduced several other inflammatory makers, that is, leucocyte infiltration, myeloperoxidase, IL-1 and IL-6 and especially prostaglandin E_2. Pallotta *et al*[14] hypothesised that the increase in COX levels by LLLT was involved in a production of inflammatory mediators related to the resolution of the inflammatory process.

LLLT is not recommended in major osteoarthritis treatment guidelines. LLLT for KOA was mentioned in the European League Against Rheumatism osteoarthritis guidelines (2018) but not recommended,[15] and in the Osteoarthritis Research Society International guidelines (2018), it was stressed that LLLT should not be considered a core intervention in the management of KOA.[16]

This may be partly due to conflicting results of two recently published systematic reviews on the current topic.[8 17] The conflicting results may arise from omission of relevant trials[8 17–23] and unresolved LLLT dose-related issues. Only Huang *et al*[17] conducted a LLLT dose–response relationship investigation in KOA, that is, by subgrouping the trials by laser dose, but they did not consider that World Association for Laser Therapy (WALT) recommends applying four times the laser dose with continuous irradiation compared to superpulsed irradiation.[22 24–26] Thus, it was unknown whether LLLT is effective in KOA, and we saw a need for a new systematic review.

The objectives of the current review were to estimate the effectiveness of LLLT in KOA regarding knee pain, disability and QoL, and we only considered placebo-controlled randomised clinical trials (RCTs) for inclusion to minimise risk of bias.

METHODS

This review is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement 2009.[27]

Literature search and selection of studies

Any identified study was included if it was a placebo-controlled RCT involving participants with KOA according to the American College of Rheumatology tool and/or a radiographic inspection with the Kellgren/Lawrence (K/L) criteria, in which LLLT was applied to participants' knee(s) and self-reported pain, disability and/or QoL was reported. There were no language restrictions.

We updated a search for eligible articles indexed in PubMed, Embase, Cumulative Index to Nursing and Allied Health Literature, Physiotherapy Evidence Database and Cochrane Central Register of Controlled Trials on 18 February 2019. The database search strings contained synonyms for LLLT and KOA, and keywords were added when optional. The PubMed search string is available in the online supplementary material. The search was continued by reading reference lists of all the eligible trial and relevant review articles,[8 17 28] citations[29–33] and a laser book[34] and involving experts in the field.

Two reviewers (MBS and JMB) each independently selected the trial articles. Both reviewers scrutinised the titles/abstracts of all the publications identified in the search, and any accessible full-text article was retrieved if it was judged potential eligible by at least one reviewer. Both reviewers evaluated the full texts of all potentially eligible retrieved articles and made an independent decision to include or exclude each article, with close attention to the inclusion criteria. When selection disagreements could not be resolved by discussion, a third reviewer (IFN) made the final consensus-based decision. Any retrieved article not fulfilling the inclusion criteria was omitted and listed with reason for exclusion.

Risk-of-bias analysis

Two reviewers (MBS and JJ) each independently evaluated all included trials for risk of bias at the outcome level, using the Cochrane Collaboration's risk-of-bias tool.[35] When risk-of-bias disagreements could not be resolved by discussion, a third reviewer (IFN) made the final consensus-based decision. Likelihood of publication bias was assessed with graphical funnel plots.[35]

Data extraction and meta-analysis

Three reviewers (MBS, JMB and KVF) each independently extracted the data for meta-analysis. Two of the reviewers (MBS and KVF) each independently collected the other trial characteristics. The data-extraction forms were subsequently compared, and data disagreements were resolved by consensus-based discussions. Summary data were extracted, unless published individual participant data were available.[21] The results from the included trials for statistical analysis were selected from outcome scales in adherence to hierarchies published by Juhl *et al*.[36]

Pain intensity was the primary outcome. As pain reported with continuous, numeric and categorical/Likert scales highly correlates with pain measured using the VAS, the scores of all pain scales were transformed to 0%–100%, corresponding to 0–100 mm VAS.[37] The pain results were combined with the mean difference (MD) method, primarily using change scores, that is, when only final scores could be obtained from a trial, change and final scores were mixed in the analysis, since the MD method allows for this without introducing bias.[35]

Self-reported disability results were synthesised with the standardised mean difference (SMD) method using change scores solely. The SMD was adjusted to Hedges' *g* and interpreted as follows: SMDs of 0.2, ~0.5 and >0.8 represent a small, moderate and large effect, respectively.[35]

Lack of QoL data prohibited an analysis of this outcome.

Random effects meta-analyses were conducted, and impact from heterogeneity (inconsistency) on the analyses was examined using I^2 statistics. An I^2 value of 0% indicates no inconsistency, and an I^2 value of 100% indicates maximal inconsistency[35]; the values were categorised as low (25%), moderate (50%) and high (75%).[38]

SDs for analysis were extracted or estimated from other variance data in a prespecified prioritised order: (1) SD, (2) SE, (3) 95% CI, (4) p value, (5) IQR, (6) median of correlations, (7) visually from graph or (8) other methods.[35]

The trials were subgrouped by adherence and non-adherence to the WALT recommendations for laser dose per treatment spot, as prespecified. WALT recommends irradiating the knee joint line/synovia with the following doses per treatment spot: ≥4 J using 5–500 mW mean power 780–860 nm wavelength laser and/or ≥1 J using 5–500 mW mean power (>1000 mW peak power) 904 nm wavelength laser.[24 25]

The main meta-analyses were conducted using two prespecified time points of assessment, that is, immediately after the end of LLLT and last time point of assessment 1–12 weeks after the end of LLLT (follow-up).

MBS performed the meta-analyses, under supervision of JMB, using the software programme Excel 2016 (Microsoft) and Review Manager Version V.5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

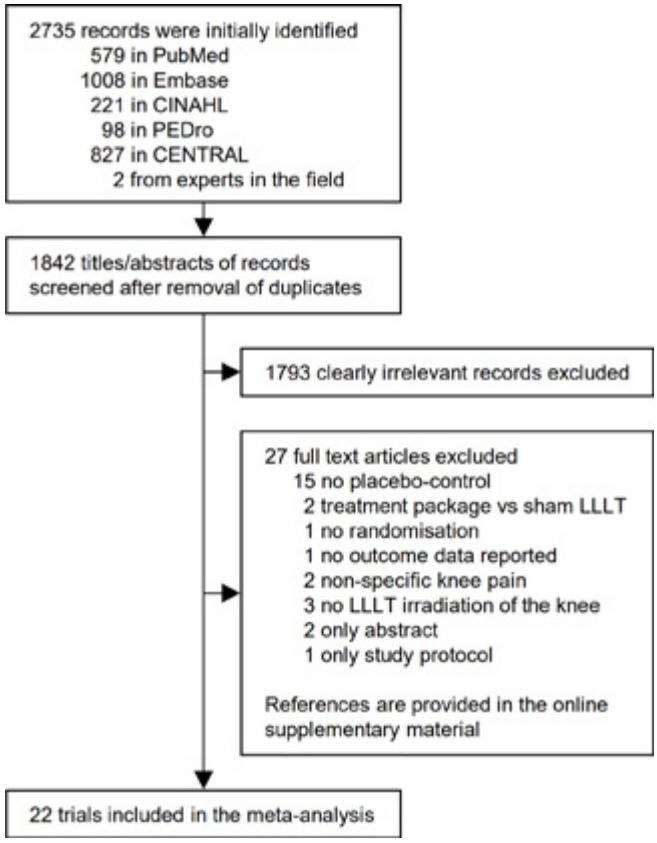

Figure 1 Flow chart illustrating the trial identification process. CENTRAL, Cochrane Central Register of Controlled Trials; CINAHL, Cumulative Index to Nursing and Allied Health Literature; LLLT, low-level laser therapy; PEDro, Physiotherapy Evidence Database.

Patient and public involvement

Patients or the public were not involved in the conceptualisation or carrying out of this research.

RESULTS

In total, 2735 records were identified in the search, of which 22 trial articles were judged eligible and included in the review (n=1089; figure 1 and tables 1–2) with data for meta-analysis (n=1063). Four included trials were not reported in the English language[19 21 23 39] and one included trial was unpublished (Gur and Oktayoglu). Excluded articles initially judged potentially eligible were listed with reasons for omission (online supplementary material).

At the group level, the mean age of the participants was 60.25 (50.11–69) years (data from 19 trials), the mean percentage of women was 69.63% (0–100%; data from 17 trials), the mean body mass index of the participants was 29.55 (25.8–38; data from 14 trials), the mean of median K/L grades was 2.37 (data from 13 trials) and the mean baseline pain was 63.61 mm VAS (35.25–92) (data from 22 trials). LLLT was used as an adjunct to exercise therapy in 11 trials. The mean duration of the treatment periods was 3.53 weeks with the recommended LLLT doses and 3.7

Table 1 Characteristics of the included trials

First author	Intervention group at baseline	Control group at baseline	Intervention versus control programme	Outcome scales, week of reassessment
Al Rashoud 2014[31]	N: 26 Women: 62% Age: 52 years BMI: 38 VAS pain: 64 mm K/L: -	N: 23 Women: 65% Age: 56 years BMI: 37.1 VAS pain: 59 mm K/L: -	3 weeks of exercise therapy, advice and LLLT versus 3 weeks of exercise therapy, advice and sham LLLT	Pain: VAS (movement) Disability: SKFS QoL: – Week of assessment: 2, **3**, **9**, 29
Alfredo 2011/2018[29 52]	N: 24 Women: 75% Age: 61.15 years BMI: 30.16 VAS pain: 53.2 mm K/L: 3	N: 22 Women: 80% Age: 62.25 years BMI: 29.21 VAS pain: 35.4 mm K/L: 2	3 weeks of LLLT followed by 8 weeks of exercise therapy versus 3 weeks of sham LLLT followed by 8 weeks of exercise therapy	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **3**, **11**, 24, 37
Alghadir 2014[32]	N: 20 Women: 50% Age: 55.2 years BMI: 32.34 VAS pain: 74.5 mm K/L: 2	N: 20 Women: 40% Age: 57 years BMI: 33.09 VAS pain: 75.5 mm K/L: 2	4 weeks of exercise therapy, heat packs and LLLT versus 4 weeks of exercise therapy, heat packs and sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **4**
Bagheri 2011[23]	N: 18 Women: 83.13% Age: 58.32 years BMI: 28.87 VAS pain: 67 mm K/L: –	N: 18 Women: 83.13% Age: 56.14 years BMI: 27.66 VAS pain: 59 mm K/L: –	2 weeks of exercise therapy, therapeutic ultrasound, TENS and LLLT versus 2 weeks of exercise therapy, therapeutic ultrasound, TENS and sham LLLT	Pain: WOMAC (VAS) 0–100 Disability: WOMAC QoL: – Week of assessment: **2**
Bülow 1994[20]	N: 14 Women: – Age: – BMI: – VAS pain: 65.08 mm K/L: –	N: 15 Women: – Age: – BMI: – VAS pain: 56.35 mm K/L: –	3 weeks of LLLT versus 3 weeks of sham LLLT	Pain: 0–121 Likert scale (movement/rest) Disability: – QoL: – Week of assessment: **3**, **6**
Delkhosh 2018[39]	N: 15 Women: 100% Age: 55.9 years BMI: 26.5 VAS pain: 57 mm K/L: –	N: 15 Women: 100% Age: 58.3 years BMI: 27.8 VAS pain: 45 mm K/L: –	2 weeks of exercise therapy, therapeutic ultrasound, TENS and LLLT versus 2 weeks of exercise therapy, therapeutic ultrasound, TENS and sham LLLT	Pain: VAS Disability: WOMAC QoL: – Week of assessment: **2**, **8**
Fukuda 2011[30]	N: 25 Women: 80% Age: 63 years BMI: 30 VAS pain: 61 mm K/L: 2	N: 22 Women: 64% Age: 63 years BMI: 30 VAS pain: 62 mm K/L: 2	3 weeks of LLLT versus 3 weeks of sham LLLT	Pain: VNSP (movement) Disability: Lequesne QoL: – Week of assessment: **3**
Gur 2003[33] (1.5 J)	N: 30 Women: 83.3% Age: 58.64 years BMI: 31.17 VAS pain: 73.2 mm K/L: 2	N: 30 Women: 80% Age: 60.52 years BMI: 30.27 VAS pain: 67.4 mm K/L: 2	14 weeks of exercise therapy and 2 weeks of LLLT versus 14 weeks of exercise therapy and 2 weeks of sham LLLT	Pain: VAS (movement) Disability: – QoL: – Week of assessment: 6, 10, **14**
Gur 2003[33] (1 J)	N: 30 Women: 76.7% Age: 59.8 years BMI: 28.49 VAS pain: 74.4 mm K/L: 2	N: 30 Women: 80% Age: 60.52 years BMI: 30.27 VAS pain: 67.4 mm K/L: 2	14 weeks of exercise therapy and 2 weeks of LLLT versus 14 weeks of exercise therapy and 2 weeks of sham LLLT	Pain: VAS (movement) Disability: – QoL: – Week of assessment: 6, 10, **14**
Gur and Oktayoglu	N: 40 Women: 75% Age: 58.2 years BMI: 29.11 VAS pain: 88 mm K/L: 3	N: 40 Women: 72.5% Age: 58.26 years BMI: 30.11 VAS pain: 92 mm K/L: 3	14 weeks of exercise therapy and 2 weeks of LLLT versus 14 weeks of exercise therapy and 2 weeks of sham LLLT	Pain: VAS (movement) Disability: – QoL: – Week of assessment: 6, 10, **14**

Continued

Table 1 Continued

First author	Intervention group at baseline	Control group at baseline	Intervention versus control programme	Outcome scales, week of reassessment
Gworys 2012[18]	N: 34 Women: – Age: 57.6 BMI: – VAS pain: 54 mm K/L: –	N: 31 Women: – Age: 67.7 BMI: – VAS pain: – K/L: –	2 weeks of LLLT versus 2 weeks of sham LLLT	Pain: VAS Disability: Lequesne QoL: – Week of assessment: **2**
Hegedűs 2009[53]	N: 18 Women: – Age: – BMI: – VAS pain: 57.5 mm K/L: 2	N: 17 Women: – Age: – BMI: – VAS pain: 56.2 mm K/L: 2	4 weeks of LLLT versus 4 weeks of sham LLLT	Pain: VAS Disability: – QoL: – Week of assessment: **4**, 6, **12**
Helianthi 2016[54]	N: 30 Women: 60% Age: 69 years BMI: 25.8 VAS pain: 60.2 mm K/L: 3	N: 29 Women: 82.8% Age: 68 years BMI: 26.3 VAS pain: 54.1 mm K/L: 3	5 weeks of LLLT versus 5 weeks of sham LLLT	Pain: VAS (movement) Disability: Lequesne QoL: – Week of assessment: 2, **5**, **7**
Hinman 2014[41]	N: 71 Women: 39% Age: 63.4 years BMI: 30.7 VAS pain: 41.5 mm K/L: –	N: 70 Women: 56% Age: 63.8 years BMI: 28.8 VAS pain: 43 mm K/L: –	12 weeks of LLLT versus 12 weeks of sham LLLT	Pain: WOMAC Disability: WOMAC QoL: AQoL-6D Week of assessment: **12**, 52
Jensen 1987[21]	N: 13 Women: – Age: – BMI: – VAS pain: 67 mm K/L: –	N: 16 Women: – Age: – BMI: – VAS pain: 72.6 mm K/L: –	1 week of LLLT versus 1 week of sham LLLT	Pain: 0–21 (movement) Disability: – QoL: – Week of assessment: **1**
Kheshie 2014[47]	N: 18 Women: 0% Age: 56.56 years BMI: 28.62 VAS pain: 76.8 mm K/L: 2.5	N: 15 Women: 0% Age: 55.6 years BMI: 28.51 VAS pain: 78.7 mm K/L: 2.5	6 weeks of exercise therapy and LLLT versus 6 weeks of exercise therapy and sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **6**
Koutenaei 2017[55]	N: 20 Women: 85% Age: 52.3 years BMI: 28.4 VAS pain: 74 mm K/L: 3	N: 20 Women: 80% Age: 53 years BMI: 28.6 VAS pain: 65.5 mm K/L: 3	2 weeks of exercise therapy and LLLT versus 2 weeks of exercise therapy and sham LLLT	Pain: VAS (movement) Disability: – QoL: – Week of assessment: **2**, **4**
Mohammed 2018[56]	N: 20 Women: 85% Age: 55.25 years BMI:≥25 VAS pain: 70 mm K/L: 2	N: 20 Women: 85% Age: 50.11 years BMI:≥25 VAS pain: 80 mm K/L: 2	4 weeks of LLLT versus 4 weeks of sham LLLT	Pain: VAS Disability: – QoL: – Week of assessment: **4**
Nambi 2016[48]	N: 17 Women: – Age: 58 BMI: 26.9 VAS pain: 78 mm K/L: 3.1	N: 17 Women: – Age: 60 BMI: 28.3 VAS pain: 76 mm K/L: 3.2	4 weeks of exercise therapy, kinesio tape and LLLT versus 4 weeks of exercise therapy, kinesio tape and sham LLLT	Pain: VAS Disability: – QoL: – Week of assessment: **4**, **8**
Nivbrant 1992[19]	N: 15 Women: 69.2% Age: 69 years BMI: – VAS pain: 67 mm K/L: –	N: 15 Women: 84.6% Age: 66 years BMI: – VAS pain: 58 mm K/L: –	2 weeks of LLLT versus 2 weeks of sham LLLT	Pain: VAS (movement) Disability: Walking disability QoL: – Week of assessment: **2**, 3, **6**

Continued

Table 1 Continued

First author	Intervention group at baseline	Control group at baseline	Intervention versus control programme	Outcome scales, week of reassessment
Rayegani 2012[43]	N: 12 Women: 83.3% Age: 61.7 years BMI: – VAS pain: 63 mm K/L:<4	N: 13 Women: 92.3% Age: 61.2 years BMI: – VAS pain: 52 mm K/L:<4	2 weeks of LLLT versus 2 weeks of sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: 6, **14**
Tascioglu 2004[40] (3 J)	N: 20 Women: 70% Age: 62.86 years BMI: 27.56 VAS pain: 68 mm K/L: 2	N: 20 Women: 65% Age: 64.27 years BMI: 29.56 VAS pain: 63.88 mm K/L: 2	2 weeks of LLLT versus 2 weeks of sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **3**, 26
Tascioglu 2004[40] (1.5 J)	N: 20 Women: 75% Age: 59.92 years BMI: 28.63 VAS pain: 65.72 mm K/L: 2.5	N: 20 Women: 65% Age: 64.27 years BMI: 29.56 VAS pain: 63.88 mm K/L: 2	2 weeks of LLLT versus 2 weeks of sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **3**, 26
Youssef 2016[42] (904 nm)	N: 18 Women: 66.7% Age: 67.5 BMI:<40 VAS pain: 51.67 mm K/L: 2	N: 15 Women: 66.7% Age: 66.3 years BMI:<40 VAS pain: 50 mm K/L: 2	8 weeks of exercise therapy and LLLT versus 8 weeks of exercise therapy and sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **8**
Youssef 2016[42] (880 nm)	N: 18 Women: 61.1% Age: 67.3 BMI: <40 VAS pain: 52.50 mm K/L: 2	N: 15 Women: 66.7% Age: 66.3 years BMI: <40 VAS pain: 50 mm K/L: 2	8 weeks of exercise therapy and LLLT versus 8 weeks of exercise therapy and sham LLLT	Pain: WOMAC Disability: WOMAC QoL: – Week of assessment: **8**

The values for age and body mass index (BMI) are means and the values for K/L grade are medians. Baseline Visual Analogue Scale (VAS) scores have been extracted or estimated as described in the Method section. Week of assessment in bold denotes time point used for the main meta-analyses.
AQoL-6D, Assessment of Quality of Life 6 Dimensions; DIQ, Disability Index Questionnaire; K/L, Kellgren/Lawrence; LLLT, low-level laser therapy; NRS, Numeric Rating Scale; QoL, quality of life; SKFS, Saudi Knee Function Scale; TENS, Transcutaneous Electrical Nerve Stimulation; VNPS, Visual Numerical Pain Scale; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

weeks with the non-recommended LLLT doses (tables 1 and 2). Non-recommended LLLT doses were applied in nine of the trials. That is, Al Rashoud *et al*,[31] Bülow *et al*,[20] Tascioglu *et al*[40] and Bagheri *et al*[23] applied too few (<4) Joules per treatment spot with 830 nm wavelength, Jensen *et al*,[21] Nivbrant *et al*[19] and Hinman *et al*[41] applied too few (<1) Joules per treatment spot with 904 nm wavelength and Youssef *et al*[42] (one group) and Rayegani *et al*[43] used continuous laser with too long of a wavelength (880 nm; table 2). No adverse event was reported by any of the trial authors. None of the trial authors stated receiving funding from the laser industry (online supplementary material).

Overall, pain was significantly reduced by LLLT compared with the placebo control at the end of therapy (14.23 mm VAS (95% CI 7.31 to 21.14); I^2=93%; n=816; figure 2) and during follow-ups 1–12 weeks later (15.92 mm VAS (95% CI 6.47 to 25.37); I^2=93%; n=581; figure 3). The dose subgroup analyses demonstrated that pain was significantly reduced by the recommended LLLT doses compared with placebo at the end of therapy (18.71 mm (95% CI 9.42 to 27.99); I^2=95%; n=480; figure 2) and during follow-ups 2–12 weeks later (23.23 mm VAS (95% CI 10.60 to 35.86); I^2=95%; n=392; figure 3). The dose subgroup analyses demonstrated that pain was significantly reduced by the non-recommended LLLT doses compared with placebo at the end of therapy (6.34 mm VAS (95% CI 1.26 to 11.41); I^2=44%; n=336; figure 2), but the difference during follow-ups 1–12 weeks later was not significant (6.20 mm VAS (95% CI −0.65 to 13.05); I^2=38%; n=189; figure 3). The between-subgroup differences (recommended versus non-recommended doses) in pain results were significantly in favour of the recommended LLLT doses regarding both time points (p=0.02 and 0.02; figures 2 and 3).

Overall, disability was significantly reduced by LLLT compared with placebo at the end of therapy (SMD=0.59 (95% CI 0.33 to 0.86); I^2=57%; n=617; figure 4) and during follow-ups 1–12 weeks later (SMD=0.66 (95% CI 0.23 to 1.09); I^2=67%; n=289; figure 5). The dose subgroup analyses demonstrated that disability was significantly reduced by the recommended LLLT doses compared with placebo

Table 2 Laser therapy characteristics of the included trials

First author	Treated area	Wavelength (nm)	Joules per treatment spot	Mean output power (mW)	Seconds per treated spot	Number of spots treated	Sessions/sessions per week
Al Rashoud 2014[31]*	Knee joint line (medial and lateral) and acupoints (SP9, SP10, ST36)	830	1.2	30	40	5	9/3
Alfredo 2011, 2018[29 52]	Knee joint line (medial and lateral)	904	3	60	50	9	9/3
Alghadir 2014[32]	Knee condyles, joint line (medial and lateral) and popliteal fossa	850	6	100	60	8	8/2
Bagheri 2011[23]*	Knee joint line	830	3	30	100	10	10/5
Bülow 1994[20]*	Painful spots in 0–10 cm radius of the knee joint line	830	1.5–4.5	25	60–180	5–15	9/3
Delkhosh 2018[39]	Knee joint	830	5	30	167	5	10/5
Fukuda 2011[30]	Front knee capsule	904	3	60	50	9	9/3
Gur 2003[33] (1.5 J)	Anterolateral and anteromedial portal of the knee	904	1.5	10	150	2	10/5
Gur 2003[33] (1 J)	Anterolateral and anteromedial portal of the knee	904	1	11.2	90	2	10/5
Gur and Oktayoglu	Anterolateral and anteromedial portal of the knee	904	1.5	10	150	2	10/5
Gworys 2012[18]	Knee joint line, patellofemoral joint and popliteal fossa	810	8	400	20	12	10/5
Hegedűs 2009[53]	Knee joint line, popliteal fossa and condyles	830	6	50	120	8	8/2
Helianthi 2016[54]	Knee joint line (lateral) and acupoints (ST36, SP9, GB34, EX-LE-4)	785	4	50	80	5	10/2
Hinman 2014[41]*	Acupoints (locations not stated)	904	0.2	10	20	6	8-12/0.67–1
Jensen 1987[21]*	Knee joint line (medial and lateral), apex and basis of patellae	904	0.054	0.3	180	4	5/5
Kheshie 2014[47]†	Front knee	830	–	160	–	–	12/2
Koutenaei 2017[55]	Front knee, popliteal fossa and femur condyles in the popliteal cavity	810	7	100	70	8	10/5
Mohammed 2018[56]	Knee joint line (lateral) and acupoints (ST36, Sp10, GB, ashi)	808	5.4	90	60	7	12/3
Nambi 2016[48]	Knee joint line, condyles and popliteal fossa	904	1.5	25	60	8	12/3
Nivbrant 1992[19]*	Knee joint line (medial and lateral) and acupoints (ST34, SP10, X32)	904	0.72	4	180	7	6/3
Rayegani 2012[43]*	Knee joint line and popliteal fossa	880	6	50	120	8	10/5
Tascioglu 2004[40] (3 J)*	Painful spots on the knee	830	3	50	60	5	10/5
Tascioglu 2004[40] (1.5 J)*	Painful spots on the knee	830	1.5	50	30	5	10/5
Youssef 2016[42] (904 nm)	Knee joint line (medial and lateral)	904	3	60	50	9	16/2
Youssef 2016[42] (880 nm)*	Knee joint line (medial and lateral), epicondyles and popliteal fossa	880	6	50	120	8	16/2

*Non-recommended low-level laser therapy dose.
†1250 Joules per session.

at the end of therapy (SMD=0.75 (95% CI 0.46 to 1.03); I^2=34%; n=339; figure 4) and during follow-ups 2–8 weeks later (SMD=1.31 (95% CI 0.92 to 1.69); I^2=0%; n=129; figure 5). The dose subgroup analyses demonstrated that disability was neither significantly reduced by the non-recommended LLLT doses compared with placebo at the end of therapy (SMD=0.36 (95% CI −0.02 to 0.73); I^2=49%; n=278; figure 4) nor during follow-ups 1–12 weeks later (SMD=0.26 (95% CI −0.06 to 0.58); I^2=0%; n=160; figure 5). The between-subgroup differences in disability results were in favour of the recommended LLLT doses over the non-recommended LLLT doses but only significantly regarding one of two time points (p=0.11 and <0.0001; figures 4–5).

No QoL meta-analysis was performed because this outcome was only assessed in a single trial, that is, by Hinman *et al* who applied a non-recommended LLLT dose and reported insignificant results.[41]

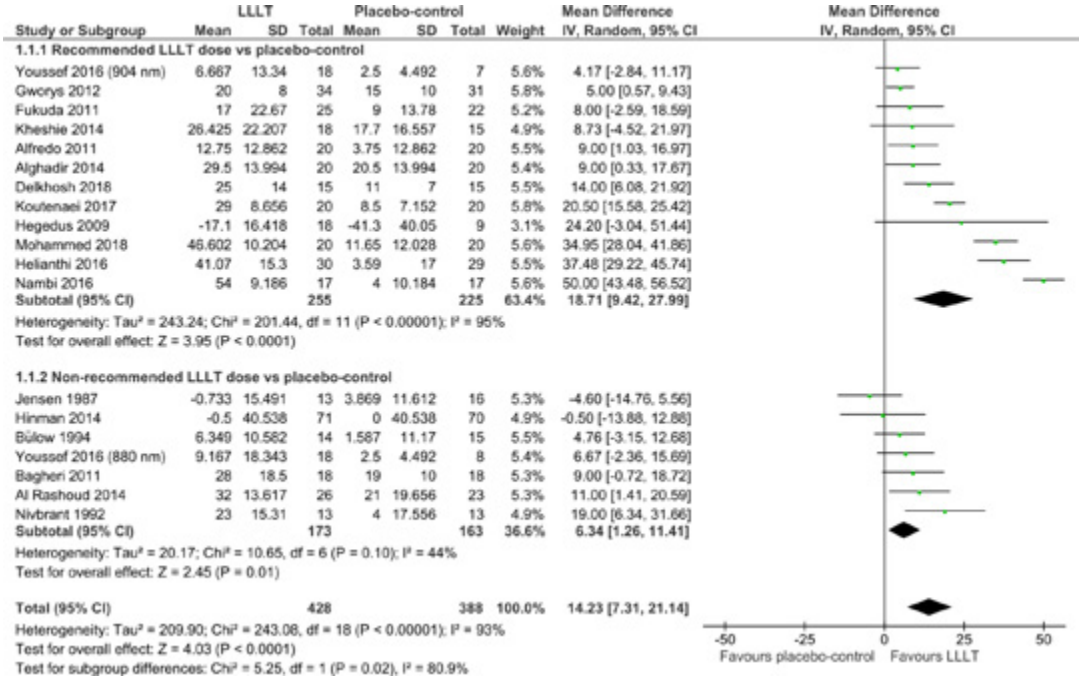

Figure 2 Pain results from immediately after the end of therapy. LLLT, low-level laser therapy.

The funnel plots indicated that there was no publication bias (online supplementary material). We additionally checked for small study bias by reducing the statistical weight of the smallest studies through a change from random to fixed effects models and this led to similar mean effect estimates, indicating that there was no small study bias (online supplementary material).[35]

Methodological quality of the included trials was judged adequate (low risk of bias), unclear (unclear risk of bias) and inadequate (high risk of bias) in 75%, 19% and 6% instances, respectively. Risk of detection bias and reporting bias appeared low in all the trials. There was a lack of information regarding random sequence generation in five trials, allocation concealment in 12 trials, blinding of therapist in four trials and incomplete outcome data in four trials. Therapist blinding was inadequate in seven trials and there was an inadequate handling of data in a single trial (figure 6). However, risk-of-bias subgroup analyses conducted post hoc revealed that there was no statistically significant interaction between the effect estimates and risk of bias, and the analyses did not display a drop in statistical heterogeneity (online supplementary material). Support for our risk of bias judgments is available (online supplementary material).

Neither did the levels of statistical heterogeneity change when we switched from the MD to the SMD method post hoc (online supplementary material).

Post hoc analyses demonstrated that LLLT was significantly superior to placebo both with exercise therapy (p=0.0009 for pain and p<0.0001 for disability) and without exercise therapy (p=0.01 for pain and p=0.008

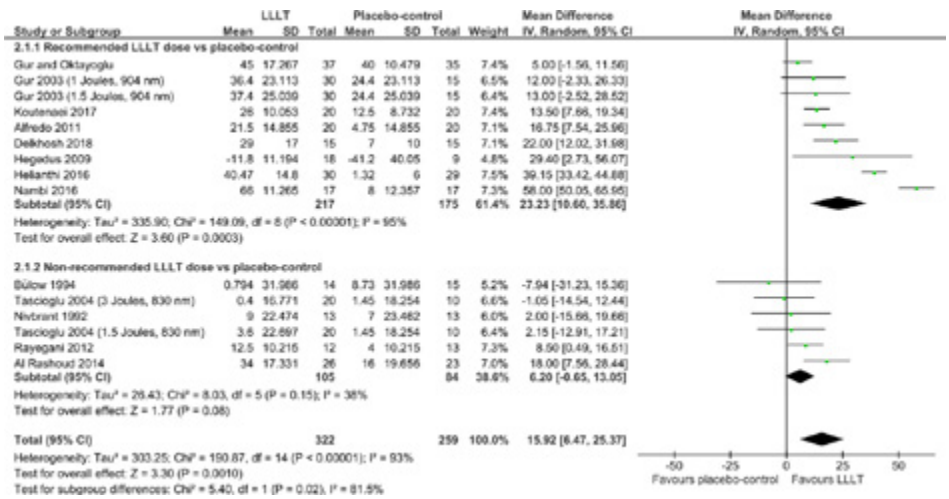

Figure 3 Pain results from follow-ups 1–12 weeks after the end of therapy. LLLT, low-level laser therapy.

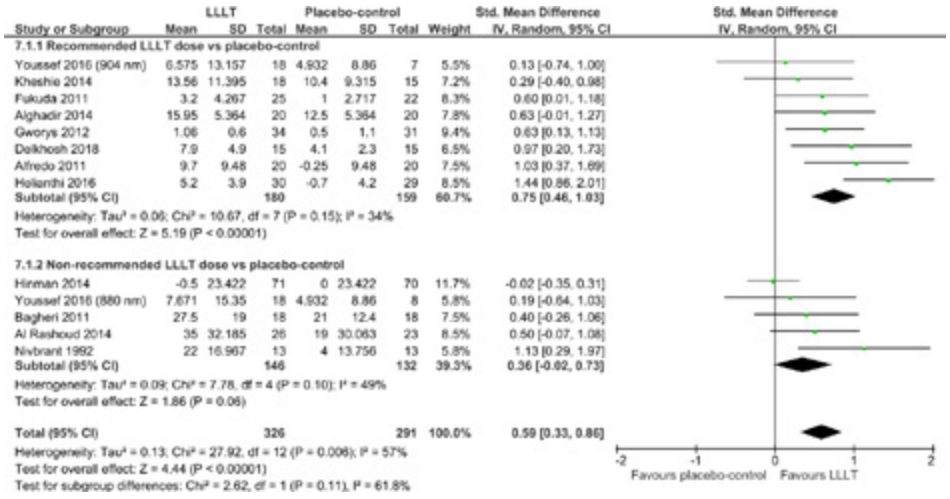

Figure 4 Disability results from immediately after the end of therapy. LLLT, low-level laser therapy.

for disability) as cointervention (online supplementary material).

Post hoc analyses were performed to more precisely estimate the pain time-effect profile for the recommended LLLT doses by imputing the results of the trials with these doses in subgroups with narrower time intervals. Pain was significantly reduced by the recommended LLLT doses compared with placebo immediately after therapy weeks 2–3 and 4–8 and at follow-ups 2–4, 6–8 and 12 weeks later; the peak point was 2–4 weeks after the end of therapy (31.87 mm VAS beyond placebo (95% CI 18.18 to 45.56); I^2=93%; n=322). The 21-week and 34-week follow-up pain results were not statistically significant (figure 7 and online supplementary material). The statistical heterogeneity in the main pain analyses of the recommended LLLT doses was high (I^2=95%; figures 2–3) but the mean statistical heterogeneity of the five subgroups covering the same time period was only moderate (I^2=58%; figure 7 and online supplementary material).

DISCUSSION

Our meta-analyses showed that pain and disability were significantly reduced by LLLT compared with placebo. We subgrouped the included trials according to the WALT recommendations (2010) for laser dose per treatment spot, and this revealed a significant dose–response relationship. Our principal finding is that the recommended LLLT doses offer clinically relevant pain relief in KOA. The non-recommended LLLT doses provided no or little positive effect.

The absolute minimally clinically important improvement (MCII) of pain in KOA has been estimated to be 19.9, 17 and 9 units on a 0–100 scale in 2005, 2012 and 2015, respectively.[44–46] It is important to note that the MCII of pain is a within-subject improvement and depends on baseline pain intensity.[44–46] The pain reduction from the recommended LLLT doses was significantly superior to placebo even at follow-ups 12 weeks after the end of therapy, and the difference was greater than 20 mm VAS from the final 4–8 weeks of therapy through follow-ups 6–8 weeks after the end of therapy. Interestingly, the pain reduction from the recommended LLLT doses peaked at follow-ups 2–4 weeks after the end of therapy (31.87 mm VAS highly significantly beyond placebo).

Disability was also significantly reduced by the recommended LLLT doses compared with placebo, that is, to

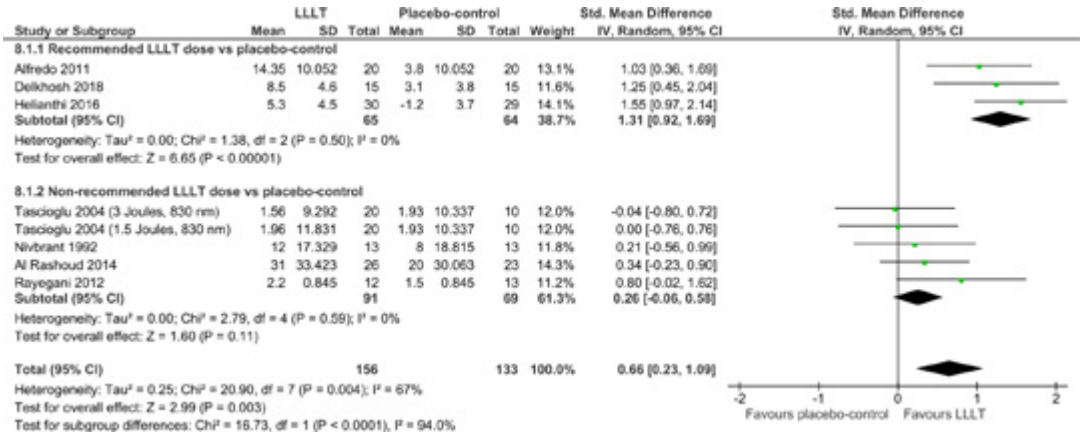

Figure 5 Disability results from follow-ups 1–12 weeks after the end of therapy. LLLT, low-level laser therapy.

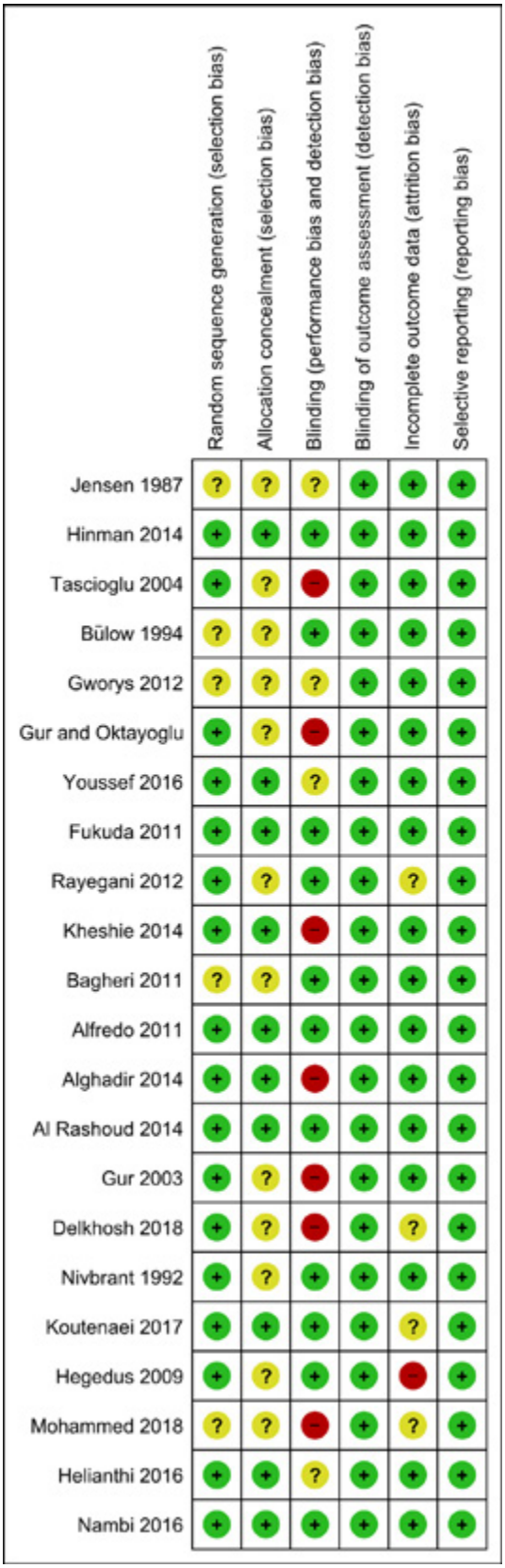

Figure 6 Risk-of-bias plot of the included trials. The trials are ranked by mean pain effect estimates, that is, more laser positive results in the bottom of the figure; the plot is based on the results from the main pain analyses (immediately after the end of therapy, primarily).

a moderate extent at the end of therapy (SMD=0.75) and to a large extent during follow-ups 2–8 weeks later (SMD=1.31). More trials with disability assessments are needed to precisely estimate the effect of LLLT on this outcome during follow-up.

Furthermore, our analyses demonstrated that LLLT is effective in KOA both with and without exercise therapy as cointervention. Strength training was seemingly only used as an adjunct to LLLT in two of the included trials,[47 48] and thus more trials with this combination of treatments are needed.

Risk of bias of the included trials appeared insignificant and could not explain the statistical heterogeneity (online supplementary material). We find it plausible that some of the statistical heterogeneity of the overall analyses is associated with the dose subgroup criteria (wavelength-specific laser doses per treatment spot) since the mean levels of statistical heterogeneity of the subgroup analyses were consistently lower than the overall levels. It is unknown to us whether other differences in the LLLT protocols impacted the results.

The statistical heterogeneity in the main pain analyses of the recommended LLLT doses was high, and some of it can be explained by the pooling of results from various time points of assessment given the pain reduction increased and subsequent decreased with time; the pain reduction time profile showed a drop in statistical heterogeneity to a moderate level.

According to WALT, the osteoarthritic knee should be laser irradiated to reduce inflammation and promote tissue repair.[24 25 49] One of the discrepancies from our review and previously published reviews of the same topic is that we omitted the RCT by Yurtkuran *et al*,[8 17 28 50] as they solely applied laser to an acupoint located distally from the knee joint (spleen 9).

In line with our findings and the WALT dose recommendations, Joensen *et al*[26] observed that the percentage of laser penetrating rat skin at 810 and 904 nm wavelength was 20% and 38%–58%, respectively. That is, to deliver the same dose beneath the skin, 2.4 times the energy on the skin surface is required with an 810 nm laser compared with a 904 nm laser device. This may be due to the different wavelengths and/or because 904 nm laser is superpulsed (pulse peak power ≥10 000 mW typically), whereas shorter wavelength laser is delivered continuously or with less intense pulsation.[26] The estimated median dose applied with the recommended LLLT was 6 and 3 J per treatment spot with 785–860 and 904 nm wavelength laser, respectively. Most of the trial authors reported LLLT parameters in detail but did not state whether the laser devices were calibrated. Therefore, in the LLLT trials with non-significant effect estimates, equipment failure cannot be ruled out.

It is important to note that no adverse events were reported by any of the trial authors and the dropout rate was minor, indicating that LLLT is harmless.

Our clinical findings that the effect of LLLT progresses over time is in line with in vivo results of Wang *et al*.[12] The

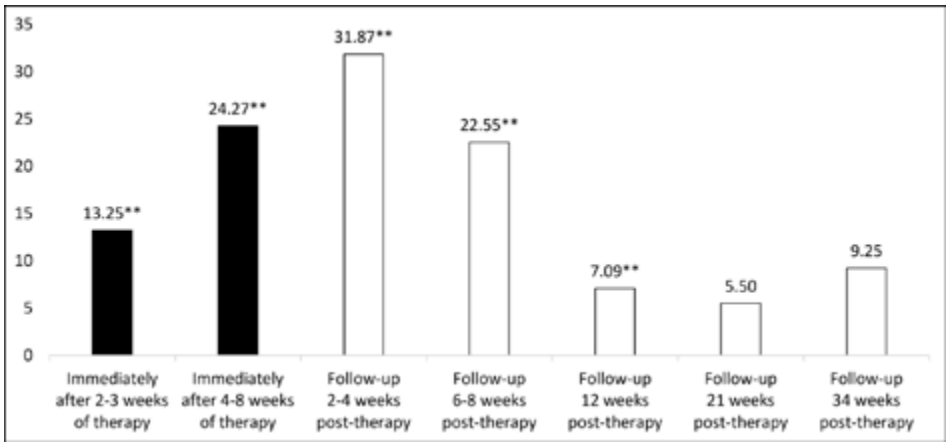

Figure 7 Pain time-effect profile (recommended low-level laser therapy (LLLT) doses versus placebo-control). Values on the y-axis are mm Visual Analogue Scale (VAS) pain results. Positive VAS score indicates that the recommended LLLT doses are superior to placebo. The related forest plot is available (online supplementary material). **The recommended LLLT doses are highly statistically significantly superior to placebo (p≤0.01).

positive effect from LLLT seems to last longer than those of widely recommended painkiller drugs.[51] The effect of using the NSAID tiaprofenic acid, for example, is probably gone within a week, unless the treatment is continued.[51] Future trials should investigate whether booster sessions of LLLT can prolong the positive effect. Comparative cost-effectiveness analyses of LLLT and NSAIDs would also be of great interest.

Strengths and limitations of this study

In contrast to previous reviews on the current topic, our review was conducted in conformance with an a priori published protocol,[8 17 28] which included a detailed plan for statistical analysis (eg, laser dose subgroup criteria). Furthermore, this is the first review on this topic without language restrictions,[8 17 28] and this expansion proved important since four (18%) of the included trials were reported in non-English language.[19 21 23 39]

We conducted a series of meta-analyses illustrating the effect of LLLT on pain over time. To ensure high reproducibility of the meta-analyses, three persons each independently extracted the outcome data from the included trial articles.

This review is not without limitations. It lacks QoL analyses, a detailed disability time-effect analysis and direct comparisons between LLLT and other interventions.

CONCLUSIONS

LLLT reduces pain and disability in KOA at 4–8 J with 785–860 nm wavelength and at 1–3 J with 904 nm wavelength per treatment spot.

Contributors MBS, JMB and HL wrote the PROSPERO protocol. MBS and JMB selected the trials, with the involvement of IFN when necessary. MBS and JJ judged the risk of bias, with the involvement of IFN when necessary. MBS and IFN did the translations. MBS, JMB and KVF extracted the data. MBS performed the analyses, under supervision of JMB. All the authors participated in interpreting of the results. MBS drafted the first version of the manuscript, and subsequently revised it, based on comments by RÁBL-M, HS and all the other authors. All the authors read and accepted the final version of the manuscript.

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Competing interests JMB and RÁBL-M are post-presidents and former board members of World Association for Laser Therapy, a non-for-profit research organization from which they have never received funding, grants or fees. The other authors declared that they had no conflict of interests related to this work.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement The dataset for meta-analysis is available from the corresponding author upon reasonable request. The corresponding author affirms that the manuscript is an honest, accurate and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Original research

Immediate pain relief effect of low level laser therapy for sports injuries: Randomized, double-blind placebo clinical trial

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ABSTRACT

Objectives: To determine the immediate pain relief effect of low-level laser therapy on sports injuries in athletes and degree of pain relief by the therapy.
Design: Double-blind, randomized, comparative clinical study.
Methods: Participants were 32 college athletes with motion pain at a defined site. Participants were randomized into two groups in which the tested or placebo laser therapy was administered to determine pain intensity from painful action before and after laser irradiation, using the Modified Numerical Rating Scale. The post-therapeutic Modified Numerical Rating Scale score was subtracted from the pre-therapeutic Modified Numerical Rating Scale score to determine pain intensity difference, and the rate of pain intensity difference to pre-therapeutic Modified Numerical Rating Scale was calculated as pain relief rate.
Results: Low-level laser therapy was effective in 75% of the laser group, whereas it was not effective in the placebo group, indicating a significant difference in favor of the laser group ($p < 0.001$). Pain relief rate was significantly higher in the laser group than in the placebo group (36.94% vs. 8.20%, respectively, $p < 0.001$), with the difference in pain relief rate being 28.74%.
Conclusions: Low-level laser therapy provided an immediate pain relief effect, reducing pain by 28.74%. It was effective for pain relief in 75% of participants.

1. Introduction

Sports injuries constitute a serious problem for many athletes and others who participate in sports because they cause pain and dysfunction, resulting in the inability to continue sports activities. Various physical therapies, including electrotherapy, thermotherapy, cryotherapy, and phototherapy, have been used to alleviate symptoms of sports injuries such as pain.[1–3] Low-level laser therapy (LLLT) has been clinically introduced as one of such physical therapies.

LLLT has been examined in clinical research and reported to be effective for its long-term effect on many diseases in the general adult population.[4–6] Bjordal et al.[7] reported that a single session of LLLT relieved tenderness at the affected site in patients with Achilles tendinitis, and they demonstrated both immediate and long-term effects on injuries in the general adult population.

Studies on the effect of LLLT on sports injuries in athletes are limited to the reports of its effect on sprained ankles[8] and Achilles tendinopathy.[9] In both of these studies, the effect of LLLT was the same as was observed in the general adult population. In the study by Stergioulas[8] in patients with sprained ankles, LLLT that was given twice daily significantly alleviated edema at 24–72 h as compared with placebo therapy. In another study by Stergioulas et al.[9] in recreational athletes with Achilles tendinopathy, the combination of eccentric exercise and LLLT for 4–12 weeks alleviated

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motion pain as compared with placebo therapy. Thus, LLLT has been demonstrated to alleviate edema and pain associated with sports injuries in a few days to weeks. However, these studies did not provide any data on the immediate pain relief effect of LLLT on sports injuries in athletes.

Because athletes with sports injuries need earlier functional recovery compared to members of the general population, the immediate effect of LLLT is important. Therefore, this study was designed to evaluate whether LLLT provides an immediate pain relief effect on sports injuries in athletes and to determine the extent of pain relief by LLLT.

2. Materials and methods

A double-blind, randomized, placebo-controlled, parallel-group comparison study was performed. Participants were randomly assigned to the laser or placebo group.

Forty-seven college athletes met the following inclusion criteria: participation in intercollege to athletic activities 5 days/week or more; treatment at Osaka University of Health and Sport Sciences Clinic between July 1, 2013, and January 31, 2015 for sports injury; and diagnosis by an orthopedist with an orthopedic sports injury for which LLLT was indicated. LLLT was indicated for sport injuries if the following criteria were met: the injuries were painful in motion; the painful area was defined; LLLT was not contraindicated; and the injuries were not associated with any neurological findings.

Exclusion criteria were the inability to define the painful area and absence of definite motion pain. Of the 47 patients enrolled in the study, 32 with a definite painful area and motion pain were included as participants.

The 32 participants were randomly assigned to one of two groups, in which either LLLT or placebo laser therapy was administered (the laser and placebo groups, respectively) according to an assignment table prepared by the coordinator using computer-generated random numbers. The following additional data were collected for each participant: name and site of injury, and period from injury to therapy (number of days after injury). The laser group includes 9 patients with ankle sprain, 1 patient with navicular stress fracture, 1 patient with plantar fasciitis, 1 patient with patella tendinitis, 1 patient with spondylolysis, 1 patient with shoulder arthroscopic surgery, 1 patient with triangular fibrocartilage complex injury, and 1 patient with proximal thumb avulsion fracture. The placebo group includes 5 patients with ankle sprain, 2 patients with meniscal injuries, 2 patients with elbow medial collateral ligament sprain, 2 patients with Achilles tendinitis, 1 patient with low back pain, 1 patient with lumbar facet arthritis, 1 patient with infraspinatus muscle injury, 1 patient with deltoid muscle injury, and 1 patient with shoulder periarthritis.

The sample size of the study was calculated at 15 per group using a statistical power of 0.9, intergroup difference of 30, standard deviation of 25, and significant level of 5% with reference to the results of Malliaropoulos et al.[10] EZR statistical software[11] (Saitama Medical Center, Jichi Medical University, Japan, http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html) was used for calculating the sample size.

This study was performed with the approval of the Research Ethics Committee of Osaka University of Health and Sport Sciences (Approval No. 12-29). Participants who received oral and written explanation of the study and provided written consent to participate were included in the study. LLLT was performed as one of the therapeutic measures after the experiment was completed. Data were collected in the physiotherapy room of Osaka University of Health and Sport Sciences Clinic. None of the participants prematurely discontinued the experiment.

Table 1
Laser parameters.

Wavelength	810 nm (GaAlAs laser)
Frequency	Continuous output
Optical output	180 mW
Spot diameter	0.0007 cm, 0.0005 cm
Spot size	0.0035 cm^2
Power density	51.4 W/cm^2
Energy	5.4 J at each spot
Energy density	1542.85 J/cm^2 at each spot
Treatment time	30 s × 20 times (total 10 min)
Irradiation site	1 cm^2
Application mode	Probe held stationary in skin contact with a 90° angle and slight pressure

Participants in the laser group received LLLT from laser therapy equipment (Softlasery JQ-W1, Minato Medical Science Co., Ltd, Japan) with an output of 180 mW, irradiation time of 30 s, and total irradiation time of 10 min (Table 1). Participants in the placebo group received placebo therapy from a placebo device (detuned laser) with an output of 0 mW, irradiation time of 30 s, and total irradiation time of 10 min. The Softlasery used for this study was contact-type laser therapy equipment with an irradiation area of 0.0035 cm^2. The most painful area during a painful motion was selected as the irradiation site. In order to find the most painful area, participants were asked to explain the most painful motion during their daily or athletic activities. Then participants were asked to identify the most painful area by their index finger during the movement.

Because the study was double-blinded, the measurer and participants were blinded as to whether they used the actual or placebo laser equipment. The output, irradiation time, and total irradiation time of the laser therapy equipment were setup by the coordinator before each participant entered the physiotherapy room. The measurer left the physiotherapy room before the coordinator setup the laser therapy equipment and was call back after the setup. Each participant operated the laser equipment independently after receiving instructions on how to use it by the coordinator. Irradiation site was kept within 1 cm^2. Therefore a laser prove was applied on the most painful area within a 1 cm^2 area for 30 s each time for 20 times, and total irradiation time of 10 min. To ensure participant safety and eliminate participant bias, participants were instructed not to look at the laser light during laser irradiation. The measurer observed the therapy procedure and measured pain intensity of the painful motion before and after laser irradiation in both groups. Pain during the painful motion was measured using the Modified Numerical Rating Scale (MNRS), which is a 10-cm scale from 0 to 10 at 1-cm intervals in millimeters, with 0 representing no pain and 10 representing the worst pain.

Injury sites were classified into upper limbs, lower limbs, and body trunk. The MNRS score after the therapy (post-MNRS) was subtracted from that before the therapy (pre-MNRS) to determine the pain intensity difference (PID). The rate of PID relative to the pre-MNRS was calculated as the pain relief rate (PRR).

$$\text{PID} = \text{Pre-MNRS} - \text{Post-MNRS}$$

$$\text{PRR} = \text{PID}/\text{Pre-MNRS} \times 100$$

Statistical analysis was performed using SPSS 21.0 J for Windows (IBM Corporation, Armonk, NY, USA) and EZR. The mean number of days after injury, mean PRR, and the 95% confidence interval (95% CI) were calculated for each group.

The difference in injury sites between the groups was tested using Fisher's exact test, with a significance level of 5%. The difference in the number of days after injury, pre-MNRS, or PRR between the groups was tested using an unpaired *t*-test with a significance level of 5%. When a significant difference in the PRR was observed between the groups, PRR was classified into poor, fair, good,

Table 2
Participants characteristics with statistical comparison.

	Laser (n = 16)	Placebo (n = 16)	Difference	p value
Age (years), mean ± SD	20.25 ± 1.18	20.88 ± 2.25		0.335*
Sex (n), male/female	7/9	7/9		1.00*
Height (cm), mean ± SD	164.86 ± 8.97	170.56 ± 9.12		0.085*
Weight (kg), mean ± SD	60.53 ± 11.66	65.68 ± 10.80		0.205*
Pain (MNRS), mean (95%CI)				
Pre	5.29 (3.98–6.61)	5.88 (5.09–6.66)		0.426*
Post	3.26 (2.35–4.18)	5.32 (4.64–6.00)	2.06 (0.97–3.15)	<0.001* (t = 3.850)
PRR (%) mean (95%CI)	36.94 (25.81–48.07)	8.20 (2.43–13.98)	28.74 (16.72–40.75)	<0.001* (t = 4.886)
Injury sites (n)				0.556†
Upper	3	5		
Lower	12	9		
Trunk	1	2		
After injury (day)	37.69 ± 43.36	32.38 ± 35.21		0.706*

MNRS: the Modified Numerical Rating Scale; PRR: pain relief rate.
* Student's t-test.
† Fisher's exact test.

excellent based on the mean value of the difference and 95% CI. A PRR value equal to or above the mean value of the difference in PRR between the groups was considered to indicate that LLLT was effective, and a PRR value below the mean value was considered to indicate that LLLT was ineffective. The difference in the rate of participants in whom LLLT was effective or ineffective was tested with the χ^2, test with a significance level of 5%.

3. Results

Their characteristics and comparison are shown in Table 2. No significant difference in injury sites (p = 0.556, Table 2) or number of days after injury (p = 0.706, t = 0.380, Table 2) was observed between the groups. The rate of participants in whom LLLT was effective was 75% in the laser group and 0% in the placebo group, and the rate of participants in whom LLLT was ineffective was 25% in the laser group and 100% in the placebo group, with a significant difference between the groups (p < 0.001, χ^2 = 19.20, Fig. 1). The PRR was significantly higher in the laser group than in the placebo group (36.94% [95% CI: 25.81–48.07] vs. 8.20% [95% CI: 2.43–13.98]; p < 0.001, t = 4.886, Table 2), with an intergroup difference of 28.74% (95% CI: 16.72–40.75, Table 2).

4. Discussion

Although an earlier study[9] showed that long-term LLLT had a pain relief effect on Achilles tendinopathy in sports injuries, no studies have specifically examined the immediate pain relief effect and PRR of LLLT in injured athletes who need early return to play. Therefore, to examine the immediate pain relief effect of LLLT on sports injuries, this study was performed as a double-blind, randomized, placebo-controlled clinical trial to compare the pain relief effect of one session of LLLT in the laser group with that of placebo therapy in the placebo group. The results showed a significantly higher rate of pain relief effect among participants in the laser group (p < 0.001). Low-level laser has been reported to provide various effects, including inhibition of nerve excitement,[12–14] anti-inflammation,[7,15,16] and tissue repair.[17–19] The immediate pain relief effect that we observed is more likely related to inhibition of nerve excitement than to chronic effects such as anti-inflammation and tissue repair. In 25% of the laser group in whom LLLT was not effective, no specific characteristics were observed and no specific causal factors could be identified. This is consistent with the results of Malliaropoulos et al.,[10] who reported that 4-weeks LLLT was not effective in 12.5% of patients with meniscal injuries and that no

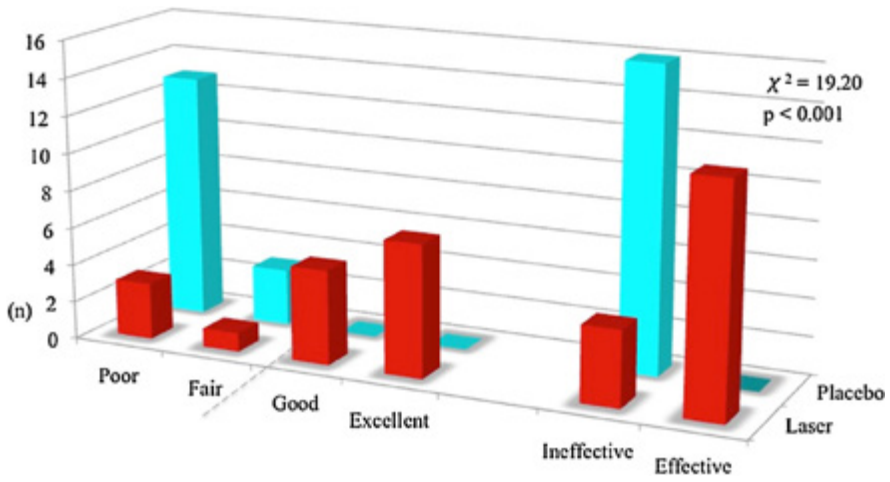

Fig. 1. PRR classification and comparison of ineffective with effective. PRR: pain relief rate (%). χ^2 test with a significance level of 5%. PRR was classified into poor, fair, good, excellent based on the mean value of the difference and 95% CI. A PRR value equal to or above the mean value of the difference in PRR between the groups was considered to indicate that LLLT was effective, and a PRR value below the mean value was considered to indicate that LLLT was ineffective.

specific causal factors could be determined. In addition to the pain relief effect of LLLT, we examined the PRR to determine the degree of pain relief. The PRR was significantly higher in the laser group than in the placebo group (36.94% vs. 8.2%, respectively, $p < 0.001$), and LLLT immediately relieved motion pain by 28.74% (Table 2). Malliaropoulos et al.[10] reported that 4-weeks LLLT and placebo laser relieved pain by approximately 65% and 22%, respectively, in a randomized controlled trial in patients with meniscal injuries. Bjordal et al.[7] investigated the change in tenderness at the affected site after a single session of LLLT and reported that LLLT significantly relieved tenderness as compared with placebo laser therapy. The results of the present study support the earlier report of Bjordal et al.[7] in terms of the comparable immediate pain relief effect of LLLT although the pain relief effect was lower than that observed with 4-weeks LLLT in the study by the Malliaropoulos et al.[10] In the present study, no significant difference was noted between the two groups for injury sites, number of days after injury, or pre-MNRS, indicating that the observed pain relief effect was attributable to low-level laser irradiation and that the effect of injury sites, number of days after injury, and pre-treatment pain intensity was limited. Moreover, LLLT relieved the pain associated with the sports injuries by 36.94%. Based on the difference from placebo therapy, the rate of pain relief from low-level laser irradiation was 28.74%, and immediate pain relief occurred in 75% of the participants.

Bjordal et al.[7] reported that a single session of LLLT reduced the accumulation of prostaglandin E2, an inflammatory substance, at 105 min. Thus, LLLT appears to have not only an immediate pain relief effect, but also late anti-inflammatory and tissue-repair promoting effects, and may be useful in combination with active exercise therapy. In fact, Stergioulas[20] reported that the combination of plyometric exercise and LLLT relieved pain more than the combination of the exercise therapy and placebo laser in patients with humeral lateral epicondylitis. Furthermore, Stergioulas et al.[9] reported that the combination of eccentric exercise and LLLT resulted in better relief of motion pain than the combination of the exercise therapy and placebo laser in recreational athletes with Achilles tendinopathy. Thus, the present study provides important data to demonstrate the uses (e.g., immediate pain relief and promotion of exercise therapy) and efficacy of LLLT.

The present study was limited by its failure to cover all sports injuries. LLLT was not shown to be effective for all sports injuries.[21]

5. Conclusions

To examine the immediate pain relief effect of LLLT on sports injuries, the present study was performed as a randomized, double-blind, placebo-controlled, clinical study comparing the pain relief effect of a single session of LLLT with that of placebo laser therapy. The results revealed that LLLT relieved pain associated with sports injuries by 36.94%. Based on the difference from the placebo therapy, the rate of pain relief from low-level laser irradiation was 28.74%, and significant immediate pain relief occurred in 75% of the participants. These results serve as important data to demonstrate the usefulness of LLLT in combination with active exercise therapy.

Practical implications

- Low-level laser therapy has immediate effect of pain relief for motion pain in sports injuries.
- Low-level laser therapy should be applied to a small treatment area for pain relief.
- Low-level laser therapy should not apply to the patients with inability to define the painful area and absence of definite motion pain.

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

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Abstract

Background

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

Methods

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

Results

18 randomised placebo-controlled trials (RCTs) were identified with 13 RCTs (730 patients) meeting the criteria for meta-analysis. 12 RCTs satisfied half or more of the methodological criteria. Publication bias was detected by Egger's graphical test, which showed a negative direction of bias. Ten of the trials included patients with poor prognosis caused by failed steroid injections or other treatment failures, or long symptom duration or severe baseline pain. The weighted mean difference (WMD) for pain relief was 10.2 mm [95% CI: 3.0 to 17.5] and the RR for global improvement was 1.36 [1.16 to 1.60]. Trials which targeted acupuncture points reported negative results, as did trials with wavelengths 820, 830 and 1064 nm. In a subgroup of five trials with 904 nm lasers and one trial with 632 nm wavelength where the lateral elbow tendon insertions were directly irradiated, WMD for pain relief was 17.2 mm [95% CI: 8.5 to 25.9] and 14.0 mm [95% CI: 7.4 to 20.6] respectively, while RR for global pain improvement was only reported for 904 nm at 1.53 [95% CI: 1.28 to 1.83]. LLLT doses in this subgroup ranged between 0.5 and 7.2 Joules. Secondary outcome measures of pain free grip strength, pain pressure threshold, sick leave and follow-up data from 3 to 8 weeks after the end of treatment, showed consistently significant results in favour of the same LLLT subgroup ($p < 0.02$). No serious side-effects were reported.

Conclusion

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

Background

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

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

Exercise therapy and stretching exercises have been used either alone or in conjunction with manipulation techniques or physical interventions. Although the sparse evidence makes it difficult to assess the separate effect of active exercises or stretching [12], four studies have found that either exercises alone [13], or in conjunction with a physiotherapy package, are more effective than placebo ultrasound therapy or wait-and-see controls. Also exercise therapy, particularly eccentric exercises, have been found effective in the intermediate term in tendinopathies of the Achilles, patellar or shoulder tendons [14, 15, 16, 17]. There is some evidence suggesting that joint manipulation or mobilisation techniques either of the wrist, elbow or cervical spine may contribute to short-term effects in LET [18, 19, 20].

Among the physical interventions, ultrasound therapy has been considered to offer a small benefit over placebo from two small trials [12], but a well-designed and more recent trial did not find significant effects of ultrasound therapy in LET [21]. Reviewers have arrived at different

conclusions for the effect of acupuncture [22, 23]. In reviews of physical interventions for LET, conclusions may vary between reviews because of differences in the treatment procedures. A good example of this is the negative conclusion of the LET review for extracorporeal shockwave therapy (ESWT) by Buchbinder et al. [24], where a later review with in-depth assessments of treatment intervention protocols [25], found that a subgroup of trials with proper treatment procedures and adequate timing of outcomes gave a positive result.

Low level laser therapy (LLLT) has been available for nearly three decades, and scattered positive results have been countered by numerous negative trial results. Several systematic reviews have found no significant effects from LLLT, in musculoskeletal disorders in general [26], and in LET in particular [12, 23, 27]. In this perspective it may seem futile to perform yet another systematic review in this area. But none of these reviews evaluated the results separately for the different LLLT treatment procedures, laser wavelengths or doses involved. Neither did they implement evidence of the newly discovered biomodulatory mechanisms which are involved when LLLT is applied. During the last 5–6 years the annual number of published LLLT reports in Medline has increased from 25 to around 200. We recently made a review of this literature, and concluded that LLLT has an anti-inflammatory effect in 21 out of 24 controlled laboratory trials, and a biostimulatory effect on collagen production in 31 out of 36 trials [28]. Both of these effects were dose-dependent and could be induced by all wavelengths between 630 and 1064 nm with slight variations in therapeutic dose-ranges according to the wavelength used. The anti-inflammatory effect was seen in higher therapeutic dose-ranges than the biomodulatory effect on fibroblast cells and collagen fibre production. Diagnostic ultrasonography of tendinopathies has revealed that partial ruptures and tendon matrix degeneration are underdiagnosed if only physical examinations are made. Consequently, the stimulatory LLLT-effect on collagen fibre production should probably be beneficial for tendon repair. Another interesting feature was that LLLT with too high power densities or doses (above 100 mW/cm^2), seemed to inhibit fibroblast activity [29] and collagen fibre production [30]. Six years ago we showed in a systematic review of tendinopathy, that the effect of LLLT is dose-dependent [31]. At the time, the accompanying editoral suggested that the advanced review design could become the new standard for reviewing empirical therapies with unknown optimal doses and procedural differences [32]. Steroids induce a down-regulation of cortisol receptors, and we recently discovered that the cortisol antagonist mifepristone completely diminished the anti-inflammatory effect of LLLT [33]. All these recent findings from the LLLT literature, prompted the World Association for Laser Therapy (WALT) to publish dosage recommendations and standards for the conductance of systematic reviews and meta-analyses last year [34]. One of the issues that has lacked attention is the validity of LLLT-application procedures in tendinopathy. To our knowledge there are only three valid irradiation techniques for LLLT in tendinopathies: a) direct irradiation of the tendon, b) irradiation of trigger points and c) irradiation of acupuncture points.

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

Methods

Literature search

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

Inclusion criteria

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

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

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

3) Design: Randomised parallel group design or crossover design

4) Blinding: Outcome assessors should be blinded

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

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

Outcome measures

Primary outcome measures

Measured after the end of treatment, either as:

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

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

Secondary outcome measures

c) painfree grip strength (dynamometer, vigorimeter)

d) pain pressure threshold (algometer)

e) sick leave (days)

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

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

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

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

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

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

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

Results

Literature search results

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

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

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

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

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

Analysis of treatment procedures

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

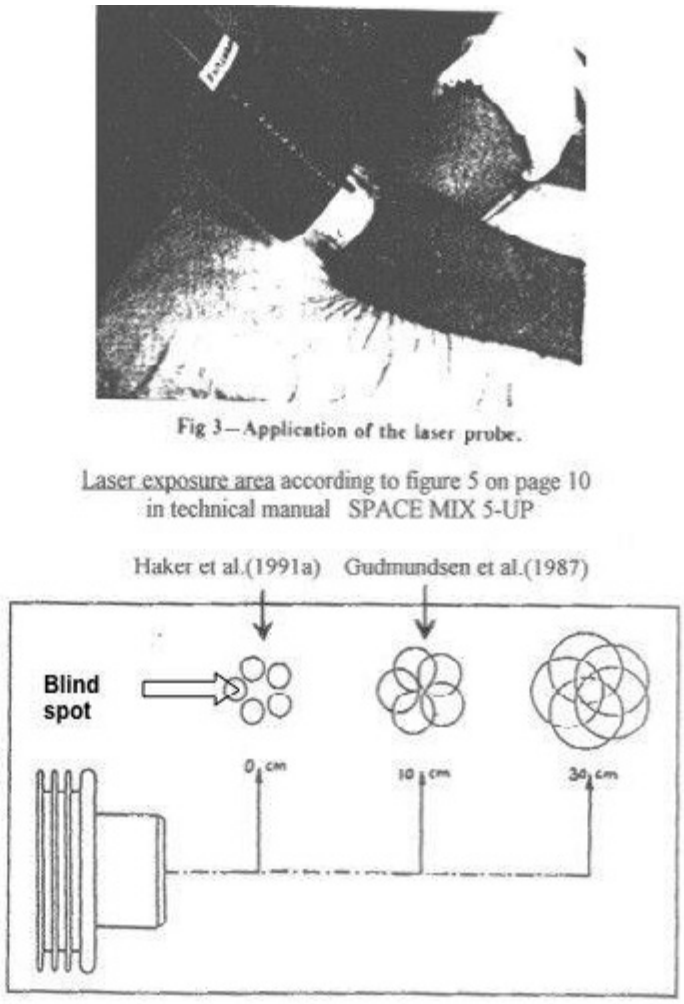

Figure 2
Photograph showing laser therapy procedure with laser head in skin contact in trial by Haker et al. The photograph is taken the trial report in from Archives of Physical Medicine 1991. The drawing of the laser spot sizes at different distances is taken from the manual of Space Mix 5 Mid-Laser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

Table 2

Study by first author	Method score	Wavelength	Application technique	Result	Reason for exclusion
Haker [43]	6	904	Tendon	No significant differences	Photograph in trial report shows that the laser probe was kept in skin contact and thereby violated the manufacturers' recommendation of a keeping the laser head at a distance of 10 cm. This violation caused a

					central blind spot of ca 3 cm^2 which left the tendon pathology unexposed to LLLT (See Figure 2)
Siebert [44]	6	904 + 632	Tendon	No significant differences	Active laser treatment to the placebo group received red 632 nm LLLT, which we calculated to be (2.25J), which again is an adequate LLLT dose. Consequently this trials lacks a placebo or non-laser control group

Randomised LLLT-trials excluded for not meeting criteria of valid procedures for active laser and placebo laser treatment.
Trial characteristics given by first author, method score, laser wavelength, laser application technique, trial results and reason for exclusion.

Publication bias

The five excluded RCTs [40, 41, 42, 43, 44] were taken into the publication bias analysis by a graphical plot as advised by Egger [45]. Four [40, 41, 42, 44] out of the five excluded trials with grave methodological and procedural flaws, were small and reported negative results. Three trials with negative results for LLLT were performed by the same research group [40, 46, 47] although this group also reported a positive outcome [50]. Three of these trials met the eligibility criteria for this review and were included in the meta-analysis [46, 47, 50]. The five largest trials [43, 48, 49, 50, 51] all presented positive results, although Simunovic et al. [43] was excluded from our meta-analyses for variable timing of endpoints as stated above. Significant asymmetry was noted in the funnel plot, indicating a considerable degree of negative publication bias (Figure 3).

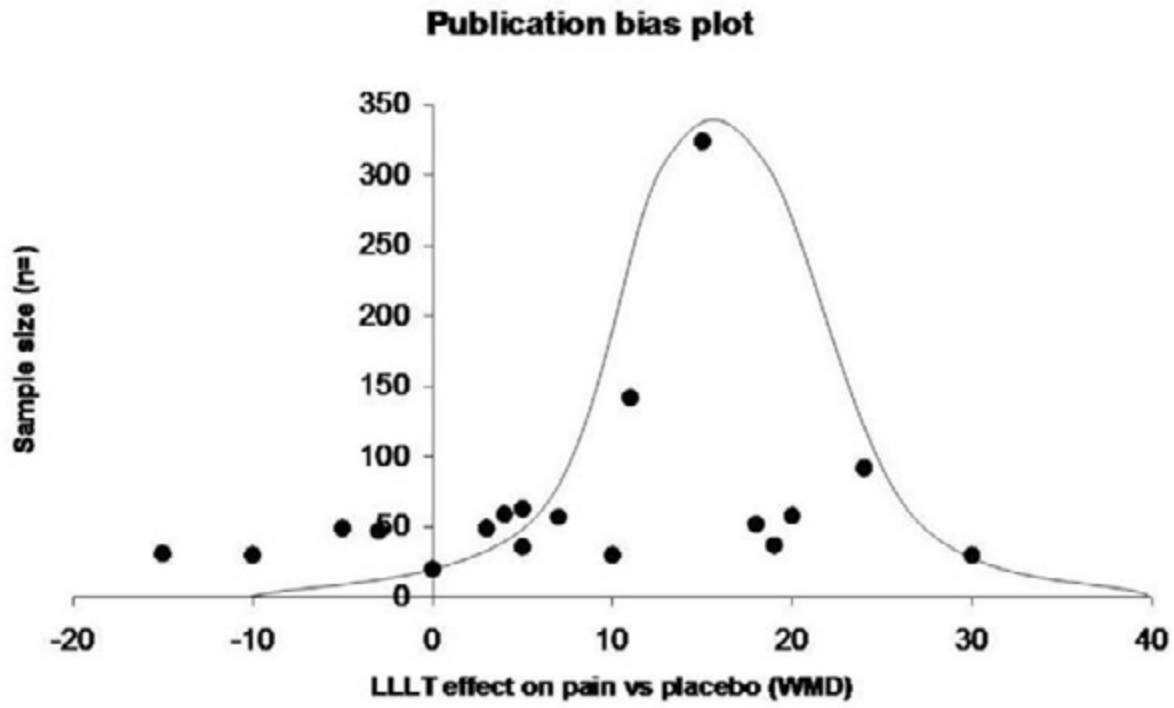

Figure 3

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

Bias analysis of 13 included trials

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

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

Table 3
Included randomised LLLT-trials.

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

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

Subgroup analysis for methodological quality

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

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

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

Assessment of LLLT procedures and treatment variables

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

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

Outcomes and effect sizes

Dichotomized trial results

Eight out of thirteen trials (62%) reported one or more outcome measures in favour of LLLT over placebo. Eleven trials used the tendon application technique, and eight (73%) of these trials reported positive results for one or more outcome measures (Table 3). All seven trials using 904 nm wavelength and the tendon application technique yielded positive results [48, 49, 50, 51, 55, 56, 57], whereas three trials using lasers with 820/30 nm [58, 52]and 1064 nm [59] wavelengths found no significant effect of LLLT. A single trial administering LLLT with a wavelength of 632 nm [60], also found significantly better results for the LLLT group. In the two trials where LLLT was administered to acupuncture points [46, 47], no significant differences between LLLT and placebo were found for any of the outcome measures.

Meta-analyses of effects

Primary outcomes

Continuous data for pain relief was available from 10 trials in a way which made statistical pooling possible. At the first observation after the end of the treatment period, LLLT was

significantly better than controls with a WMD of 10. 2 mm [95% CI: 3.0 to 17.5] in favour of LLLT on a 100 mm VAS (p = 0.005). In a subgroup of five trials [48, 50, 55, 56, 57] where 904 nm LLLT was administered directly to the tendon, LLLT reduced pain by 17.2 mm [95% CI: 8.5 to 25.9] more than placebo (p = 0.0001). One trial [60] with 632 nm LLLT, showed significantly better results for LLLT than a wrist brace and ultrasound therapy, but none of the results from trials with wavelengths of 820 nm or 1064 nm, or acupoint application technique were significantly different from placebo. The results are summarized in Figure 4.

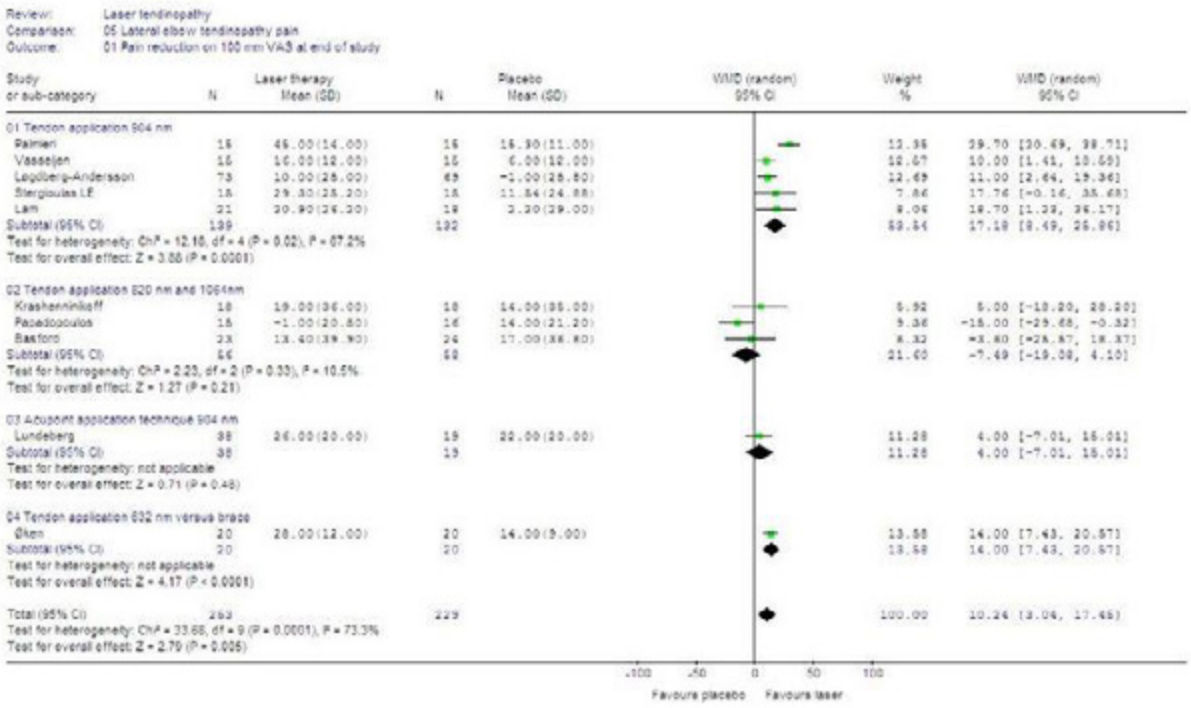

Figure 4
End of treatment results for LLLT measured as the WMD pain reduction on 100 mm VAS. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

Seven trials [46, 49, 50, 51, 55, 57, 58] presented data in a way which allowed us to pool data for global improvement. LLLT was significantly better than placebo with an overall relative risk for improvement at 1.36 [95% CI: 1.16 to 1.60] (p = 0.002). In a subgroup of five trials [49, 50, 51, 55, 57] where 904 nm LLLT was used to irradiate the symptomatic tendon, the relative risk for global improvement was significantly better than placebo at 1.53 [95% CI 1.28 to 1.83] (p < 0.0001). In the remaining two trials [46, 58] where LLLT was administered to acupoints or with 820 nm wavelength, the relative risk for global improvement was not significantly different from placebo at 0.80 [95% CI 0.50 to 1.22]. The results are summarized in Figure 5.

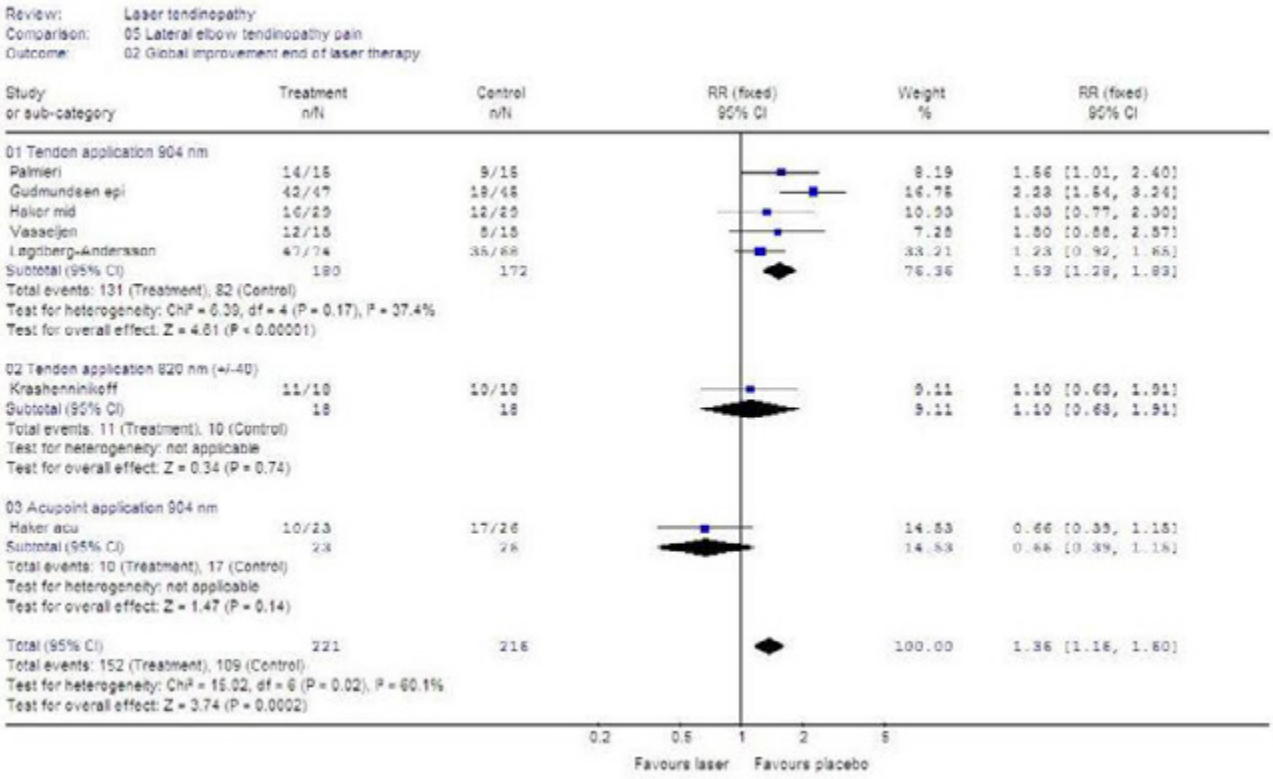

Figure 5
End of treatment results for LLLT measured as global improvement. Trials are subgrouped by application technique and wavelengths, and their combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

Secondary outcomes

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

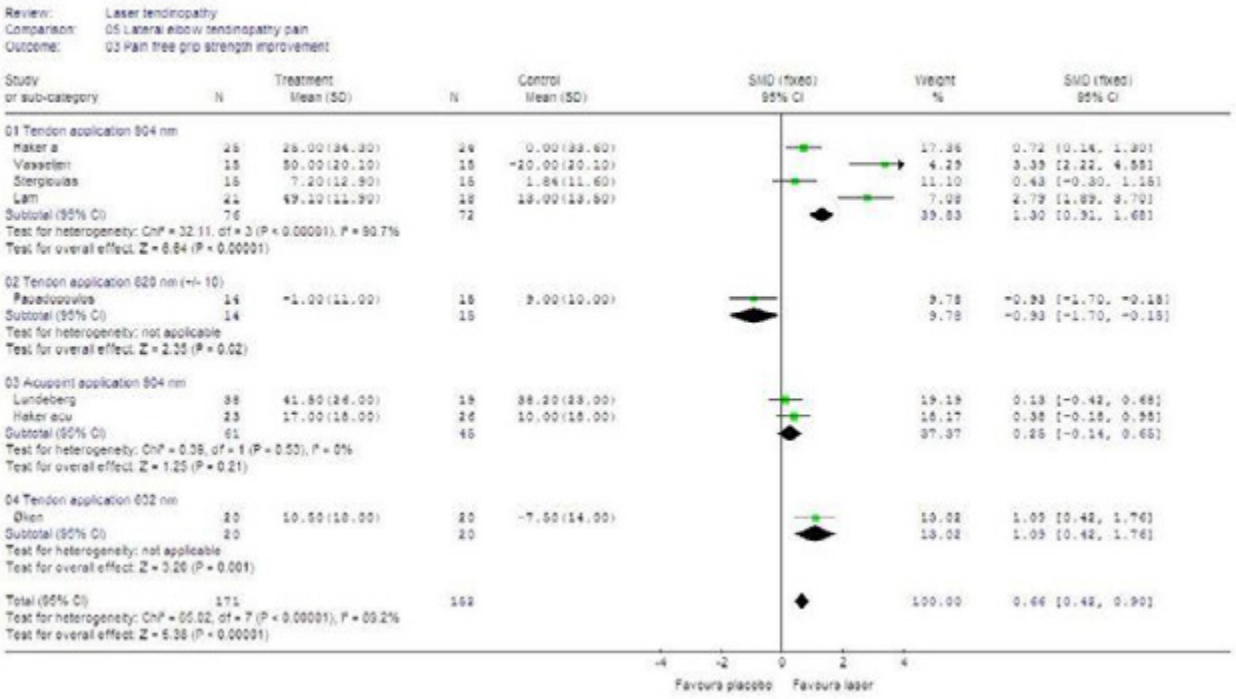

Figure 6
End of treatment results for LLLT measured as the SMD for pain-free grip strength. Trials are subgrouped by application technique and wavelengths, and their combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

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

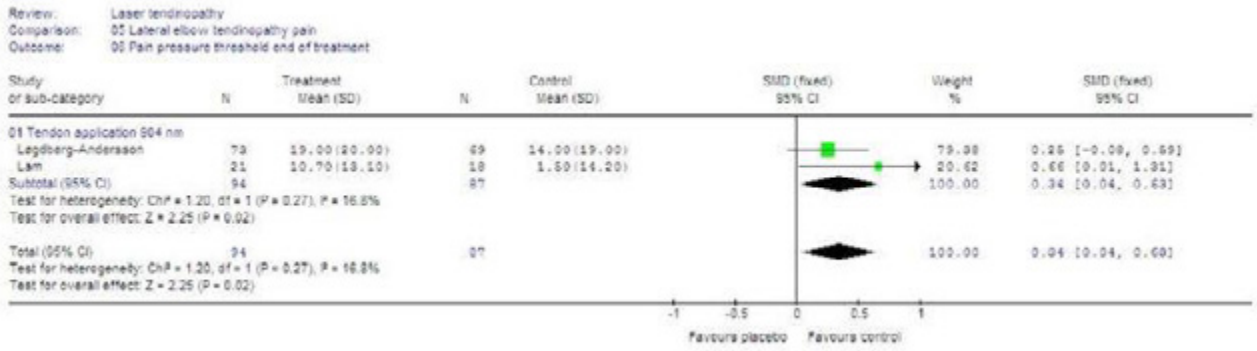

Figure 7
End of treatment results for LLLT measured as the SMD for pain pressure threshold. Only trials using the tendon application technique and 904 nm wavelength were available, and their

combined results are shown as the total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

Sick leave

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

Follow-up

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

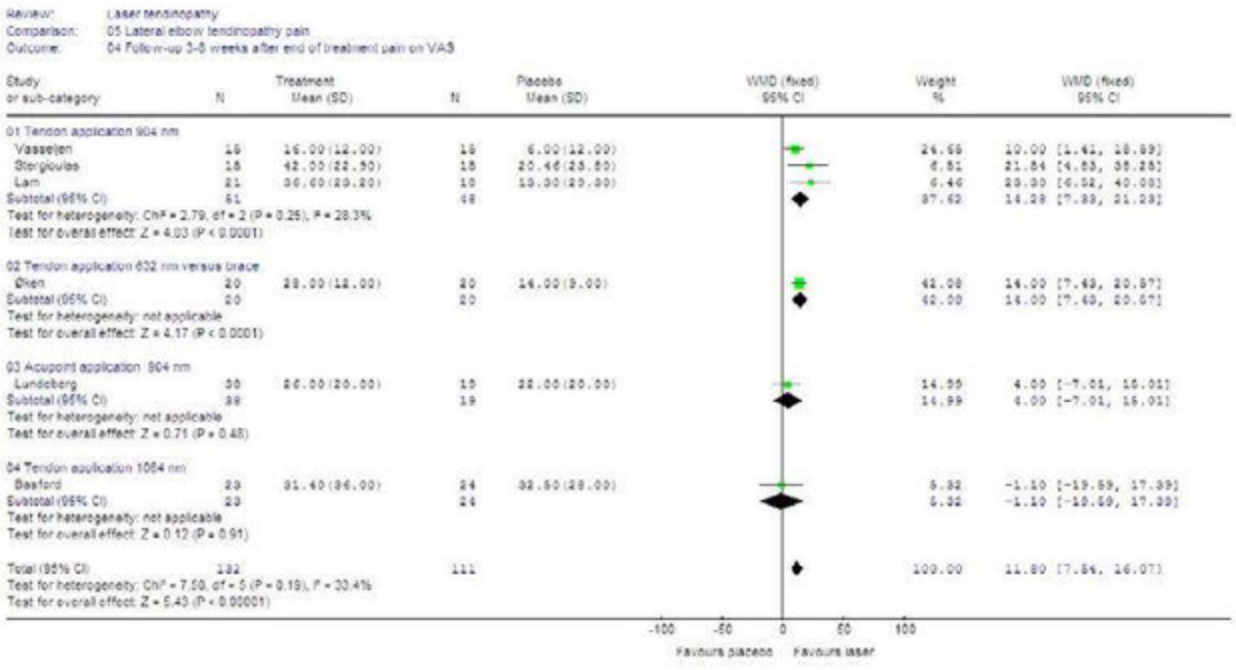

Figure 8
Follow-up results at 3–8 weeks after end of treatment for LLLT measured as the WMD for pain reduction on 100 mm VAS. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle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

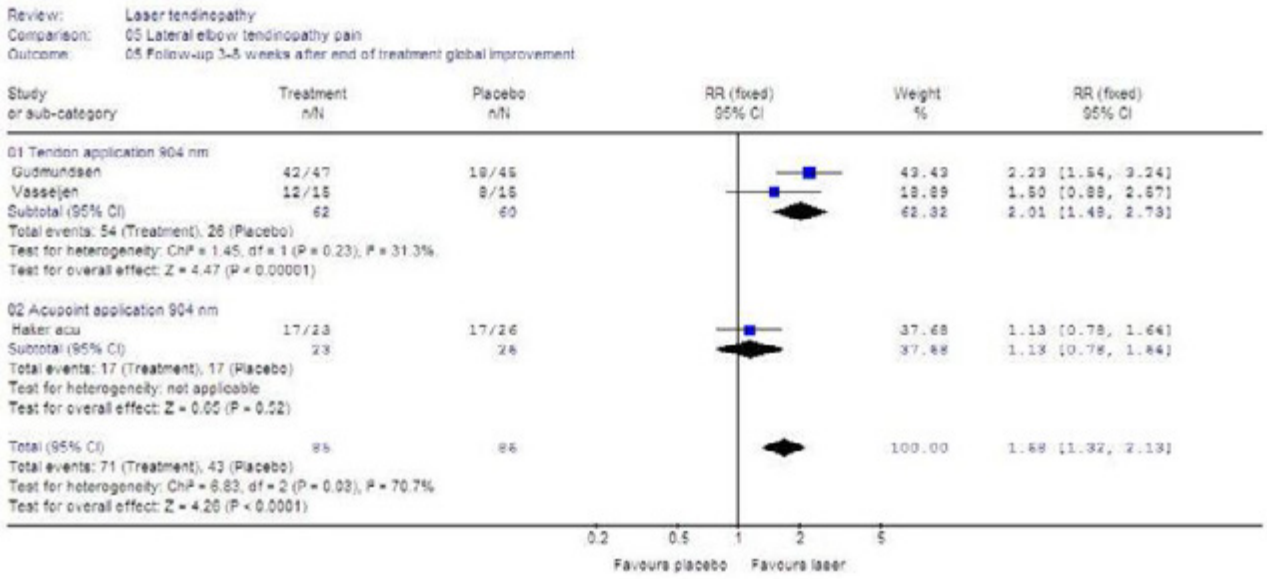

Figure 9
Follow-up results at 3–8 weeks after the end of treatment measured as the relative risk for global improvement for LLLT compared to placebo. Trials are subgrouped by application technique and wavelengths, and combined results are shown as total at the bottom of the table. Plots on the right hand side of the middle line indicate that the LLLT effect is superior to the control treatment.

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

Side-effects and compliance

Treatment was generally well tolerated and no adverse events were reported. Compliance was high ranging from 100% to 91% in all but two trials [48, 58]. One of these trials [48] had a considerably longer treatment period (8 weeks) than the other trials (median 3 weeks), and all withdrawals were caused by lack of effects. In another trial [58] using 830 nm wavelength, an exceptionally high withdrawal/dropout rate of 15% occurred after a single treatment session without any given reason.

Discussion

In this review, we found that most RCTs of LLLT for LET were of acceptable methodological quality. This finding is in line with previous reviews [12, 23, 27], although there were some differences between reviewers in methodological scores for individual trials. RCTs of LLLT are of similar methodological quality and include similar sample sizes as RCTS included in recent

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

Previous LET-reviews of LLLT [12, 23, 27] and pharmacological interventions like NSAID [8] or corticosteroid injections [5] have not assessed possible bias from for-profit funding sources or publication bias. Our analysis revealed that bias from for-profit funding was largely absent in the available LLLT material and that trials were performed by independent research groups receiving funding from internal sources or non-profit organisations. This feature of the LLLT literature is definitely different from pharmacological pain treatments where up to 83% of trials may be industry-funded [62]. A second feature of the LLLT-literature is that publication bias seems to go in a negative direction. This is distinctly different from the drug trials [63, 64] where positive results have been found to account for up to 85% of the published trials in single journals [63], although this bias seems to be lesser or absent in high impact journals [64]. Our review suggests that LLLT trials reporting negative results are more likely to be published than trials with positive results. To our knowledge we are the first to demonstrate such bias, but such negative publishing bias is probably not unique to LLLT, and it may also be present for other electrophysical agents including TENS and acupuncture. We were surprised to see how large well-designed positive trials of LLLT [51, 50] were published in unlisted journals or journals with low-impact factor, and how small negative trials [46], often with grave methodological [42] or procedural flaws [40] were published in higher ranking journals. This may reflect a predominance of RCTs designed using drug-research methodology paradigms without due consideration given to adequacy of the technique used in delivering LLLT, leading to under dosing and negative outcome bias [65]. In addition, it has been that documented drug sponsorship of research activities may influence guideline panels, journal editors and referees [66, 67] leading to negative views on non-drug treatments such as LLLT as reflected in editorials in pain journals [68] and national medical journals [69].

Despite these concerns, we believe that the positive overall results of this review need to interpreted with some caution. They arise from a subgroup of 7 out of the 13 included trials [48, 49, 50, 51, 55, 56, 57]. These 7 trials had a narrowly defined LLLT regimen where lasers of 904 nm wavelength with low output (5–50 mW) were used to irradiate the tendon insertion at the lateral elbow using 2–6 points or an area of 5 cm^2 and doses of 0.25–1.2 Joules per point/area. The positive results for this subgroup of trials were consistent across outcomes of pain and function, and significance persisted for at least 3–8 weeks after the end of treatment, in spite of several factors which may have deflated effect sizes.

For the red 632 nm wavelength which has a poorer skin penetration ability [70], a single trial [60] with a higher dose (6 Joules) seemed to be equally effective as the lower doses of 904 nm used in the seven positive trials. These LLLT-doses are well within the therapeutic windows for reducing inflammation, increasing fibroblast activity and collagen fibre synthesis, and the dosage recommendations suggested by WALT [71].

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

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

Based on the above findings, LLLT should be considered as an alternative therapy to commonly used pharmacological agents in LET management. Cochrane-based reviews of NSAIDs [8] and corticosteroid injections [5] have found evidence of short-term effects within 4 and 6 weeks, respectively. The short-term reduction in pain intensity after corticosteroid injections may appear to have a more rapid onset and may also be larger in effect size than after LLLT. But on the other hand, the available LLLT-material is confounded by factors capable of deflating effect sizes. In this perspective, there is a need for more high quality trials with head-to-head comparison of short-term effects between LLLT and corticosteroid injections. In the longer term, NSAIDs seems to be ineffective and corticosteroid injections seem to be harmful both at 26 and at 52 weeks [6]. For LLLT there are some significant long-term effects found at 8, 12 and 24 weeks after the end of treatment.

Conclusion

The available material suggests that LLLT is safe and effective, and that LLLT acts in a dose-dependent manner by biological mechanisms which modulate both tendon inflammation and tendon repair processes. With the recent discovery that long-term prognosis is significantly

worse for corticosteroid injections than placebo in LET, LLLT irradiation with 904 nm wavelength aimed at the tendon insertion at the lateral elbow is emerging as a safe and effective alternative to corticosteroid injections and NSAIDs. LLLT also seems to work well when added to exercise and stretching regimens. There is a need for future trials to compare adjunctive pain treatments such as LLLT with commonly used pharmacological agents.

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Magnetic Resonance Imaging and Clinical Outcomes of Laser Therapy, Ultrasound Therapy, and Extracorporeal Shock Wave Therapy for Treatment of Plantar Fasciitis: A Randomized Controlled Trial

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ABSTRACT

We determined and compared the effectiveness of low-level laser therapy (LLLT), therapeutic ultrasound (US) therapy, and extracorporeal shock wave therapy (ESWT) using magnetic resonance imaging (MRI). We performed a randomized, prospective, comparative clinical study. A total of 60 patients with a diagnosis of chronic plantar fasciitis were divided randomly into 3 treatment groups: group 1 underwent 15 sessions of LLLT (8 J/cm^2; 830 nm); group 2 underwent 15 sessions of continuous US (1 mHz; 2 W/cm^2); and group 3 underwent 3 sessions of ESWT (2000 shocks). All patients were assessed using the visual analog scale (VAS), heel tenderness index (HTI), American Orthopaedic Foot and Ankle Society (AOFAS) ankle-hindfoot scale, Roles–Maudsley score, and MRI before and 1 month after treatment. The primary efficacy success criterion was the percentage of decrease in heel pain of >60% from baseline at 1 month after treatment for ≥2 of the 3 heel pain (VAS) measurements. Significant improvement was measured using the mean VAS, AOFAS scale, and HTI scores for all 3 groups. The thickness of the plantar fascia had decreased significantly on MRI in all 3 groups. The treatment success rate was 70.6% in the LLLT group, 65% in the ESWT group, and 23.5% in the US group. LLLT and ESWT proved significantly superior to US therapy using the primary efficacy criterion ($p = .006$ and $p = .012$, respectively), with no significant difference between the LLLT and ESWT groups ($p > .05$). The treatment of chronic plantar fasciitis with LLLT and ESWT resulted in similar outcomes and both were more successful than US therapy in pain improvement and functional outcomes.

Plantar fasciitis is the most common diagnosis (10% to 15%) for patients with foot and ankle pain (1). Plantar fasciitis has a multifactorial etiology. It was previously thought to be an inflammatory syndrome; however, recent studies have emphasized that a degenerative process is more dominant (2–4). The factors thought to be associated with the disease include biomechanical dysfunction, mechanical overload, obesity, overuse, Achilles tendon strain, decreased ankle dorsiflexion, atrophy of the intrinsic muscles, and a pronated foot type (5,6). The patient's history and physical examination findings are usually sufficient to diagnose plantar fasciitis. Patients typically present with a throbbing, burning, or piercing type of inferior heel pain, especially with the first few steps in the morning. However, the pain will decrease after a few steps but will return during the day with prolonged weightbearing activity. Sometimes, the pain will persist for months or even years (4).

Although not routinely necessary, imaging can be used to verify recalcitrant plantar fasciitis or to rule out other foot pathology. Magnetic resonance imaging (MRI), although expensive, is very sensitive and has been accepted as the standard imaging method to evaluate plantar fascia morphology and bone marrow edema. The MRI findings of plantar fasciitis include thickening of the plantar fascia, perifascial and intrafascial edema pattern at T_2-weighted images, intrafascial T_1-weighted signal enhancement, and a limited bone marrow edema pattern at the calcaneal tuberosity (7,8).

The treatment options include numerous methods focusing on the anatomic and biomechanical problems and pain management. The recommended first-tier treatment options are nonsteroidal anti-inflammatory drugs, therapeutic orthotic insoles, limitations of extended physical activities, and Achilles and plantar fascia stretching

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exercises (4,9). Patients will usually have a clinical response within 6 weeks. However, if the symptoms persist, second-tier treatment, including physical therapy, orthotic devices, steroid injections, and night splints be added to the ongoing first-tier treatment. Extracorporeal shock wave therapy (ESWT) and surgery are recommended as the third tier of treatment for patients with chronic (6-month) plantar fasciitis recalcitrant to treatment (4).

Low-level laser therapy (LLLT) can be used to accelerate wound healing and reduce pain and inflammation (10). The studies investigating the molecular effects of LLLT have focused on the photobiomodulation and photobiostimulation phenomena, which promote cell proliferation and tissue regeneration (11). An important factor associated with the effectiveness of LLLT is tissue penetration capability and the optimum dosage of energy. Therefore, standardization of the treatment protocols and dosages according to the disease directly affects the success of the treatment (12). In that context, the World Association for Laser Therapy group published disease-specific dosage recommendations for LLLT (13). Another treatment modality used for >60 years extensively in the treatment of acute and chronic pain is therapeutic ultrasound (US). Therapeutic US is used to produce thermal or nonthermal effects by high-frequency acoustic energy (14). US therapy is usually used in combination with the other conventional therapies for the treatment of the plantar fasciitis in daily practice. ESWT is another treatment recommendation for chronic plantar fasciitis approved in 2000 by the Food and Drug Administration. The possible effect of ESWT is stimulation of the wound healing cascade, allowing chronic damage to become acute damage and initiate the normal wound healing process by application of high-intensity pressure waves into the body. Previous studies reported a success rate for ESWT for plantar fasciitis ranging from 34% to 88% (15).

Despite the increasing popularity of LLLT and ESWT, randomized controlled trials comparing the efficacy of the treatment modalities are lacking. The aims of the present study were to determine and compare the clinical effects of LLLT, ESWT, and therapeutic US objectively using MRI at 1 month of follow-up for patients with chronic recalcitrant plantar fasciitis.

Patients and Methods

Participants

From December 2012 to December 2014, the present study included 60 patients with chronic recalcitrant plantar fasciitis. All patients agreed to participate in the study and freely signed an informed consent statement after being informed about the purpose of the study, examination, and treatment application. The local medical ethics committee approved the present study, which was supported by University Scientific Research Project Coordination.

The inclusion criteria were the presence of symptoms of a chronic recalcitrant plantar painful heel of ≥6 months duration that was unresponsive to 6 weeks of first-tier conservative treatment (nonsteroidal anti-inflammatory drug, home exercise program, and standard insoles). The diagnosis was confirmed clinically by the physical examination finding of tenderness to palpation with local pressure at the origin of the plantar fascia on the medial tubercle of the calcaneus and an indication of significant pain by a score of ≥5 for ≥1 of 3 visual analog scales (VASs; intensity of pain measured by the VAS for the first few steps in the morning, during daily activities, and during exercise) before treatment.

The exclusion criteria were previous local trauma, foot surgery, local steroid injection within the previous 3 months, local infection, abnormalities in the knees or ankles, vascular disease, diabetes mellitus, malignancy, peripheral neuropathy, pacemaker, metal implants, rheumatic inflammatory disease, and plantar fascial rupture.

The trial design was a prospective, randomized, comparative, clinical study with the investigators kept unaware of the treatment groups. The patients were advised to continue their standard home exercise program (plantar fascia stretching, calf muscle stretching, Achilles tendon stretching, and strengthening of the intrinsic muscles of the foot) previously implemented during the treatment course (2,4). Sixty patients were randomized into 3 treatment groups using the stratified block randomization method according to gender and body mass index by one of us (A.U.). The 3 groups were the LLLT group, US therapy group, and ESWT group. All patients were assessed using the VAS for heel pain (first steps in the morning, during daily activities, and during exercise), foot functionality (pain and range of motion domains) using the American Orthopaedic Foot and Ankle Society (AOFAS) ankle-hindfoot scale and Roles–Maudsley score (RMS; patient-administered scoring system regarding activity limitations), and the sensitivity of the heel using the heel tenderness index (HTI) before and after 1 month of treatment. The same investigator (L.C.) administered these measures and was kept unaware of the treatment groups. MRI was performed using a SIGNA™ HDXT 1.5 Tesla MRI system (GE Healthcare, Chicago, IL) before and 1 month after treatment. The maximum thickness of the proximal plantar fascia where it attaches to the calcaneus was measured using electronic calipers on fluid-sensitive MRI sequences in the sagittal and coronal planes (Fig. 1). The intrafacial and perifacial soft tissue edema and calcaneal bone marrow edema were assessed in the sagittal plane on short tau inversion recovery sequences, and the presence of the calcaneal spurs was evaluated on T_1-weighted sequences. After all the patients had completed therapy, the pre- and post-treatment MRI scans were interpreted simultaneously by a radiologist (S.O.), who was unaware of the treatment groups.

LLLT, US therapy, and ESWT were performed by the same investigator (A.U.) using the BTL-5000 SWT combined device (BTL Turkey, Ankara, Turkey). All subjects were placed in the prone position during treatment.

Group 1 underwent LLLT. All patients in group 1 received a total of 15 LLLT sessions (5 sessions each week during a consecutive 3-week period). All patients were treated with a gallium-aluminum-arsenide (GaAlAs) laser, with 830 nm of laser light with 50 mW. The laser probe was scanned into the areas of the painful heel, insertion of the plantar fascia on the medial calcaneal area, and the myofascial junction at the dorsum of the heel, for a total dose of 8 J/cm^2 for 200 seconds.

Group 2 underwent US therapy. All the patients in group 2 received a total of 15 US sessions (5 sessions each week during a consecutive 3-week period). US therapy was applied using the following parameters: continuous mode, base frequency of 1 MHz to produce a deeper penetration, power of 2 W/cm^2 into the areas of the painful heel, insertion of the plantar fascia on the medial calcaneal area, and the myofascial junction at the dorsum of the heel for 5 minutes using an ultrasound gel.

Group 3 underwent ESWT. All the subjects in the ESWT group received 3 sessions of ESWT weekly for 3 weeks. Ultrasound gel was applied for better transmission between the ESWT head and the skin. The patients received 2000 shock waves with a 2.5-bar

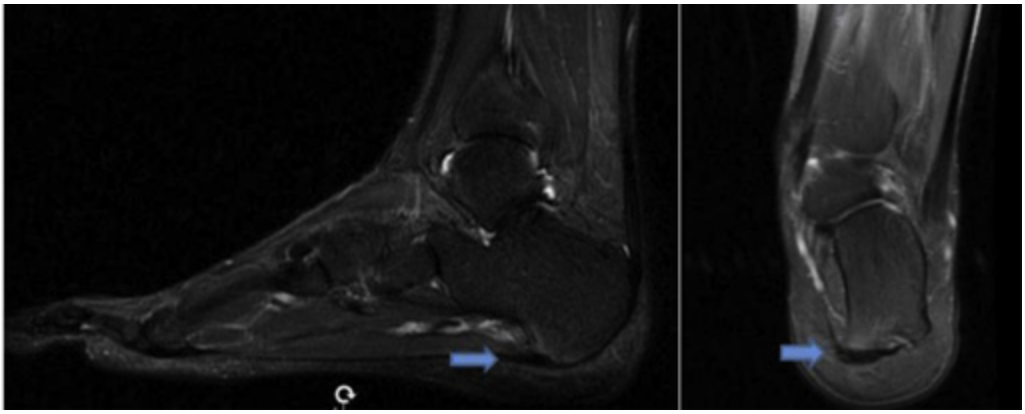

Fig. 1. Evaluation of the plantar fascia thickness by magnetic resonance imaging on sagittal (5.4 mm) and coronal (5.6 mm) planes. *Arrows* indicate measurement location.

pressure, 10-Hz frequency during sessions into the areas of the painful heel, insertion of the plantar fascia on the medial calcaneal area, and myofascial junction at the dorsum of the heel.

Outcome Measures

The primary efficacy success criterion was defined as a ≥60% decrease in heel pain for ≥2 VAS measurements (16,17). The secondary outcome measures were a functional response to treatment as defined by the RMS (1 indicating excellent; 2, good; 3, fair; and 4, a poor response), HTI (0 indicating excellent; 1, good; 2, fair; and 3, a poor response), and improvement in the AOFAS scale score and a reduction in plantar fascial thickness on MRI.

Statistical Analysis

In the present study, we calculated that the required minimum number of patients would be 45 for 3 groups, with an effect size of 0.48, an α of 0.05, and power of 0.80. Allowing for withdrawals, we determined that the number of participants should be 60. Statistical analyses were performed using the SPSS for Windows, version 15.0, software (IBM Corp., Armonk, NY). Descriptive statistics and frequency analysis were performed for categorical variables using counts and percentages, and the minimum, maximum, mean, and standard deviation are presented for the numerical variables. We performed the Wilcoxon test to compare the pretreatment and post-treatment findings within the groups; the McNemar test was used for categorical data. The Kruskal-Wallis test was used to assess the differences among the 3 groups and the crosstab chi-square test for categorical data. The Mann-Whitney *U* test used for pairwise comparisons. Correlations between continuous variables were analyzed using the Pearson correlation test. A p value of $< .05$ was considered statistically significant.

Results

Sixty patients fulfilled the inclusion criteria and were included in the present study. Of the 60 patients, 2 withdrew from the study during the treatment period (both from group 2), 4 were unable to complete the follow-up examination by 1 month after treatment (3 from group 1 and 1 from group 2), and 2 patients refused the second MRI examination at the follow-up visit because they reported their symptoms had completely improved (1 from group 1 and 1 from group 3; Fig. 2). Thus, the data from 54 patients were analyzed for the primary outcome and 52 for the MRI evaluations. Side effects were not observed in any patient. No significant differences were found in age, body mass index, or symptom duration in months among the 3 groups ($p > .05$) before treatment (Table 1). Also, no significant differences were found in the initial clinical parameters as determined using the VAS (pain intensity), HTI, AOFAS scale, and RMS among the 3 groups ($p > .05$).

In all 3 groups, significant differences were found between the pre- and post-treatment clinical values. The VAS score had significantly decreased and the AOFAS scale scores had significantly improved after treatment in all 3 groups ($p < .05$; Table 2).

The primary efficacy measure of success (decreasing heel pain >60% for ≥2 of the 3 heel pain VAS measurements) was detected in 70.6% of the LLLT group, 65% of the ESWT group, and 23.5% of the US

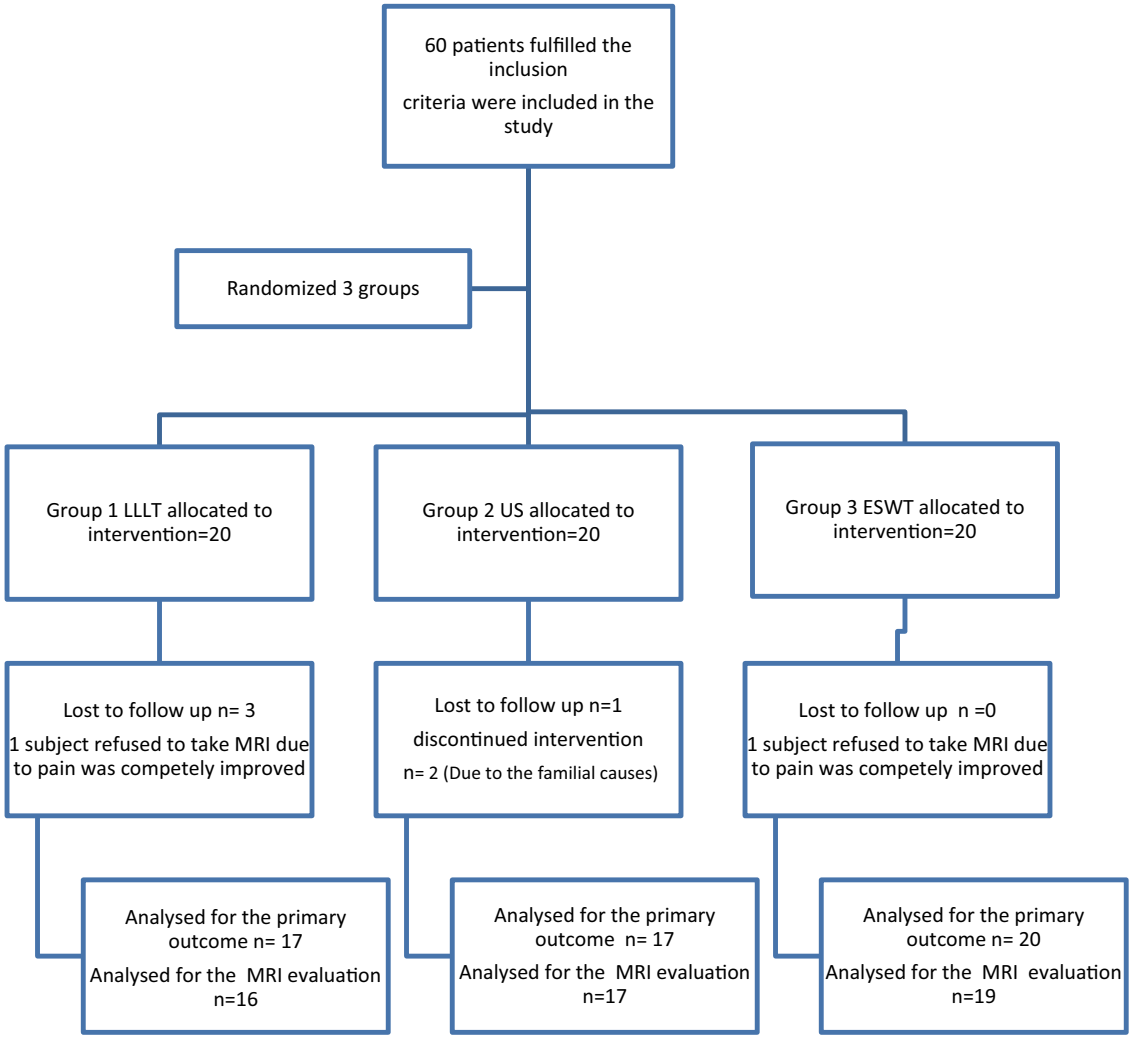

Fig. 2. Flowchart of the study design. Abbreviations: ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; MRI, magnetic resonance imaging; US, ultrasound.

Table 1
Patient characteristics at enrollment stratified by treatment group

Characteristic	LLLT (n = 20)	US Therapy (n = 20)	ESWT (n = 20)	*p* Value
Age (y)				.388
Mean ± SD	53.40 ± 14.71	50.95 ± 9.62	54.45 ± 6.90	
Range	19–75	34–75	43–66	
Gender				.895
Female	16	17	16	
Male	4	3	4	
Pain duration (mo)				.389
Mean ± SD	14.40 ± 9.00	17.30 ± 14.71	27.00 ± 29.79	
Range	6–36	6–60	6–120	
BMI (kg/m^2)	31.94 ± 5.55	30.20 ± 4.45	32.01 ± 4.06	.353

Abbreviations: BMI, body mass index; ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; SD, standard deviation; US, ultrasound.

group (Table 3). In the comparison of the 3 groups, LLLT and ESWT were found to be more effective than US therapy, with no significant difference found between LLLT and ESWT (group 1 versus 2, $p = .006$; group 2 versus 3, $p = .012$; group 1 versus 3, $p = .717$) in the success rate (VAS score 60%).

The RMSs for the functional responses are listed in Table 4. The RMSs improved in all 3 groups. The comparison showed that 2 treatment modalities (LLLT, $p = .03$; ESWT, $p = .014$) were more effective than US therapy, with no significant differences found between LLLT and ESWT ($p = .82$).

The HTI score changes before and after treatment are listed in Table 5. In the comparison of the 2 groups, ESWT was found to be more effective than US therapy ($p = .004$). No significant difference was found between LLLT and ESWT ($p = .115$) or between LLLT and US therapy ($p = .106$).

The initial MRI findings and measurements are listed in Table 6. Soft tissue edema was present in 88.3% and bone marrow edema in 36.7%. The fascia thickness was ≥4 mm in 86.7% on ≥1 plane. A significant decrease was revealed in the thickness of the fascia in all 3 groups after treatment (LLLT, $p = .001$; US, $p < .001$; ESWT, $p < .001$; Table 7). No statistically significant difference was found between the groups in the reduction of the fascia thickness measured on MRI. The reduction in plantar fascia thickness correlated moderately with the reduction in the pain with the first steps in the morning after treatment ($p = .027$; r = 0.306). Soft tissue edema persisted in 32 of the 52 patients at the 1-month follow-up MRI. Also, in these patients, the

Table 2
Results for mean values of American Orthopaedic Foot and Ankle Society scale and visual analog scale scores before and after treatment

Variable	Before Treatment	After Treatment	*p* Value
AOFAS scale score			
LLLT (n = 17)	60.85 ± 15.90	85.70 ± 14.51	<.001*
US therapy (n = 17)	58.90 ± 13.33	82.00 ± 10.71	<.001*
ESWT (n = 20)	63.60 ± 15.74	83.70 ± 8.37	<.001*
VAS score, daily activities			
LLLT (n = 17)	6.87 ± 1.25	2.93 ± 1.84	<.001*
US therapy (n = 17)	6.66 ± 1.11	3.56 ± 1.68	<.001*
ESWT (n = 20)	6.60 ± 1.12	2.74 ± 1.41	<.001*
VAS score, first steps in morning			
LLLT (n = 17)	7.09 ± 1.34	2.75 ± 1.91	<.001*
US therapy (n = 17)	7.14 ± 1.74	3.77 ± 2.38	<.001*
ESWT (n = 20)	7.12 ± 1.12	2.81 ± 1.27	<.001*
VAS score, exercise			
LLLT (n = 17)	6.95 ± 1.45	2.90 ± 1.93	<.001*
US therapy (n = 17)	7.26 ± 0.88	4.20 ± 1.64	<.001*
ESWT (n = 20)	6.69 ± 1.47	2.41 ± 1.58	<.001*

Abbreviations: AOFAS, American Orthopaedic Foot and Ankle Society; ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; US, ultrasound; VAS, visual analog scale.
* Statistically significant.

Table 3
Primary efficacy success rate* for the 3 groups

Variable	LLLT	US Therapy	ESWT	*p* Value
Primary efficacy success rate	12 (70.6)	4 (23.5)	13 (65)	.01
Failure rate	5 (29.4)	13 (76.5)	7 (35)	
p Value	<.001	.125	<.001	

Abbreviations: ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; US, ultrasound.
Data presented as n (%).
* Decreasing in heel pain of >60% for ≥2 of the 3 heel pain (visual analog scale) measurements.

reduction in the VAS score for morning first step pain ($p = .040$) and VAS score for pain with daily activity ($p = .037$) was lower than that of the patients with complete regression of soft tissue edema. However, no significant differences were found in pain reduction with (n = 13) or without (n = 39) ongoing bone marrow edema after treatment ($p > .05$). The reduction in plantar fascia thickness in the excellent-good group using the RMS score was significantly greater than that in the fair-poor group on the sagittal ($p = .042$) and coronal ($p = .023$) planes.

When the patients were invited to attend the follow-up clinic at the first year, they reported no serious symptoms that would require the patients to undergo physical therapy again.

Discussion

Plantar fasciitis is the most common cause of plantar heel pain in adults. The goals of treatment are pain relief and restoration of function (17). However, many different treatment strategies have been recommended, and limited evidence are available to support any of the common treatments (18). The findings from the present investigation showed that LLLT, US therapy, and ESWT all significantly reduced the pain with no side effects and provided an objective reduction in fascial thickness on MRI. However when we evaluated the effectiveness and functionality of the 3 treatment modalities, we found that ESWT and LLLT were more effective than US therapy at 1 month after the intervention.

LLLT has gained popularity in recent years and has been supported by new evidence resulting from the standardization of dosing recommendations according to the disease being treated. The first study of the effectiveness of LLLT in plantar fasciitis was reported in 1998 by Basford et al (19). In their study, 28 subjects underwent irradiation with 830-nm GaAlAs laser in 12 sessions, for 1 month, with a dosage of 1 J to the origin of the plantar fascia and 2 J to the medial side of the fascia. They detected no clinically significant differences between the LLLT and placebo groups. The investigators concluded that laser therapy is ineffective in the treatment of plantar fasciitis (19). However, this failed result likely resulted from the low therapeutic dosage they used, because the World Association for Laser Therapy recommended a treatment dose of a minimum of 8 J for LLLT for plantar fasciitis (13). Kiritsi et al (10) applied GaAlAs (904 nm) LLLT at 8.4 J to

Table 4
Roles–Maudsley scores after treatment

Functional Response	LLLT (n = 17)		US Therapy (n = 17)		ESWT (n = 20)	
	Before	After	Before	After	Before	After
Excellent-good (1–2)	0 (0)	14 (82.4)	0 (0)	8 (47.1)	0 (0)	17 (65)
Fair-poor (3–4)	17 (100)	3 (17.6)	17 (100)	9 (52.9)	20 (100)	3 (35)

Abbreviations: ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; US, ultrasound.
Data presented as n (%).

Table 5
Heel tenderness index scores after treatment

Heel Tenderness Index Score	LLLT (n = 17)		US Therapy (n = 17)		ESWT (n = 20)	
	Before	After	Before	After	Before	After
Excellent-good (0–1)	4 (17.6)	15 (88.2)	1 (5.9)	11 (64.7)	8 (40)	20 (100)
Fair-poor (2–3)	13 (82.4)	2 (11.8)	16 (94.1)	6 (35.3)	12 (60)	0 (0)

Abbreviations: ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; US, ultrasound.
Data presented as n (%).

the tendon insertion and 8.4 J to the medial side of the fascia or placebo to 30 individuals, recorded the pain on the VAS, and used US to measure the plantar fascia thickness before and after treatment. Pain had improved significantly in the LLLT group compared with the placebo group; however, the plantar fascia thickness was similar in both groups that showed significant changes (10). In a 2015 study, Macias et al (20) reported on a randomized placebo-controlled study of LLLT in 69 subjects were treated with a wavelength of 635 nm and 17 MW output for 6 sessions. The patients experienced an improvement in pain and the plantar fascial thickness decreased significantly with LLLT compared with the placebo group (20). In the present study, the clinical parameters improved and the plantar fascia thickness decreased significantly in the LLLT group, similar to the findings from Macias et al (20). We used the BTL GaAlAs laser for the present study, which has a wavelength of 830 nm and 50 MW output, with the recommended dose of 8 J/cm^2, 5 times per week for 3 weeks. The treatment success rate was 70.6% in the LLLT group. These data have demonstrated that LLLT is an efficient and reliable treatment method for chronic plantar fasciitis.

Therapeutic US treatment is one of the most commonly used physical therapy modalities; however, conflicting results have been reported regarding its effectiveness in the treatment of plantar fasciitis. Both Crawford and Snaith (21) and Zanon et al (22) reported that therapeutic US was not superior to placebo in plantar fasciitis treatment. In addition, the 2014 plantar fasciitis treatment guidelines did not include therapeutic US among the recommendations (9). In contrast, Aydoğ et al (23) compared US therapy (10 sessions, 2 W/cm^2, for 10 minutes) plus infrared therapy versus infrared therapy alone. They showed increased effectiveness in the US therapy plus infrared therapy group (23). Cheing et al (24) applied US treatment in continuous mode for 5 minutes at 1 MHz, 1 W/cm^2 for 3 days each week for 3 weeks and showed significant improvements in the VAS pain scores after treatment. These conflicting findings resulted from the lack of standardization of dosages, sessions, and implementation

Table 6
Initial magnetic resonance imaging findings and measurements (n = 60)

Variable	Value
Plantar fascia thickness, coronal (mm)	
Mean ± SD	4.75 ± 0.813
Range	3.40–6.50
Plantar fascia thickness sagittal	
Mean ± SD	4.75 ± 0.867
Range	3.40–6.60
Soft tissue edema (intrafacial, perifacial)	
Yes	53 (88.3)
No	7 (11.7)
Bone marrow edema	
Yes	22 (36.7)
No	38 (63.3)
Calcaneal spur	
Yes	50 (83.3)
No	10 (16.7)

Abbreviation: SD, standard deviation.
Data presented as n (%), unless noted otherwise.

Table 7
Fascial thickness measured on magnetic resonance imaging scans before and after treatment

Group	Fascial Thickness (mm)	
	Coronal Plane	Sagittal Plane
LLLT		
Before	4.33 ± 0.59	4.31 ± 0.68
After	3.75 ± 0.69	3.76 ± 0.73
US therapy		
Before	4.76 ± 0.72	4.79 ± 0.68
After	3.99 ± 0.62	4.03 ± 0.65
ESWT		
Before	5.17 ± 0.89	5.16 ± 1.00
After	4.31 ± 0.82	4.31 ± 0.87

Abbreviations: ESWT, extracorporeal shock wave therapy; LLLT, low-level laser therapy; US, ultrasound.
Data presented as mean ± standard deviation.

periods, making it difficult to compare the results of clinical trials. In our investigation, we found statistically significant improvements in the clinical parameters and VAS pain scores in the US treatment group (1 MHz, 2 W/cm^2, 5 minutes, 15 sessions), which also experienced significant reductions in the thickness of the plantar fascia. However, when we evaluated the effectiveness of treatment, the success rate in the US therapy group was 23.5%, and 47.1% of patients had functional improvement according to the RMS. These data suggest that therapeutic US alone for plantar fasciitis treatment is unable to provide sufficient success in the short term.

A large number of randomized controlled trials were conducted to investigate ESWT efficacy in the treatment of plantar fasciitis. In the present study, the ESWT group showed significant improvement in all clinical parameters after treatment, and the functional and success rate was 65%. In 2013, a meta-analysis investigated the effect of ESWT compared with placebo to treat chronic plantar fasciitis and reported a >60% reduction in pain scores and improvement in RMSs. In the 5 of the 6 studies, ESWT was significantly superior to placebo (25). However, some studies could not show the superiority of ESWT to placebo. The trial by Haake et al (26) randomized 272 patients to 3 sessions of ESWT or sham ESWT. Treatment success was defined as achieving an RMS of 1 or 2. The success rate did not differ between groups at 12 weeks (34% for ESWT versus 30% for placebo) or at 1 year (81% for ESWT versus 76% for placebo) of follow-up (26).

Studies of the effectiveness of plantar fasciitis treatment have often been designed as placebo-controlled trials, and the number of studies comparing different treatments have been quite limited. Also, the comparison of these studies is often difficult owing to differences in the methods used. The design of the present study was scientifically rigorous, comparative, randomized, and prospective and included blinding of the investigator. To the best of our knowledge, the present study is the first to evaluate LLLT, US therapy, and ESWT and compare the results objectively using MRI. In the present study, each of the 3 treatments improved the pain VAS scores, heel sensitivity, RMS, and AOFAS scale scores compared with before treatment. However, ESWT and LLLT resulted in greater success rates than therapeutic US.

The measurement of the plantar fascia thickness provide an objective finding regarding the effect of the treatment used. MRI also allowed for the assessment of bone marrow edema and facial and perifacial edema. Grasel et al (27) showed that perifascial edema was the most common feature of plantar fasciitis. Zhu et al (28) reported a similar incidence in both facial thickening and soft tissue edema. Also, in our study, soft tissue edema was the most frequent MRI sign (increased facial and perifascial signal in 88.3%), and facial thickening (86.7% with fascia thickness >4 mm) was the second. The least common MRI feature in our series was limited bone marrow edema in the calcaneal region at 36%.

We detected significantly greater pain scores for daily activities and the first steps in the morning for the subjects with persistent soft tissue edema at the follow-up examination compared with those without soft tissue edema. These results suggest that the pain is associated with soft tissue edema persistence after treatment. Despite this, we could not demonstrate this association between pain and the presence of bone marrow edema at 1 month after the intervention. Probably owing to the short follow-up period, we failed to show any MRI change, because the expected time for visible regression of bone marrow edema on MRI is ≥6 weeks (29).

Fabrikant and Park (30) measured the plantar fascia thickness before and after treatment but found no correlation between a reduction in thickness and clinical improvement. However, Mahowald et al (31) showed statistically significant correlations between the reduction in facial thickness (0.82 ± 1.04 mm) and improvement in pain VAS scores (3.64 ± 2.7). In the present study, we have confirmed their findings by showing a correlation between the fascial thickness reduction and decreased first step in the morning pain. Also, the fascial thickness reduction was greater for patients with a good functional response (according to the RMS) after treatment. These results suggest that the changing thickness on MRI of the plantar fascia is associated with clinical success and is a valid objective measure to assess the effectiveness of treatment. MRI is a valid, but expensive, tool. However, the cost/benefit ratio for MRI in standard practice has shown that clinical improvement will be sufficient during follow-up.

Study Limitations

The present study had several limitations. The first and most important limitation was the short follow-up period. Second, the sample size of the study was relatively limited. Also, we could not include a control group because we included patients who had been experiencing pain for ≥6 months that had been unresponsive to first-line treatment for 6 weeks. Thus, we could not include a placebo group because of ethical concerns.

In conclusion, we found that LLLT, US therapy, and ESWT significantly reduced the pain experienced with plantar fasciitis, providing clinical and radiologic improvement. However, when we evaluated the success rates, LLLT and ESWT were more successful in providing pain improvement and functional outcomes compared with US therapy at 1 month after treatment.

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OFFICIAL REVIEW OF **ASACAMPUS**

The affect of MLS therapy on nerve conduction parameters in developing diabetic sensory peripheral neuropathy.

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ABSTRACT

The MLS laser is composed of an 808nm continuous emission laser and a 905nm pulsed emission laser that are synchronized. The purpose of this study was to determine the effect of the MLS laser on the injured tibial and peroneal nerves in diabetic sensory neuropathy. The sural nerve was chosen as an untreated control nerve.

A controlled prospective study was performed on ten patients with documented type 2 diabetes and peripheral sensory neuropathy. Nerve conduction parameters were determined prior to therapy and reevaluated post therapy. The course of therapy was three weeks. F-wave chronodispersion (Fc) measurements at the completion of the study showed significant improvement with this therapy. Peroneal Fc went from 8.99ms to 6.19ms (p=.015). Tibial Fc went from 10.30ms to 6.97ms (p=.001). The MLS laser therapy produced objective improvement in nerve function for persons with developing diabetic sensory neuropathy.

INTRODUCTION

As the prevalence of diabetes mellitus continues to rise throughout the world, so do the complications associated with this disease. Neuropathy is a common and serious complication associated with diabetes. The peripheral sensory type of diabetic neuropathy (DPN) is implicated as a causal factor in the development of foot ulcerations, infections and amputations. The loss of sensation in DPN has been shown to be a key component in the formation of foot ulcerations [1]. DPN is part of a triad of neuropathy, deformity and trauma that predispose the individual to pedal ulceration. Removal of one or more of the causal pathways is a goal in the prevention of foot ulcer development and healing of existing wounds [2]. In the United States, the cost associated with treating the sequelae of diabetic neuropathy is in the billions of dollars [3]. Often research and treatment are aimed at providing symptomatic pain relief. However, sensory restoration, not pain relief, is needed to interrupt the causal pathway leading to foot ulceration in DPN. Novel treatments have been tried that attempt to provide healing of the injured peripheral nerve. Subjective responses to these treatments have been published. Unfortunately, little objective evidence of reparation of the peripheral nerve in response to treatment has been demonstrated [4].

The MLS laser is a novel treatment for a variety of maladies causing pain and inflammation. The MLS laser is characterized by an 808 nm continuous emission laser and a 905 nm pulsed emission laser that are synchronized. In vitro and in vivo research has shown a beneficial effect of this technology on peripheral nerve injury [5]. Nerve conduction studies (NCS) are an objective measurement of peripheral nerve function. This controlled prospective pilot study was devised to look at the effect of the MLS laser on NCS parameters in developing DPN.

MATERIALS AND METHODS

Study subjects were taken from a cohort the author previously studied regarding the characteristics of developing diabetic sensory polyneuropathy [6]. 10 subjects were enrolled in this MLS laser pilot study.

Prior to inclusion in the study, subjects completed a subjective neuropathy screening questionnaire which was a modification of the Michigan neuropathy screening instrument. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Local IRB approval was obtained for the study design and informed consent was obtained from all subjects.

Exclusion criteria for the group included

any evidence of coronary artery disease or peripheral arterial disease including past surgical or medical intervention, claudication symptoms, rest pain or ischemia associated ulceration history. Exclusion criteria also included any disease diagnosis that may cause peripheral nerve dysfunction. The list of diseases were: thyroid disorders, vitamin B12 or folate deficiency, seronegative or seropositive spondyloarthropathy, hepatic or renal disease, lumbosacral pathology, toxin exposure including chemotherapeutic agents, familial polyneuropathy, any existing diagnosis of neuromuscular disorders, history of chronic alcohol abuse, previous medical or surgical intervention for peripheral nerve pathology or previous back or extremity surgery.

Inclusion criteria for the experimental group included a mandatory diagnosis of type 2 diabetes for less than 10 years prior to the test date. Subjects additionally had to be willing to discontinue their medications for symptomatic treatment of neuropathic pain for twenty-four hours prior to sensory testing. All included subjects provided pertinent medical histories and laboratory work along with their list of prescription and over-the-counter medications. All ten subjects were diagnosed with DPN according to the guidelines from the 1988 San Antonio Joint Consensus Statement requiring an elimination of confounding factors with a multiplicity of signs and symptoms. Additional factors monitored included age, height, weight, BMI and hemoglobin A1c.

NCS was performed on the tibial, peroneal and sural nerves of the left lower extremity. A single certified technician administered all of the testing. Testing was performed according to the manufacturer's instructions for the Neurometrix® NCS equipement. The tibial and peroneal nerves were treated with the MLS laser and the sural nerve

INDIVIDUAL PRE AND POST TREATMENT NCS RESULTS

#	1	2	3	4	5	6	7	8	9	10	Mean	p =	Ref. range
Ht	65	64	61	60	65	72	66	65	70	71	66		
Sex	F	F	F	F	F	M	F	F	M	M			
Age	58	82	64	39	64	63	74	39	64	49	59.6		
BMI	31.6	25.7	46.1	24.4	33.3	29.8	29.0	22.6	33.7	30.4	30.7		<25
A1c	7.8	6.3	11.3	11.0	7.1	7.7	6.8	7.0	6.9	6.1	7.8		4.0-5.7
pPFc	4.73	9.77	A	A	2.93	8.59	15.56	9.85	14.88	5.59	8.99		
3PFc	0.43	10.16	6.64	7.03	0	0.98	12.77	6.96	12.96	5.26	6.19	0.015	<18.05
pTFc	12.11	4.30	A	6.25	6.45	14.84	16.80	10.16	16.48	4.98	10.30		
3TFc	8.40	1.76	A	2.54	6.05	9.57	10.62	6.20	12.41	5.15	6.97	0.001	<14.49
pPA	1.42	2.79	0.25	1.84	1.41	3.67	0.44	2.26	0.96	1.60	1.67		
3PA	2.61	2.68	1.03	1.85	0.97	3.51	0.74	2.12	1.14	1.67	1.83	0.30	>1.15
pTA	4.53	2.54	A	3.04	2.63	3.04	2.88	2.41	2.90	4.74	3.19		
3TA	3.98	3.87	A	3.00	2.54	2.67	3.02	3.66	2.77	4.64	3.35	0.49	>1.41
pSV	42.73	A	A	A	50.50	46.29	A	43.66	38.70	57.84	46.62		
3SV	39.68	A	A	A	46.29	42.73	A	45.82	34.56	48.80	42.98	0.24	variable
pSA	6.09	A	A	A	4.92	9.27	A	2.22	6.04	1.76	5.05		
3SA	5.08	A	A	A	7.81	10.48	A	1.80	5.58	7.95	6.45	0.34	>3.39

Legend:
(subject number),
Ht (height inches),
BMI (body mass index),
A1c(hemoglobin A1c),
pPFc(pre treatment peroneal Fc),
3PFc(post treatment peroneal Fc),
pTFc (pre treat-ment tibial Fc),
3TFc (post treatment tibial Fc),
pPA (pre treatment peroneal CMAP),
3PA (post treatment peroneal CMAP),
pTA(pre treatment tibial CMAP),
3TA(post treatment tibial CMAP),
pSV(initial sural CV),
3SV(3rd week sural CV),
pSA(initial sural ampli-tude),
3SA(3rd week sural amplitude),
Ref. range(normal reference range),
A(absent).

was not treated. In this way, the sural nerve acted as an internal control.

Treatment was administered three times per week for 3 weeks. Each treatment session consisted of 1.50 J/cm2 (2.5 min) at the tarsal tunnel, 1.5 J/cm2 (2.5 min) at the fibular neck, and 4.00 J/cm2 at the dorsal foot. NCS parameters were taken prior to the first treatment and immediately following the final treatment. Statistical analysis was computed with mean, standard deviation, paired t-testing and p-value computation.

RESULTS

All subjects completed the full course of therapy and returned for post study NCS evaluation. None of the study subjects reported any adverse reaction to the therapy.

The means are as follows: age 59.6 years (39-82), height 66 inches (60-72), weight 191 lbs (125-252), A1c level 7.8% (6.3-11.3). All ten subjects had a diagnosis of type 2 diabetes. Seven subjects were female and three were male. None of the enrolled subjects took medication for pain.

Individual results are reported in table I. F-wave chronodispersion (Fc) for the peroneal nerve pre treatment was 8.99ms. Post treatment the Fc was 6.19ms yielding a p- value of .015. Fc for the tibial nerve was 10.30ms pre treatment and 6.97ms post treatment. This yielded a p-value of .001. The amplitudes (CMAP) of the peroneal and tibial nerve pre and post treatment did not reach a $p<.05$. Similarly, the p values for the untreated sural nerve (CMAP and conduction velocity (CV) were followed) did not reach a $p<.05$. The sural nerve CMAP and CV was not obtainable 40% of the time leading to less reliable evaluation; however, the CVs were generally slower at the end of the study and the CMAPs were slightly increased.

DISCUSSION

NCS are objective, quantitative and reproducible evaluations of the function of peripheral nerves. Reproducibility of nerve conduction requires consistency of methods, including electrode locations, distances, and temperature [7]. These parameters are well controlled and permit reproducible results with the Neurometrix® testing equipment [8]. F-wave latencies are the most reproducible, with only a 2-3% variation. CMAPs have the lowest reproducibility (10-15% variation) and CV and distal latency are intermediate (4-7% variation) [9]. F-waves are the most sensitive measure of diabetic neuropathies [10].

Fc is a measure of the variability of conduction in different axons in the whole nerve. This makes Fc uniquely useful for detecting even mild abnormalities. For the relative diagnostic sensitivity of all F-wave parameters, Fc is the most often abnormal parameter. Fc is ideally suited for monitoring the treatment of DPN [11]. F-waves have been used as frequently as every two weeks to follow peripheral nerve healing in previously published studies. In this study, tibial ($p=.001$) and peroneal ($p=.015$) Fc improved significantly ($p<.05$) over 3 weeks. This improvement is much greater than the published 2-3% variation. This finding is indicative of nerve healing.

CMAP provides an indication of the number of functioning axons in a nerve and the amount of muscle that is still innervated. CMAPs and CVs are dependent upon the persistent myelinated fibers in the nerve conducting the applied stimulus [7]. An injured nerve will heal at approximately 1mm per day. Because of the relatively slow healing of the peripheral nerve, no change in the CMAP or CV over the course of a 3 week treatment is expected. The length of these nerves being tested would lead one to expect months not weeks of healing before the CMAP or CV would be profoundly affected. While CMAP did increase in both the tibial ($p=.49$) and peroneal ($p=.30$) nerves, it was within the reported 15% variability associated with this parameter.

The sural nerve served as the control in this study. Sural nerve CV did trend slower, but fell within the 7% published variation rate. In the same way, the sural CMAP results pre and post study fell within the published 15% variation rates. The control was statistically unchanged. Previous evaluation of sensory loss in DPN found the axonal pathology is not entirely length dependent and not purely of metabolic cause. An anatomic component for sensory loss was implied [6]. The anatomic regions chosen for application of MLS laser therapy were the tarsal tunnel, fibular neck region and dorsal foot. These regions represent anatomic sites predisposed to peripheral nerve entrapment and damage in DPN [12,13,14].

The small sample size, short period of treatment and immediate follow up are limitations of this pilot study. Future research should look at variable treatment parameters with the MLS laser and the effect of this promising therapy over a longer period of time. Evaluation of the NCS parameters over months instead of weeks should yield more dramatic improvements in the CMAP and CV of the treated nerves if regeneration and healing of the nerve fibers persists.

CONCLUSIONS

MLS laser therapy applied to the tibial and peroneal nerves in persons with documented DPN will lead to objective improvement in nerve function as demonstrated by NCS evaluation. A reasonable expectation is that this improvement in function will lead to improved sensibility in the feet. Improved sensibility interrupts the causal pathway leading to ulceration, infection and amputation. In this pilot study, MLS therapy appears to be uniquely capable of healing the injured nerve in DPN and shows great promise in the battle against the devastating sequelae of this disease.

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Effectiveness of low-level laser on carpal tunnel syndrome

A meta-analysis of previously reported randomized trials

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Abstract

Background: Low-level laser therapy (LLLT) has been applied in the treatment of carpal tunnel syndrome (CTS) for an extended period of time without definitive consensus on its effectiveness. This meta-analysis was conducted to evaluate the effectiveness of low-level laser in the treatment of mild to moderate CTS using a Cochrane systematic review.

Methods: We conducted electronic searches of PubMed (1966–2015.10), Medline (1966–2015.10), Embase (1980–2015.10), and ScienceDirect (1985–2015.10), using the terms "carpal tunnel syndrome" and "laser" according to the Cochrane Collaboration guidelines. Relevant journals or conference proceedings were searched manually to identify studies that might have been missed in the database search. Only randomized clinical trials were included, and the quality assessments were performed according to the Cochrane systematic review method. The data extraction and analyses from the included studies were conducted independently by 2 reviewers. The results were expressed as the mean difference (MD) with 95% confidence intervals (CI) for the continuous outcomes.

Results: Seven randomized clinical trials met the inclusion criteria; there were 270 wrists in the laser group and 261 wrists in the control group. High heterogeneity existed when the analysis was conducted. Hand grip (at 12 weeks) was stronger in the LLLT group than in the control group (MD = 2.04; 95% CI: 0.08–3.99; $P = 0.04$; $I^2 = 62\%$), and there was better improvement in the visual analog scale (VAS) (at 12 weeks) in the LLLT group (MD = 0.97; 95% CI: 0.84–1.11; $P < 0.01$; $I^2 = 0\%$). The sensory nerve action potential (SNAP) (at 12 weeks) was better in the LLLT group (MD = 1.08; 95% CI: 0.44–1.73; $P = 0.001$; $I^2 = 0\%$). However, 1 included study was weighted at >95% in the calculation of these 3 parameters. There were no statistically significant differences in the other parameters between the 2 groups.

Conclusion: This study revealed that low-level laser improve hand grip, VAS, and SNAP after 3 months of follow-up for mild to moderate CTS. More high-quality studies using the same laser intervention protocol are needed to confirm the effects of low-level laser in the treatment of CTS.

Abbreviations: CI = confidence interval, CMAP = compound muscle action potential, CTS = carpal tunnel syndrome, FSS = functional status scores, LLLT = low-level laser therapy, MD = mean difference, MDL = motor distal latency, MNV = motor nerve velocity, RCTs = randomized controlled trials, SNAP = sensory nerve action potential, SDL = sensory distal latency, SNV = sensory nerve velocity, SSS = symptom severity scores, US = ultrasound, VAS = visual analog scale.

Keywords: carpal tunnel syndrome, low-level laser, meta-analysis

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Z-jL and YW contributed equally to this study.

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1. Introduction

Carpal tunnel syndrome (CTS) is an important mononeuropathy that is mainly caused by entrapment of the median nerve by a swollen transverse carpal ligament resulting from chronic inflammation. The change in the median nerve in CTS is a process. Early compression causes a block in venous outflow leading to the nerve becoming hyperemic and edematous;[1] this process is followed by inflammatory reaction, fibrosis, demyelination, and axonal loss over the next 30 days.[2] Additionally, increased expression of prostaglandin E2, vascular endothelial growth factor,[3] and interleukin-6[4] might play a role in CTS. Diagnosis is based on clinical symptoms, physiological tests, and electrodiagnostic examination.[5,6] Clinical symptoms and signs are characterized by numbness and tingling of the first 3 fingers and the radial side of the ring finger, nocturnal awakening from pain, and weakness or atrophy of the thenar muscle. Phalen's maneuver and Tinel's sign are positive in some patients. Nerve conduction studies show longer latency and slower conduction velocity than in normal conditions.

For serious cases, surgical intervention is an effective choice for relieving pressure around the median nerve, although there is a risk of recurrence.[7,8] Recurrent symptoms of CTS have been shown to occur in 0% to 19% of patients following surgery, and up to 12% of cases require re-exploration.[9] The natural history of CTS typically progresses slowly, and some patients can recover spontaneously.[10] Therefore, conservative treatments are welcome in mild and moderate patients and have less expense and less frequent complications. Nonsurgical treatments are available, including exercise, wrist splinting, nonsteroidal anti-inflammatory drugs, local injection of corticosteroid, and ultrasound (US).

Low-level lasers were first studied by Padua et al,[11] and studies have shown that increasing myelin production and reducing retrograde degeneration of motor neurons were found in a rat spinal cord crushing model.[12] Other possible mechanisms of the benefits of low-level lasers include anti-inflammatory effects,[13] selective inhibition of nociceptive activation at peripheral nerves,[14] increased ATP production and cellular respiration,[15,16] and improvement of blood circulation to remove algesic substances.[13,17] Weintraub suggested that 9 J of energy over 5 points (7–15 treatments) reversed CTS in 77% of cases.[18] However, these studies were uncontrolled. The safety profile of LLLT was later established for clinical use.[19]

In recent years, some placebo-controlled studies have shown beneficial effects of LLLT on clinical and electrophysiological parameters in the treatment of CTS.[20–25] However, these findings are not consistent because of different laser intervention protocols. Moreover, the functional mechanism of low-level lasers is not clear, and some studies suggested that laser irradiation did not change the functional properties of peripheral nerves.[26,27] Thus, this study was conducted to critically review and summarize the literature regarding low-level lasers to obtain a clear answer concerning the effectiveness of LLLT as a promising treatment for CTS.

2. Methods

2.1. Search strategy

Electronic searches of PubMed (1966–2015.10), Medline (1966–2015.10), Embase (1980–2015.10), and ScienceDirect (1985–2015.10) were performed to identify trials according to the Cochrane Collaboration guidelines. We used the following search terms and different combinations of the terms: "low level or low intensity," "laser," "carpal tunnel syndrome" with the Boolean operators AND or OR. Manual searches including those of the reference lists of all the included studies were used to identify trials that the electronic search might have failed to identify. There was no restriction on language. Two reviewers independently assessed the titles and abstracts of all the reports identified by the electronic and manual searches. When inclusion was unclear based on the abstracts, full text articles were retrieved. Disagreements were resolved through discussion. This study is a meta-analysis, which need not the ethics committee or institutional review board to approve the study.

2.2. Inclusion and exclusion criteria

Trials with the following characteristics were included: (1) randomized clinical trials; (2) comparison of low-level laser with or without splinting for CTS; (3) mild or moderate CTS; (4) full text articles; and (5) available data to be used. Exclusion criteria were as follows: (1) patients who received nonsteroidal anti-inflammatory drugs, and oral corticosteroids or local injection of corticosteroids before LLLT; (2) studies comparing LLLT with other conservative treatment; (3) articles that were duplicate reports of earlier trials, post-hoc analyses of randomized controlled trials (RCTs) data, and articles for which we were unable to obtain the full text.

2.3. Quality assessment

A quality assessment was conducted according to the Cochrane Collaboration's tool for assessing the risk of bias and included the following key domains: adequate sequence generation, allocation of concealment, blinding, incomplete outcome data, and an absence of selective reporting and other bias. Disagreements were resolved by discussion or by consultation with the senior reviewer.

2.4. Data extraction

Two authors independently extracted the data from the included articles. Data regarding the authors, year, patient demographics, inclusion and exclusion criteria, interventions, outcomes, and follow-up tests for each group were extracted. We attempted to contact the authors for supplementary information when the reported data were inadequate.

2.5. Data analysis and statistical methods

The meta-analysis was undertaken using RevMan 5.1 for Windows (Cochrane Collaboration, Oxford, United Kingdom). Statistical heterogeneity was assessed using a standard chi-square test (the statistical heterogeneity was considered significant at $P < 0.05$) and the I^2 statistic (I^2 value of 50% or higher was considered to indicate substantial heterogeneity).[28] When heterogeneity occurred, the pooled data were meta-analyzed using a random-effects model. Otherwise, a fixed-effects model was used for the analysis. The mean difference (MD) and 95% confidence interval (CI) were calculated for the continuous outcomes.

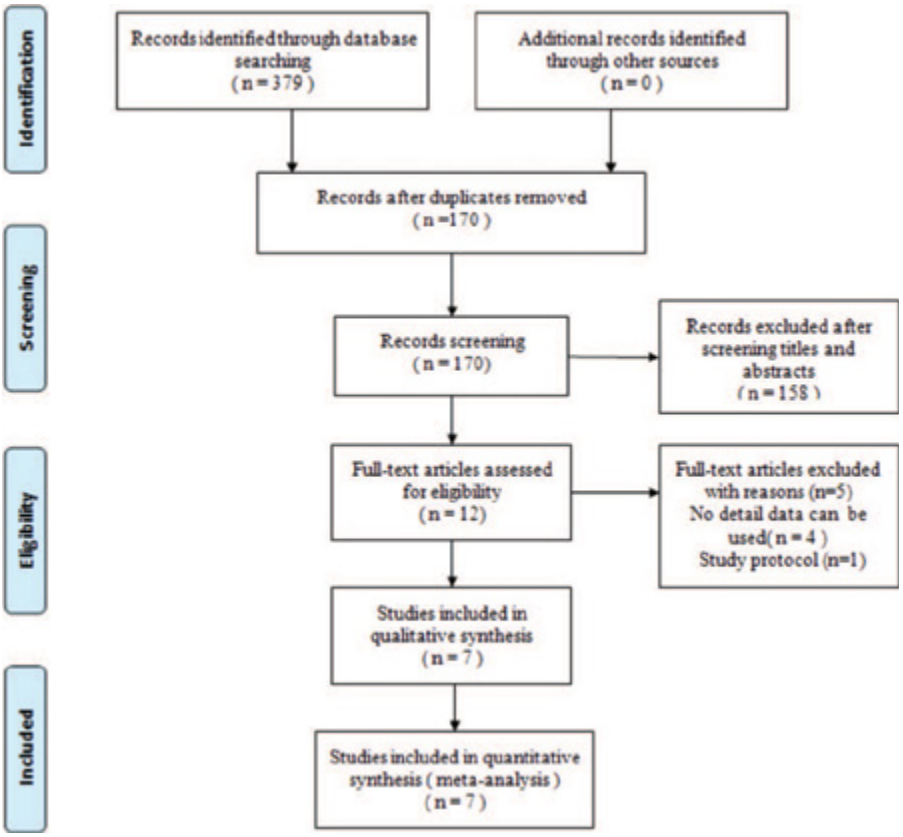

Figure 1. Flowchart showing the identification and selection of studies.

3. Results

3.1. Literature search

Figure 1 shows the flowchart of the study selection and inclusion process. A total of 170 potential studies were identified with the first search strategy. Of these, 161 reports were excluded, based on the eligibility criteria. One RCT by Lazovic was excluded because no available data can be pooled to calculate together.[29] No additional studies were obtained after the reference review. The search strategy ultimately identified 7 randomized clinical trials satisfying the predefined inclusion criteria; there were 270 wrists in the laser group and 261 wrists in the control group.[22–24,30–33] Individual patient data were available from these articles, except for data for the subjects lost to follow-up.

3.2. Study characteristics

The characteristics of the included studies are summarized in Table 1. Statistically similar baseline characteristics were observed between the 2 groups. The sample sizes in the studies ranged from 15 to 141 wrists. Among these studies, a splint was used in the patients in 3 studies.[22,30,33] The laser treatment methods were different in all of these studies.

3.3. Risk of bias assessment

Random sequence generation and allocation were not described in 2 studies.[23,24] The blindness of the participants and personnel was not clear in 2 studies,[23,32] and the blindness of the outcome assessment was not described in 4 studies.[23,24,31,32] The details of the methodological quality of the included studies are presented in Fig. 2.

3.4. Outcomes for meta-analysis

The clinical parameters of hand grip strength, visual analog scale (VAS), symptom severity scores (SSS), and functional status scores (FSS) of the patients were calculated according to the test time. Because different follow-up times for clinical or electrophysiological tests were adopted in the included studies, we defined a "short" time as less than 6 weeks after treatment and a "long" time as 12 weeks. The meta-analysis results of clinical parameters are summarized in Table 2. No significant differences between the 2 groups were observed in most of the parameters with the exception of hand grip (long) and VAS. The hand grip (long) was stronger in the LLLT group than in the control group (MD = 2.04; 95% CI: 0.08–3.99; $P = 0.04$; $I^2 = 62\%$);[22,30,33] better improvements in VAS (long) were found for the LLLT group (MD = 0.97; 95% CI: 0.84–1.11; $P < 0.01$; $I^2 = 0\%$).[32,33] However, the study by Fusakul et al[33] was weighted as >95% in the calculation of hand grip strength and VAS at 12 weeks.

Different electrodiagnostic parameters examining the effects of lasers on nerves were tested and are summarized in Table 3. Similar to the clinical tests, most of the nerve conduction studies showed no significant differences between the 2 groups with apparent heterogeneity. The only significant difference was noticed for SNAP, and the study by Fusakul et al occupied >95%

Table 1

Characteristics of included studies.

Study	Group	Gender (M/F)	Age, y	Wrists	Duration, m	Group information	Laser information				
							Pause (yes/no)	Location	Laser	Wavelength	Power output	Tests (w)
Evcik et al[22]	LLLT	33/8	47.7±10.0	72	N	Laser + splint	Yes 1000 Hz	Two points over the carpal tunnel area	GaAlAs	830	450	0–6–14
	C	37/3	51.0±11.8	69		Splint		7 J/point, 5 times a wk for 2 wk				
Chang et al[24]	LLLT	N	46.0±11.7	20	>12	Laser	Yes 10 Hz	Two laser diodes above the transverse carpal ligament	N	830	60	0–2–4
	C		49.1±11.3	20		Sham laser		9.7 J/cm^2, 2 wk for 10 min a day, 5 d per wk				
Shooshtari et al[23]	LLLT	73/7	48.1±10.7	40	N	Laser	Yes 1168–4672 Hz	Over the carpal tunnel area	N	785	400	0–3
	C			40		Sham laser		9–11 J/cm^2 5 times a wk for 3 wk				
Yagci et al[30]	LLLT	0/24	49.5±6.3	24	13.1±9.7	Laser + splint	No	Three points over the course of the median nerve at the wrist	GaAlAs	830	30	0–14
	C	0/21	51.8±12.1	21	12.8±7.2	Splint		8.1 J 5 times a wk for 2 wk				
Tascioglu et al[31]	LLLT	17/3	47.3±7.4	20	5.1±1.0	Laser	No	Five points across the median nerve trace	GaAlAs	830	50	0–3
	C	15/5	50.9±9.1	20	4.4±1.4	Sham laser		3 J /point, 5 times a wk for 3 wk				
Rayegani et al[32]	LLLT	N	52±12	18	13±9	Laser	No	Five points over the course of the median nerve at the wrist	indium	880	N	0–5–14
	C		49±11	15	13±9	Sham laser		6 J/cm^2,10 min per time, 5 times a wk for 2 wk				
Fusakul et al[33]	LLLT	2/54	50.7±1.4	56	14.0±2.0	Laser + splint	No	Linear laser beam parallel with distal wrist crease	GaAlAs	810	50	0–10-17
	C	2/54	50.8±1.4	56	13.8±2.0	Splint		18 J per time, 3 times a wk for 5 wk				

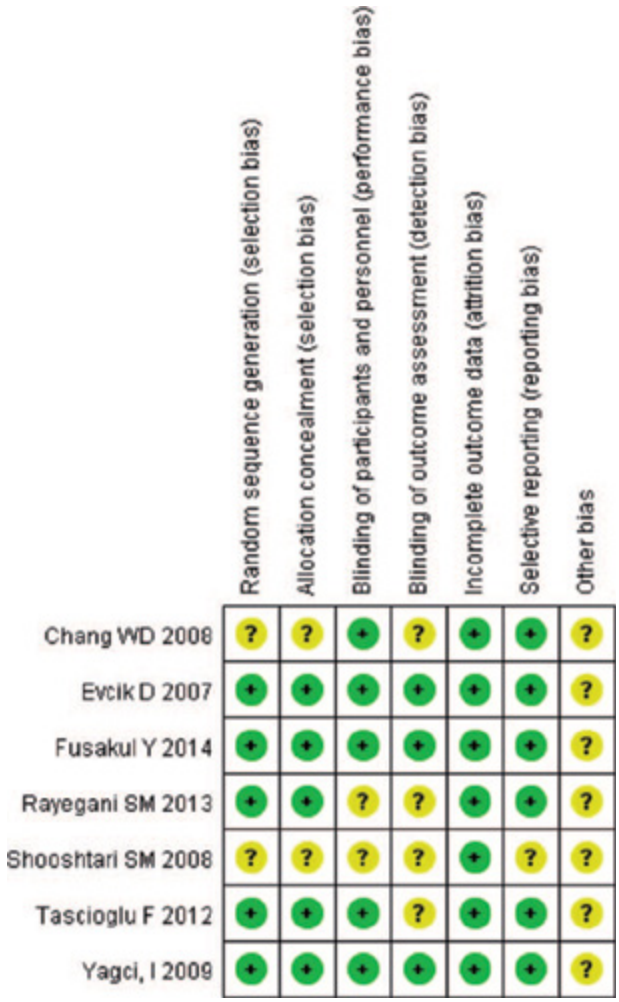

Figure 2. Methodological qualities of the included studies.

of the weight. The SNAP (long) was better in the LLLT group than in the control group (MD = 1.08; 95% CI: 0.44 to 1.73; $P = 0.001$; $I^2 = 0\%$).[30,32,33]

4. Discussion

Although LLLT has been reportedly used in clinical practice with good performance, no statistically significant differences were found in most clinical parameters or nerve conduction studies between the groups in our meta-analysis based on 7 randomized controlled trials. This study revealed that low-level laser improves hand grip, VAS, and SNAP after 3 months of follow-up for mild to moderate CTS. No statistically significant differences were found in other clinical parameters or nerve conduction studies between these 2 groups.

Whether significant differences were found in these parameters, an important problem is that high heterogeneity existed in most of the calculations, which would lower the persuasive power of this meta-analysis. Many factors would influence the precision of the results, such as heterogeneous participants, different interventions, and different follow-up times for conducting clinical or electrophysiological tests. In the included studies, the inclusion criteria of the patients were similar; mild to moderate cases were recruited without surgery of the wrist, rheumatoid arthritis, a history of metabolic disease, paralyzed limbs, or similar conditions.

The LLLT factors were important, including wavelength, power, frequency, pulse or not, action position, and treatment schedule.[34,35] Different laser irradiation doses for patients in the included studies were adopted; the doses are expressed as energy from 2.7 to 11 J for each point or as total energy from 81 to 300 J for the entire treatment. Three or 5 points over the course of the median nerve at the wrist was the most commonly used action position, whereas 2 laser diodes above the transverse carpal ligament were used in the study of Chang et al.[24] These differences resulted in heterogeneity in the meta-analysis. We were unable to find a good method to conduct subgroup analyses based on 1 factor. The difficulties in the analysis made it impossible to determine which low-level laser treatment protocol was best and should be adopted.

Another factor that contributed to high heterogeneity is the test time during the follow-up. The evaluation times were different in the included studies. In our study, long follow-up tests were performed 3 months after treatment and short tests were

Table 3

Results of electrodiagnostic testing.

Outcome (time)	Studies	MD	95% CI	*P*	I^2
MDL (short)	3	−0.07	−0.34 to 0.20	0.61	63%
MDL (long)	3	−0.62	−1.89 to 0.65	0.34	98%
SDL (short)	2	−0.03	−0.25 to 0.18	0.75	75%
SDL (long)	2	−0.06	−0.33 to 0.21	0.67	84%
CMAP (long)	3	−0.51	−1.58 to 0.57	0.35	59%
SNAP (long)	3	1.08	0.44 to 1.73	0.001	0
MNV (short)	2	−0.58	−2.73 to 1.56	0.59	0
SNV (long)	2	1.31	−0.56 to 3.18	0.17	0

CMAP = compound muscle action potential amplitude, MDL = motor distal latency, MNV = motor nerve velocity, SDL = sensory distal latency, SNAP = sensory nerve action potential amplitude, SNV = sensory nerve velocity.

Table 2

Results of clinical parameters.

Outcome (time)	Studies	MD	95% CI	*P*	I^2
Hand grip (short)	5	1.46	−0.85 to 3.77	0.22	89%
Hand grip (long)	3	0.98	0.59 to 1.37	<0.001	62%
VAS (short)	4	−0.02	−2.63 to 2.58	0.99	100%
VAS (long)	2	0.97	0.84 to 1.11	<0.001	0
SSS (short)	4	−1.40	−8.15 to 5.34	0.68	100%
SSS (long)	3	0.11	−0.36 to 0.58	0.65	62%
FSS (long)	4	−1.58	−3.29 to 0.13	0.07	96%
FSS (long)	3	−0.05	−0.44 to 0.35	0.81	56%

FSS = functional status scores, SSS = symptom severity scores, VAS = visual analog scale.

conducted immediately, 2, 4, or 5 weeks after treatment. Calculating the data from different test times together results in heterogeneity. Subgroup analyses based on different test times are a good choice to allow further understanding, although these analyses could not be performed in this study because of the number of inadequate studies. Detecting the actual effects of low-level laser treatment on CTS during different processes is difficult.

In addition to the limitations mentioned above, the application of a splint in some studies would influence the results. Immobilization of the wrist in a neutral position with a splint could maximize carpal tunnel volume, facilitating the release of pressure on the median nerve.[36] The effect of a splint on CTS might confuse the power of LLLT. Additional RCTs with a similar laser treatment protocol are needed to minimize bias and confirm the effect of LLLT in the treatment of CTS.

5. Conclusions

The results of this review show that low-level laser improves hand grip, VAS, and SNAP after 3 months of follow-up for mild to moderate CTS. However, more high-quality studies with the same laser intervention protocol and follow-up time are needed to decrease heterogeneity and to confirm the effects of LLLT on CTS. Besides, we also need double-blind studies to evaluate the effects of applying LLLT comparing with conventional therapies including anti-inflammatory medication on improving clinical and electrophysiologic findings in patients with mild to moderate CTS.

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