Case Studies

Contents

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BIOLOGICAL RESPONSES OF STEM CELLS TO PHOTOBIOMODULATION THERAPY

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ARTICLE

KEYWORDS:

Stem cell, low-level laser therapy, regenerative medicine, photobiomodulation, mesenchymal stem cells.

ABSTRACT:

Background: Stem cells have attracted the researchers interest, due to their applications in regenerative medicine. Their self-renewal capacity for multipotent differentiation, and immunomodulatory properties make them unique to significantly contribute to tissue repair and regeneration applications. Recently, stem cells have shown increased proliferation when irradiated with low-level laser therapy or Photobiomodulation Therapy (PBMT), which induces the activation of intracellular and extracellular chromophores and the initiation of cellular signaling. The purpose of this study was to evaluate this phenomenon in the literature.

METHODS:

The literature investigated the articles written in English in four electronic databases of PubMed, Scopus, Google Scholar and Cochrane up to April 2019. Stem cell was searched by combining the search keyword of “low-level laser therapy” OR “low power laser therapy” OR “low-intensity laser therapy” OR “photobiomodulation therapy” OR “photo biostimulation therapy” OR “LED”. In total, 46 articles were eligible for evaluation.

RESULTS:

Studies demonstrated that red to near-infrared light is absorbed by the mitochondrial respiratory chain. Mitochondria are significant sources of reactive oxygen species (ROS). Mitochondria play an important role in metabolism, energy generation, and are also involved in mediating the effects induced by PBMT. PBMT may result in the increased production of (ROS), nitric oxide (NO), adenosine triphosphate (ATP), and cyclic adenosine monophosphate (cAMP). These changes, in turn, initiate cell proliferation and induce the signal cascade effect.

CONCLUSION:

The findings of this review suggest that PBMT-based regenerative medicine could be a useful tool for future advances in tissue engineering and cell therapy.

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CAN PHOTOBIOMODULATION THERAPY (PBMT) CONTROL BLOOD GLUCOSE LEVELS AND ALTER MUSCLE GLYCOGEN SYNTHESIS?

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ABSTRACT

HIGHLIGHTS

- First time that photobiomodulation therapy (PBMT) controlled blood glucose levels (systemic effect) after muscle irradiation without exercise;
- PBMT controlled blood glucose levels and its variability with a biphasic dose-response (10 J/cm^2 best dose);
- PBMT increased muscle glycogen synthesis with a triphasic dose-response (10 and 60 J/cm^2 best doses);
- Very strong and negative correlation between blood glucose control and muscle glycogen synthesis (supposed mechanism of action).

ABSTRACT

Photobiomodulation therapy (PBMT) has many effects on the energy metabolism of musculoskeletal tissue, such as increased glycogen and adenosine triphosphate synthesis. In addition, these effects may be due to a systemic blood glucose control. Twenty-four Wistar rats were randomly and equally allocated into four groups: sham, PBMT 10 J/cm^2, PBMT 30 J/cm^2 and PBMT 60 J/cm^2. The animals were fasting for 6 h for blood glucose evaluations during pre-irradiation period, 1 h, 3 h and 6 h after PBMT. Muscle glycogen synthesis was measured 24 h after PBMT. This PBMT used a cluster of 69 LEDs (light-emitting diodes) with 35 red (630 ± 10 nm) and 34 infrared (850 ± 20 nm); 114 mW/cm^2 for 90s (10 J/cm^2), 270 s (30 J/cm^2), 540 s (60 J/cm^2) applied on large muscle areas (back and hind legs) of the animals. The 10 J/cm^2 group showed lower blood glucose levels and glucose variability over 6 h (5.92 mg/dL) compared to the sham (13.03 mg/dL), 30 J/cm^2 (7.77 mg/dL) and 60 J/cm^2 (9.07 mg/dL) groups. The PBMT groups had the greatest increase in muscle glycogen (10 J/cm^2 > 60 J/cm^2 > 30 J/cm^2 > sham), characterizing a triphasic dose-response of PBMT. There was a strong negative correlation between blood glucose variability over 6 h and muscle glycogen concentration for 10 J/cm^2 group ($r = -0.94$; $p < .001$) followed by 30 J/cm^2 group ($r = -0.84$; $p < .001$) and 60 J/cm^2 group($r = -0.73$; $p < .006$). These results suggest that PBMT can play a very important role in the control of blood glucose levels, and its possible mechanism of action is the induction of greater muscle glycogen synthesis independently of physical exercise.

KEYWORDS:

Blood glucose; Diabetes mellitus; Glycogen; LED therapy; Low-level laser therapy.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DOES PHOTOTHERAPY ENHANCE SKELETAL MUSCLE CONTRACTILE FUNCTION AND POSTEXERCISE RECOVERY? A SYSTEMATIC REVIEW

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REPORT

ABSTRACT

Context: Recently, researchers have shown that phototherapy administered to skeletal muscle immediately before resistance exercise can enhance contractile function, prevent exercise-induced cell damage, and improve postexercise recovery of strength and function.

Objective: To critically evaluate original research addressing the ability of phototherapeutic devices, such as lasers and light-emitting diodes (LEDs), to enhance skeletal muscle contractile function, reduce exercise-induced muscle fatigue, and facilitate postexercise recovery.

Data Sources: We searched the electronic databases PubMed, SPORTDiscus, Web of Science, Scopus, and Rehabilitation & Physical Medicine without date limitations for the following key words: ***laser therapy***, ***phototherapy***, ***fatigue***, ***exercise***, ***circulation***, ***microcirculation***, and ***photobiomodulation***.

Study Selection: Eligible studies had to be original research published in English as full papers, involve human participants, and receive a minimum score of 7 out of 10 on the Physiotherapy Evidence Database (PEDro) scale.

Data Extraction: Data of interest included elapsed time to fatigue, total number of repetitions to fatigue, total work performed, maximal voluntary isometric contraction (strength), electromyographic activity, and postexercise biomarker levels. We recorded the PEDro scores, beam characteristics, and treatment variables and calculated the therapeutic outcomes and effect sizes for the data sets.

Data Synthesis: In total, 12 randomized controlled trials met the inclusion criteria. However, we excluded data from 2 studies, leaving 32 data sets from 10 studies. Twenty-four of the 32 data sets contained differences between active phototherapy and sham (placebo-control) treatment conditions for the various outcome measures. Exposing skeletal muscle to single-diode and multidiode laser or multidiode LED therapy was shown to positively affect physical performance by delaying the onset of fatigue, reducing the fatigue response, improving postexercise recovery, and protecting cells from exercise-induced damage.

Conclusions: Phototherapy administered before resistance exercise consistently has been found to provide ergogenic and prophylactic benefits to skeletal muscle.

Key Words: photobiomodulation, laser therapy, skeletal muscle fatigue

KEY POINTS

- Phototherapy administered before resistance exercise may enhance contractile function, reduce exercise-induced muscle damage, and facilitate postexercise recovery.
- The effectiveness of phototherapy is dose dependent, so selecting appropriate treatment variables, such as wavelength and output power, is important.
- In attempting to reproduce clinical outcomes, clinicians and researchers should use evidence-based decision making when selecting treatment variables in phototherapy.

- Given the increased beam reflection and attenuation at the skin interface, a larger treatment dose may be necessary when using light-emitting diodes (LEDs) instead of a semiconductor laser.

Phototherapy involves the therapeutic use of light to treat various pathologic conditions and musculoskeletal injuries. Research addressing the ability of light therapy to modulate physiologic processes associated with injury and healing has yielded promising results. Such modulatory processes associated with phototherapy often are called photobiomodulation (PBM), which involves the use of light to induce biochemical changes in tissue in a stimulatory or inhibitory manner.[1]

The use of light as a clinical modality has increased greatly over the past decade. The beneficial outcomes of phototherapy for the treatment of acute and chronic musculoskeletal disorders include pain control,[2,3] enhanced blood circulation,[4] and improved tissue repair.[5]Although evidence is available on how light is absorbed by tissue and cells, the biochemical translation to alter clinical outcomes in humans remains poorly understood. The biological effects of phototherapy are mediated by the absorption of photons (light particles) by endogenous chromophores and the subsequent transduction of light energy into chemical energy inside the plasma membrane or cytosolic organelle.[6] Membrane-bound chromophores act as photosensitizers that induce changes in membrane permeability and transport mechanisms that give rise to intracellular changes in pH, ion concentrations, and membrane excitability.[7,8] Photons that penetrate the cell membrane often will enter mitochondria, where they readily are absorbed by cytochrome enzymes (eg, cytochrome c oxidase), generating physiologic responses conducive to the production of reactive oxygen species and increased rates of adenosine 5′-triphosphate (ATP) and protein synthesis.[1,9] The reactive oxygen species concentrations below cytotoxic levels have been shown to create biostimulatory effects for the cell.[10]

Recently, researchers have begun to explore the ergogenic effects of phototherapy in delaying the onset or resisting the effects of muscle fatigue and exhaustion. Acutely, fatigue impairs muscular strength and motor control and reduces a muscle's capacity to perform work over a designated period.[11] The decrease in muscle function associated with fatigue is believed to be a result of metabolic alterations, such as substrate depletion (lack of ATP and glycogen), oxidative stress, tissue hypoxia, and blood acidification.[11] Researchers also have indicated that specific doses of phototherapy reduce blood lactate and inflammatory biomarker levels after strenuous upper and lower extremity exercise.[12,13] Based on these findings, one may infer that phototherapy also provides a prophylactic effect to tissue by limiting exercise-induced cellular damage. Limiting inflammation and cell damage during exercise also can improve recovery of muscle strength and function postexercise. Therefore, the purpose of our systematic review was to determine the ability of phototherapeutic devices, such as lasers and light-emitting diodes (LEDs), to enhance muscle contractile function, reduce exercise-induced muscle fatigue, and facilitate postexercise recovery.

METHODS

Data Sources

We searched for articles in the electronic databases of PubMed, SPORTDiscus, Web of Science, Scopus, and Rehabilitation & Physical Medicine without date limitations for the following key words: *laser therapy, phototherapy, fatigue, exercise, circulation, microcirculation,* and *photobiomodulation*. The articles had to be original research involving human participants and written in English. Citations from related articles also were retrieved and reviewed to identify additional articles for possible inclusion.

Selection Criteria

We identified research articles in which investigators evaluated the effect of phototherapy on modulating physiologic functions that result in reducing muscle damage, delaying the onset of muscle fatigue, and improving postexercise recovery during resistance exercise (Figure). Articles in which comparisons were made between phototherapy and a control condition or an alternative therapy were selected for this review. Articles were included in the screening process if the investigators studied humans, evaluated physical function changes from baseline, and assessed changes in muscle function between the control condition and phototherapy. Articles were excluded if they were reviews, meta-analyses, or animal studies.

Figure 1 Criteria for selection of articles for review.

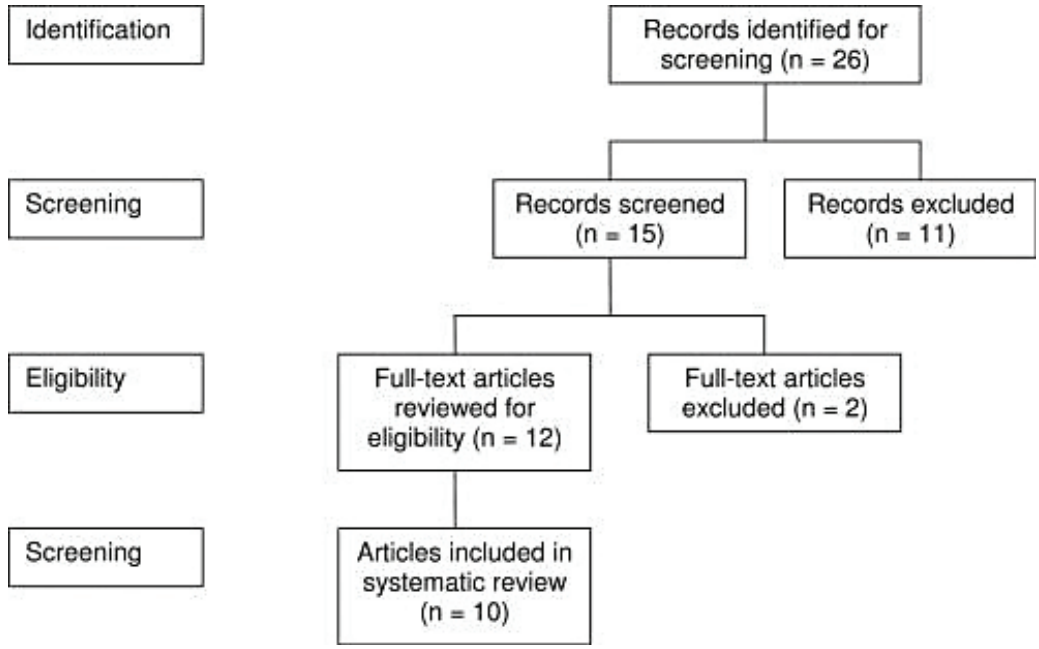

Table 1. Physiotherapy Evidence Database (PEDro) Scale[14a]

Criteria	Yes/No	Points
1. Eligibility criteria were specified.	Yes	0
2. Subjects were randomly allocated in groups.		1
3. Allocation was concealed.	Yes	1
4. The groups were similar at baseline regarding the most important prognostic indicators.	Yes	1
5. There was blinding of all subjects.	Yes	1
6. There was blinding of all therapists who administered therapy.	Yes	1
7. There was blinding of all assessors who measured at least one key outcome.	Yes	1
8. Measures of at least one key outcome were obtained from more than 85% of the subjects initially allocated to groups.	Yes	1
9. All subjects from whom outcome measures were available received the treatment or control condition as allocated or, when this was not the case, data for at least one key outcome were analyzed by "intention to treat."	Yes	1
10. The results of between-groups statistical comparisons were reported for at least one key outcome measure.	Yes	1
11. The study provides both point measures and measures of variability for at least one key outcome.	Yes	1
TOTAL		10

[a] Adapted from the Centre for Evidence-Based Physiotherapy (PEDro) scale (http://www.pedro.org.au).

Physiotherapy Evidence Database Scale (PEDro)

The 12 articles that were deemed eligible for inclusion in the review were rated and scored using the Physiotherapy Evidence Database (PEDro) Scale[14] (Table 1). The PEDro scale was developed as an instrument of evaluation for the Physiotherapy Evidence Database by the Centre for Evidence-Based Physiotherapy. The database provides access to literature on clinical trials and systematic reviews within the field of physiotherapy. The PEDro scale was developed to help users identify studies with the highest methodologic quality. The scale grades research reports based on study design, "believability," and "interpretability" of the research conducted. It considers all aspects of research, including blinding of research participants, examiners and assessors; group allocation; comparability of groups at baseline; between-groups statistical comparability; and adequacy of follow-up measures. The scale consists of an 11-item checklist that yields a numeric score, with a maximum of 10 points if all criteria are met. No points are awarded for the first criterion. A lower score on the PEDro scale indicates a lack of methodologic techniques, such as randomization, blinding, and other controls that maintain internal or external validity.

Table 2. Physiotherapy Evidence Database (PEDro) Scores, Beam Characteristics, and Treatment Variables

Authors	PEDro Score[a]	Light Source	Wavelength, nm	Power Output, mW	Power Density, W/cm^2	Energy Density, J/cm^2	Treatment Points, n	Energy, Joules per Point	Treatment Time, s	Cumulative Dose, J
Leal Junior et al[16] (2008)	9	Single-diode laser	655	50	5	500	4	5	400	20
Leal Junior et al[17] (2009)	10	Single-diode laser	830	100	35.7	1785	4	5	200	20
Leal Junior et al[18] (2009)	10	Cluster-diode LED	660 (34 diodes)	10	0.05	1.5	1	41.7	30	41.7
			850 (35 diodes)	30	0.15	4.5				
Leal Junior et al[19] (2009)	10	Single-diode laser	810	200	5.5	165	2	41.7	60	12
		Cluster-diode LED	660 (34 diodes)	10	0.05	1.5	2	41.7	60	41.7
			850 (35 diodes)	30	0.15	4.5				41.7
Leal Junior et al[20] (2009)	9	Single-diode laser	830	100	35.7	1071–1429	10	3–4	300–400	30–40
Leal Junior et al[21] (2010)	10	Multidiode laser	810 (5 diodes)	200	5.5	165	2	30	60	60
Leal Junior et al[22] (2011)	10	Cluster-diode LED	660 (34 diodes)	10	0.05	1.5	10	41.7	300	417
			850 (35 diodes)	30	0.15	4.5				
Baroni et al[23] (2010)	7	Multidiode laser	810 (5 diodes)	200	5.5	165	6	30	180	180
Baroni et al[24] (2010)	7	Cluster-diode LED	660 (34 diodes)	10	0.05	1.5	3	41.7	90	125.1
			850 (35 diodes)	30	0.15	4.5				
Kelencz et al25(2010)	7	Single-diode LED	640	0.116	0.222	2	8	1.044	72	8.4
				0.116	0.222	4	8	2.088	144	16.7
				0.116	0.222	6	8	3.132	216	25.1

[a] The PEDro scale scores range from 1 to 10.

Data Extraction

We used the PEDro scale due to its tested reliability. Data of interest were elapsed time to fatigue, total number of repetitions to fatigue, total work performed, maximal voluntary isometric contraction (strength), electromyographic activity, and postexercise biomarker levels (blood lactate, creatine kinase [CK], C-reactive protein [CRP], lactate dehydrogenase). Creatine kinase and CRP are measured commonly and are used as indicators of exercise-induced muscle damage. Two authors (P.A.B. and K.A.L.) rated all studies. They independently evaluated the 12 randomized controlled trials that met the criteria of evaluation. They scored each of the 12 articles individually based on the PEDro scale criteria and then met to review the final scores for all articles. Individual PEDro scores for all articles yielded an interrater reliability score of $\kappa = 0.94$. After the authors met and reviewed the discrepancies, full agreement was achieved with $\kappa = 1.00$.

Data Synthesis

Effect sizes for outcome measures were calculated as the difference between the mean score for the active phototherapy treatment and the mean score for the sham treatment (placebo control). The mean difference then was divided by the average of the standard deviation for the 2 measures, giving us the effect size (Cohen d).[15] Effect sizes were interpreted as small (0.2), medium (0.5), or large (0.8).[15]

RESULTS

The PEDro scores ranged from 5 to 10 out of a maximum 10 points. We included only those papers with a PEDro score of 7 or greater. Ten of the 12 research articles met our level of acceptance for this review and contained a total of 32 data sets. Data sets are summarized in Tables 2 through 5. The effect sizes with corresponding P values for all data sets with differences between groups are shown in Table 5. Of the 32 data sets, 24 contained differences between active phototherapy and sham (placebo-control) treatment conditions for the various outcome measures.

Table 3. Effectiveness of Laser Therapy on Selected Outcome Measures Used to Characterize Ergogenic Effects of Laser

Authors	Journal	Type of Fatigue Protocol	Muscles Exercised	Time to Fatigue	Repetitions to Fatigue	Strength Loss or Work Performed	Electromyographic Activity
Leal Junior et al[16] (2008)	*Photomed Laser Surg*	Isotonic arm curl	Biceps brachii	Effective	Effective	Not applicable	Not applicable
Leal Junior et al[17] (2009)	*Lasers Med Sci*	Isokinetic arm curl	Biceps brachii	Ineffective	Effective	Not applicable	Not applicable
Leal Junior et al[18] (2009)	*Lasers Surg Med*	Isokinetic arm curl	Biceps brachii	Effective	Effective	Not applicable	Not applicable
Leal Junior et al[19] (2009)	*Photomed Laser Surg*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Not applicable	Not applicable	Ineffective	Not applicable
Leal Junior et al[20] (2009)	*Lasers Med Sci*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Not applicable	Not applicable	Ineffective	Not applicable
Leal Junior et al[21] (2010)	*J Orthop Sports Phys Ther*	Isokinetic arm curl	Biceps brachii	Effective	Effective	Not applicable	Not applicable
Leal Junior et al[22] (2011)	*Lasers Med Sci*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Not applicable	Not applicable	Ineffective	Not applicable
Baroni et al[23] (2010)	*Eur J Appl Physiol*	Isokinetic (eccentric) leg extension	Quadriceps femoris	Not applicable	Not applicable	Effective	Not applicable
Baroni et al[24] (2010)	*Photomed Laser Surg*	Isokinetic (concentric) leg flexion-extension	Quadriceps femoris	Not applicable	Not applicable	Effective	Not applicable
Kelencz et al[25] (2010)	*Photomed Laser Surg*	Isometric jaw clench	Masseter	Effective (2.09 J only)	Not applicable	Not applicable	Effective (1.04 J only)

DISCUSSION

The important factors related to phototherapy that we discuss include (1) the type of light source used and its treatment variables (wavelength and dose), (2) when the light treatment was applied (before or after exercise), (3) the exercise protocol used, (4) the muscle groups targeted for exercise and testing, and (5) the outcomes of each study.

Beam Characteristics and Tissue Interaction

The phototherapy devices used in the 10 studies were semiconductor or solid-state diode lasers or LEDs. Semiconductor or solid-state laser diodes contain a lasing medium to stimulate photon emission. They emit photons in a more collimated and coherent manner than do LED sources. Another important characteristic of photon emission is the diameter of the beam or spot size. Smaller beam diameters and spot sizes produce greater irradiance of energy density at the point of skin contact. In the studies we included, the laser diodes that were used delivered light using spot sizes[16,17,19–21,23] ranging from 0.003 to 0.01 cm^2, whereas LEDs used spot sizes[18,19,22,24] ranging from 0.2 to 0.5 cm^2 and delivered light onto the skin in a much greater divergent pattern. Data on the beam characteristics appear in Table 2.

Pigmentation is known to affect light transmission through skin. Light penetration through darker-pigmented skin is reduced due to the absorption of photons by melanin.[26] If the rate of photoabsorption by the skin exceeds the capacity of the tissue to dissipate the light energy, the energy will be converted to heat and may cause thermal discomfort. Practical guidelines recommend that when treating darker-pigmented individuals, the risk of tissue heating can be minimized while maintaining the same treatment dose by reducing the average output power or energy density of the beam and increasing the treatment time. In addition, if more light energy is absorbed by the skin in darker-pigmented individuals, then a larger treatment dose is required to transmit the equivalent amount of light energy to deeper tissues. In clinical settings, the therapist usually chooses the increased treatment dose based on past experience.

Wavelength plays an important role in the ability of light to penetrate soft tissue.[27,28] When light therapy

Table 4. Effectiveness of Laser Therapy on Selected Outcome Measures Used to Characterize Prophylactic and Recovery Effects of Laser

Authors	Journal	Type of Fatigue Protocol	Muscles Exercised	Postexercise Biomarker Levels			Strength Recovery[a]		Pain and Soreness[b]
				Immediately	24 h	48 h	24 h	48 h	
Leal Junior et al[16] (2008)	*Photomed Laser Surg*	Isotonic arm curl	Biceps brachii	Ineffective	Not	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et al[17] (2009)	*Lasers Med Sci*	Isokinetic arm curl	Biceps brachii	Ineffective	Not	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et al[18] (2009)	*Lasers Surg Med*	Isokinetic arm curl	Biceps brachii	Effective—blood lactate, creatine kinase, and C-reactive protein	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et al[19] (2009)	*Photomed Laser Surg*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Effective—creatine kinase	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et al[20] (2009)	*Lasers Med Sci*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Effective—blood lactate and creatine kinase	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et a[l21] (2010)	*J Orthop Sports Phys Ther*	Isokinetic arm curl	Biceps brachii	Effective—blood lactate, creatine kinase, and C-reactive protein	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Leal Junior et al[22] (2011)	*Lasers Med Sci*	Wingate cycle ergometer	Quadriceps femoris (bilateral)	Effective—blood lactate and creatine kinase	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Baroni et al[23] (2010)	*Eur J Appl Physiol*	Isokinetic (eccentric) leg extension	Quadriceps femoris	Not applicable	Effective—creatine kinase	Effective—creatine and lactate dehydrogenase	Effective	Effective	Ineffective
Baroni et al[24] (2010)	*Photomed Laser Surg*	Isokinetic (concentric) leg flexion-extension	Quadriceps femoris	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable
Kelencz et al[25](2010)	*Photomed Laser Surg*	Isometric jaw clench	Masseter	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable

[a] Measured with maximal voluntary isometric contraction.

[b] Measured with a visual analog scale.

Table 5. Effect Sizes With P Values for Data Sets With Differences Between Groups

Authors	No. of Participants and Age (Mean ± SD, y)	Time to Fatigue	Repetitions to Fatigue	Strength Loss, Work, or EMG	Blood Lactate	Creatine Kinase	C-Reactive Protein	Lactate Dehydrogenase	Strength Recovery
Leal Junior et al[16] (2008)	12 male professional volleyball players (22 ± 3.0)	Cohen d = 2.32, *P* = .0001	SD not provided	Not applicable	Not different	Not applicable	Not applicable	Not applicable	Not measured
Leal Junior et al[17] (2009)	10 healthy male professional volleyball players (22.3 ± 6.1)	Cohen d = 0.63, *P* = .04	Not different	Not applicable	Not different	Not applicable	Not applicable	Not applicable	Not measured
Leal Junior et al[18] (2009)	10 healthy male professional volleyball players (23.6 ± 5.6)	Cohen d = 0.50, *P* = .02	Cohen d = 0.39, *P* =.04	Not applicable	Cohen d = 0.92, *P* = .04	Cohen d = 1.12, *P* = .04	Cohen d = 0.80, *P* = .03	Not applicable	Not measured
Leal Junior et al[19] (2009)	8 male volleyball players (18.5 ± 0.93)	Not applicable	Not applicable	Not different	Not applicable	Cohen d = 1.62, *P* < .01	Not applicable	Not applicable	Not measured
Leal Junior et al[20] (2009)	9 male professional volleyball players (20.7 ± 2.96) and 11 male soccer players (16.2 ± 0.75)	Not applicable	Not applicable	Not different	Cohen d = 0.99; *P* < .01	Cohen d = 1.77, *P* = .01	Not applicable	Not applicable	Not measured
Leal Junior et al[21] (2010)	9 male volleyball players (18.6 ± 1.0)	Cohen d = 1.01, *P* = .04	Cohen d = 0.75, *P* =.03	Not applicable	Cohen d = 1.67, *P* < .01	Cohen d = 1.01, *P* = .02	Cohen d = 1.52, *P* = .047	Not applicable	Not measured
Leal Junior et al[22] (2011)	6 futsal players (20.7 ± 2.96)	Not applicable	Not applicable	Not different	Cohen d = 1.94, *P* = .004	Cohen d = 2.07, P= .006	Not different	Not applicable	Not measured
Baroni et al[23] (2010)	36 healthy men (24.8 ± 4.4)	Not applicable	Not applicable	Cohen d = 0.90, *P* = .01	Not applicable	24 h[a]: Cohen d = 0.89, *P* = .02 48 h[a]: Cohen d = 1.50, P= .001	Not applicable	24 h: not different 48 h: Cohen d = 0.89, *P* = .02	24 h: Cohen d = 1.03, *P* =.00448 h: Cohen d = 1.16, *P* = .001
Baroni et al[24] (2010)	17 physically active men (26.3 ± 4.3)	Not applicable	Not applicable	Cohen d = 0.23, *P* = .03	Not applicable	Not applicable	Not applicable	Not applicable	Not measured
Kelencz et al[25] (2010)	30 men and women	Right: Cohen d = 0.28, *P* < .05 Left: Cohen d = 0.29, *P* < .05	Not applicable	Cohen d = 0.65, *P* < .05	Not applicable	Not applicable	Not applicable	Not applicable	Not measured

Abbreviation: EMG, electromyography.

[a] 24 h refers to 24 hours postexercise; 48 h refers to 48 hours postexercise.

is administered directly to the patient's skin, some light is attenuated by the superficial layers. Tunér and Hode[7] indicated that from 50% to 90% of energy is absorbed by the skin and subcutaneous tissues and the remaining light energy penetrates into deeper tissue layers (muscle, deep fascia, ligament). Esnouf et al[29] used a near-infrared (NIR), 850-nm laser diode with an output power of 100 mW to irradiate a 0.784-mm-thick section of human skin and found that 66% of the initial beam intensity was attenuated by the skin section. Similarly, Kolari and Airaksinen[30] exposed dermal tissue to both visible red and NIR lasers and found that a substantial amount of light energy was absorbed within 0.5 mm after penetration. Researchers[5,6,28] have indicated that diodes with wavelengths ranging from 820 to 904 nm can transmit light energy from 2 to 4 cm beyond the skin interface and, therefore, are best suited for treating deep soft tissue disorders, such as those involving muscles, ligaments, and tendons. Diodes with wavelengths ranging from 400 to 700 nm can transmit light energy only to the epidermal and dermal tissue layers (<1 cm) and, therefore, are best suited for targeting superficial wounds and skin disorders.[31,32]

Dose Dependency

Therapeutic dose is reported to have the greatest influence on tissue healing and clinical outcome.[28,33,34] Achieving a therapeutic dose without understimulating or overstimulating the target tissues is often the most difficult component of clinical phototherapy practice.[33]The Arndt-Schultz principle has been adopted from early toxicology studies of yeast culture to explain the optimal therapeutic dose level of laser. Optimal doses have been established experimentally in cell and tissue cultures. This therapeutic laser dose or level of photostimulation must be attained; if the amount of energy absorbed is insufficient to stimulate the absorbing tissues, no reactions or changes can occur in body tissues.[35] Weak stimuli (underdosing) produce no effect or only a minimal effect on cellular function, moderate to strong stimuli positively enhance cellular function, and very strong stimuli (overdosing) suppress or inhibit cellular function.[34]

The optimal doses that are photostimulating for many human tissues are not known; however, results from animal and cell studies[13,36,37] have indicated that the therapeutic effects of light therapy are dose dependent and operate within a therapeutic window when treating musculoskeletal injuries. Therefore, one could surmise that the same dose dependency exists for treating musculoskeletal pathologic conditions in humans. However, problems exist when attempting to translate light therapy studies from animal models to human participants. No known or universally accepted method is available to calculate a comparable treatment dose in humans from those doses used in cell and animal models. In our review, light treatment doses and the type of light source varied among studies, with the trend favoring a higher dose when using an LED device than when using a laser diode. This finding makes sense because the light emanating from LEDs has a wider bandwidth, is not coherent, and is more divergent than the light emanating from laser diodes, resulting in more reflection and less transmission of LED-generated light through the skin. Therefore, a higher dose when using LED therapy may compensate for beam reflection and divergence.

Contractile Function, Fatigue Resistance, and Postexercise Recovery

Recently, researchers have shown that phototherapy can provide an ergogenic effect by improving the contractile function of a skeletal muscle.[12,13,16–18,21,23,24,38] In several studies, researchers have investigated the effects of laser and LED therapy on skeletal muscle fatigue and muscle damage using both animal[12,13] and human populations.[16–18,20] Lopes-Martins et al[13] and Leal Junior et al[12] showed a delay in the fatigue response to repeated, electrically evoked tetanic contractions in the tibialis anterior muscle of rats exposed to laser therapy. They also showed that skeletal muscle exposed to laser displayed lower levels of blood lactate and CK activity after repeated tetanic contractions.[12,13] In human studies, skeletal muscle exposed to selected doses of laser[16,17,21] or LED[18,24] therapy demonstrated enhanced performance by maintaining contractile force output and delaying the onset of fatigue when challenged with resistance exercise. Skeletal muscle exposed to laser or LED

therapy also had less cell damage after exercise, indicating that phototherapy provided protection from exercise-induced damage.[12,18–23]

In a series of studies, Leal Junior et al[16–18,21] administered active phototherapy treatments or sham treatments to the biceps brachii muscle of professional volleyball players and instructed them to perform repeated, near-maximal arm-curl exercises until voluntary exhaustion. The phototherapy intervention consisted of having 20 J (5 J per point at 4 points),[16,17] 42 J (1 point),[18] or 60 J (30 J per point at 2 points)[21] of light delivered with either a laser diode or multidiode LEDs to the biceps brachii muscle of the dominant upper extremity before exercise. The biceps brachii muscle that was treated with active laser or LED therapy performed more arm-curl repetitions until voluntary exhaustion than did the muscle receiving the sham treatment.[16–18,21] Blood lactate levels compared before and after exercise were slightly lower to much lower after exercise when an active light treatment was administered to the biceps.[17,18,21] Creatine kinase and CRP levels also were lower after the active light treatment than in the sham (control) condition.[18,21] Leal Junior et al[16,17] initially assessed the effects of laser therapy on biceps brachii muscle function by comparing 655-nm[16] and 830-nm[17] single-diode lasers. The cumulative dose level for both studies was 20 J (5 J per point at 4 points). Although both wavelengths produced similar results for muscle performance and postexercise recovery, we found the results interesting because the red (655-nm) lasers emitted light in shorter wavelengths and therefore had poorer light penetration than NIR (830-nm) lasers.[32] Researchers[28,32] have indicated that less light can be transmitted to muscle tissue using a red 655-nm laser, so we would expect the muscle receiving laser therapy with the 655-nm wavelength to fatigue at a faster rate and incur more exercise-induced cell damage than the muscle receiving the NIR 830-nm laser condition. Because light attenuation and absorption were not measured directly in these studies, we could not determine the actual dose of light absorbed by the muscles that were treated.

In later studies, Leal Junior et al[18,21] compared the ergogenic and prophylactic effects of phototherapy using a 5-diode NIR laser (810 nm) and a cluster multidiode (34 red and 35 NIR diodes) LED probe. The cluster LED probe delivered 42 J at 1 point to the biceps, and the multidiode NIR laser delivered 60 J (30 J per point at 2 points). Again, both devices produced similar results for performance enhancement and postexercise recovery. Active LED or laser therapy applied pre-exercise increased the number of repetitions to exhaustion by 12.9% and 14.5%, respectively, and the elapsed time to exhaustion by 11.6% and 8%, respectively.[18,21] In addition, the muscle exposed to active light therapy displayed less exercise-induced cell damage than the muscle in the sham treatment condition.

Leal Junior et al[19,20] assessed the effects of laser or LED therapy on muscle fatigue, exercise-induced muscle damage, and postexercise recovery after strenuous lower extremity exercise. Participants completed a single bout of resistance exercise for the lower extremity using the Wingate cycle ergometry test. Male volleyball and soccer players were evaluated after receiving either a controlled dose of laser or LED therapy or a sham dose to the rectus femoris muscle before completing the Wingate test. Researchers found no differences in peak or mean power or the amount of muscular work performed between the active-light and sham-control treatment conditions; however, postexercise blood biomarker levels were lower when the athletes received the active dose of light therapy. This finding indicated that light therapy may protect muscles from damage or enhance postexercise recovery mechanisms after strenuous exercise. The doses delivered to the target muscle varied considerably among studies. Laser diode (NIR 810–830 nm) treatment doses consisted of either 12 J (6 J per point at 2 points)[19] or 30 to 40 J (3–4 J per point at 10 points)[20] per muscle, whereas the multidiode LED dose consisted of 83.4 J (41.7 J per point at 2 points)[19] delivered per muscle.

In 2 studies using isokinetic dynamometry, Baroni et al[23,24] evaluated muscle fatigue and postexercise recovery of the quadriceps femoris muscle group in response to phototherapy or sham therapy. They investigated the protective effects of laser or LED therapy on exercise-induced muscle damage and postexercise strength loss and recovery using a

knee-extensor model. The quadriceps femoris muscle was exposed to an active or sham laser or LED treatment, then the participants performed a challenging resistance exercise regimen for the quadriceps muscle group. The authors then treated the quadriceps muscle group (rectus femoris, vastus medialis, and vastus lateralis) with a multidiode NIR 810-nm laser[23] or a cluster (34 at 660 nm or 35 at 850 nm) multidiode LED[24] before exercise at cumulative doses of 180 J (30 J per point at 6 points) and 125.1 J (41.7 J per point at 3 points), respectively. In both studies, Baroni et al[23,24] showed that active laser or LED therapy before exercise reduced postexercise levels of markers of muscle damage, limited the extent of muscle fatigue, and improved postexercise recovery compared with sham light therapy.

Kelencz et al[25] studied the effects of LED (640 nm) therapy on muscle activity and fatigue resistance. The masseter muscle was irradiated under 4 dose conditions (sham and 8.3 J [1.04 J per point at 8 points], 16.7 J [2.09 J per point at 8 points], and 25.1 J [3.13 J per point at 8 points] per muscle). After treatment, the participant bit down on a pressure plate that was equipped with a load cell embedded into the platform. The electromyography (EMG) signals and maximal voluntary isometric contraction levels were recorded before and after treatment and mandibular occlusion. A dose-dependent increase in muscle activity and time before fatigue was observed after treatment. The 8.3-J dose produced greater muscle activity posttreatment, whereas the 16.7-J dose increased the time to fatigue.

In most of the studies in our review, the investigators administered the laser treatments before completing the exercise protocol. In one of these studies, laser therapy was applied after the exercise protocol was completed.[22] Leal Junior et al[22] compared the effects of cold-water immersion therapy (CWIT), LED therapy, and sham LED therapy on postexercise recovery after high-intensity resistance exercise. Participants completed a single bout of resistance exercise for the lower extremity using the Wingate cycle ergometer test. Multidiode LED therapy was administered to the major muscle groups of the lower extremity (quadriceps, hamstrings, triceps surae) 5 minutes after completing the exercise bout. The cumulative dose for both legs was 417 J (41.7 J per point at 10 points). The LED therapy was more effective than CWIT or sham LED therapy in facilitating muscle recovery postexercise. Blood lactate levels and CK activity were lower after treatment with active light therapy than with CWIT and sham light therapy.

Mechanisms of Actions

Ergogenic Effects. The authors of the reviewed studies postulated that phototherapy could provide an ergogenic effect during exercise by enhancing intramuscular microcirculation,[24]decreasing lactic acid production,[18,19,21,22] improving mitochondrial function,[1,9] and improving the antioxidant capacity[16,17,23] of exercising muscle. However, microcirculation and oxidative stress levels were not assessed directly in the studies we reviewed.

Lower levels of blood lactate after strenuous upper and lower extremity exercise for the active laser or LED condition indicate that fewer anaerobic or glycolytic metabolic pathways were used during exercise or that lactic acid was being used as a substrate for oxidative metabolism by the mitochondria.[39,40] Well-oxygenated muscle cells are better able to oxidize lactic acid to pyruvate, which then is used by the mitochondria to produce ATP.[39]Phototherapy, especially in the NIR spectrum, activates the respiratory chain through the photoacceptor cytochrome c oxidase.[9] Respiratory chain activation results in a cascade of biochemical reactions, leading to increased rates of ATP synthesis for sustained muscle function.

Prophylactic Effects. Exercise-induced muscle damage occurs in 2 distinct phases: primary and secondary. Primary muscle damage results from the mechanical stress of exercise, and secondary muscle damage is caused by the cascade of biochemical reactions that occur due to the inflammatory response.[41,42] Phototherapy administered before exercise is thought to protect muscle cells from primary and secondary damage, whereas phototherapy administered after injury protects cells from secondary damage only.[23] The exact mechanisms by which phototherapy protects muscle from exercise-induced damage are not fully understood; however, the results from in vivo animal studies have revealed important information about how phototherapy can protect muscle from secondary damage after trauma.

Authors of in vivo animal studies have shown that phototherapy administered to injured muscle tissue produces anti-inflammatory and antioxidant effects that protect muscle from secondary damage. Avni et al[43] investigated the cytoprotective effect of laser therapy using an ischemia-reperfusion injury (IRI) model in rat gastrocnemius muscle. They showed that laser therapy applied immediately and 1 hour after arterial occlusion blunted the extent of tissue degeneration and improved the antioxidant capacity of injured muscle hours after IRI compared with control muscle.[43] Similarly, in a series of experiments, Oron et al[44] and Ad and Oron[45] investigated the effects of laser therapy on muscle damage and scar tissue formation using an experimentally induced myocardial infarction (MI) model in rats and dogs. Myocardial infarction was induced by occlusion of the left anterior descending coronary artery. In the canine model, laser therapy was applied to the infarcted region of the heart at 15 minutes and again at 3 days after MI. A total dose of 1.08 J/cm^2 was delivered at each time using the wavelength of 803 nm with a power density of 6 mW/cm^2. Tissue samples were analyzed with computerized morphometry software. The hearts that received laser therapy had a 50% to 70% reduction in infarct size and less scar tissue formation 4 to 6 weeks postinfarction compared with the hearts that did not receive laser therapy. The authors concluded that laser therapy after MI and IRI substantially increased the production of heat shock proteins and preserved mitochondrial integrity, which in turn protected the heart muscle from the damaging effects of ischemia, inflammation, and oxidative stress. Rizzi et al[46] investigated the effects of laser therapy on inflammation and repair using male Wistar rats with an induced injury to the gastrocnemius muscle. Laser therapy was administered daily to the injured muscle. Results revealed that laser therapy reduced inflammatory signaling pathways and blocked the damaging effects of reactive oxygen species. Inflammatory and oxidative stress levels were reduced, thereby minimizing secondary muscle damage.

In several of the phototherapy studies in our review, postexercise reductions in the levels of CK, lactate dehydrogenase, and CRP were shown when active laser or LED treatment was administered before exercise.[18–23] These reductions indicate that less exercise-induced muscle damage and inflammation occurred during the bout of upper and lower extremity resistance exercise. The enhanced cellular protection during strenuous exercise due to the phototherapy treatment likely had a direct effect on the improved postexercise recovery.[22,23]

Sample and Effect Sizes

For 9 of the 10 studies that we reviewed, considerable overlap of investigators occurred.[16–24]In addition, the studies contained relatively small sample sizes with very similar inclusion and exclusion criteria. Seven of the 9 studies contained sample sizes ranging from 6 to 12 participants and consisted primarily of male professional athletes between the ages of 18 and 36 years who competed in volleyball, soccer, or futsal.[16–22] Therefore, an overlap of participant samples may have been present among several of the studies. The small sample sizes and similar participant characteristics among studies severely limit the generalizability of the findings.

The effect sizes were rather robust, ranging from small to large depending on the outcome measure (Table 5). Effect sizes for the muscle performance outcomes (repetitions and time to fatigue, work, strength loss, strength recovery, and EMG) averaged 0.78 (range, 0.28–2.32) and for the outcomes of the biomarkers of muscle damage (CK, lactate dehydrogenase), inflammation (CRP) and recovery (blood lactate) averaged 1.34 (range, 0.80–2.07). The collective strength of the effect sizes lend further support to the effectiveness of phototherapy in providing ergogenic and prophylactic benefits for skeletal muscle during resistance exercise.

Clinical Implications

Skeletal muscle fatigue is a new avenue for research in phototherapy. The traditional use of phototherapy in clinical settings has been directed toward treating injured tissue to control pain and enhance healing. However, new paradigms for clinical practice are expanding the traditional model for phototherapy in clinical situations. We provided an in-depth look at the effects of phototherapy administered pre-exercise on limiting the extent of exercise-induced fatigue and muscle damage and on facilitating postexercise

recovery. Our review is novel because we examined phototherapy from a proactive standpoint as an ergogenic aid to therapeutic exercise and prophylaxis to exercise-induced muscle damage.

Phototherapy appears to be a viable treatment modality for skeletal muscle. It is safe, easy to administer, and noninvasive and has no known side effects and few reported contraindications. Study outcomes consistently demonstrated ergogenic and prophylactic benefits to skeletal muscle after a treatment dose of phototherapy. Positive outcomes occurred when phototherapy was administered pre-exercise[12,16–21,23–25] and postexercise.[22]Investigators could conclude that exposing skeletal muscle to single-diode and multidiode laser or multidiode LED therapy positively affects physical performance by delaying the onset of fatigue,[16–18,21] reducing the fatigue response,[23–25] improving postexercise recovery,[21–23] and protecting cells from exercise-induced damage.[18–23] The results we discussed may directly affect how phototherapeutic modalities are prescribed by therapists and used in clinical practice settings.

With respect to light source, laser diodes typically emit light in a very narrow bandwidth compared with LED.[47] A more narrow emission intensifies the beam, creating a greater energy density or fluence level (J/cm^2). Laser diodes emit light at higher energy densities than LEDs, and this may affect the rate of absorption, depth of penetration, and transmission patterns of light into deeper tissues, regardless of the wavelength.[28,47–49] Light-emitting diodes are less expensive to manufacture than semiconductor or solid-state diodes and have been shown to be safe and effective treatment modalities.[18,19,24]

Being able to quickly and effectively treat an entire muscle or muscle group is an important factor when using laser therapy as an ergogenic aid. Multidiode lasers and LEDs may have an advantage over single-diode lasers for treating large muscles because they can cover a larger surface area over the muscle.[18,19] Single-diode laser treatments deliver tight beams of light to small areas on the skin, and consequently the applicator has to be moved constantly to cover multiple points on a muscle or muscle group. Because multidiode LED applicators have greater beam divergence than diode lasers, they do not have to be moved as often during treatment while still covering a large surface area of the muscle per application point. The number of treatment points per muscle or muscle group varied among studies. The biceps brachii was treated on 1 to 2 points with multidiode laser/LEDs and 4 points with a single-diode laser. For the lower extremity, the number of treatment points for the quadriceps femoris ranged from 2 to 6 for multidiode lasers/LEDs and included 5 points for single-diode lasers.

Phototherapy administered immediately before resistance exercise extended the elapsed time to fatigue and the total number of repetitions to fatigue.[16–18,21] In addition, phototherapy reduced the fatigue response of skeletal muscle during a bout of resistance exercise,[23,24] and it enhanced postexercise recovery and reduced exercise-induced damage when applied before and after strenuous resistance exercise.[18,21–23] The effective dose for the biceps brachii ranged from 20 to 60 J, whereas larger muscles in the lower extremity displayed ergogenic and prophylactic effects when higher treatment doses ranging from 125 to 180 J were administered. On average, the treatment doses were higher in the LED therapy studies than in the single-diode and multidiode laser studies to achieve a similar therapeutic outcome.[49]Therefore, clinicians may need to adjust the cumulative treatment dose when using LED devices to compensate for the higher beam divergence and reduced energy density.

Individuals recovering from musculoskeletal impairment often experience changes in physical performance, such as early-onset muscle fatigue with associated pain and soreness due to disuse atrophy, degenerative changes (eg, osteoarthritis), and neuromuscular deficits. In clinical situations where progressive resistance exercise is indicated for structural and functional recovery (eg, with postoperative or postinjury rehabilitation), exercise-induced muscle fatigue and pain can be limiting factors.[50] This is especially true for muscle groups that maintain balance and locomotor function, such as the quadriceps femoris.[51]

During a normal rehabilitation session, an injured athlete usually undergoes a combination of therapeutic modality treatments coupled with therapeutic exercise. Therapeutic modalities typically are administered during a treatment session to control pain and other symptoms that may limit the intensity and volume of therapeutic exercise. The aims of therapeutic exercise are for the athlete to regain range of motion, strength, and function so he or she can return safely to participation. Enhanced muscle performance and fatigue resistance would benefit athletes during rehabilitation and recovery from injury when muscle atrophy and weakness can impair muscle function. If phototherapy is found to be beneficial in the rehabilitation and postexercise recovery process, it may improve clinical outcomes and the overall quality of health care for injured athletes.

CONCLUSIONS

Phototherapy administered immediately before a bout of resistance exercise consistently was shown to provide an ergogenic effect to skeletal muscle by improving physical performance (extending the elapsed time and total number of repetitions to fatigue, reducing the deficit in maximal voluntary isometric contraction pre-exercise to postexercise) and improving the clearance of blood lactate immediately after exercise. It also consistently was shown to provide a protective effect for skeletal muscle by reducing postexercise plasma levels of CK and CRP. The information gained from this novel use of laser therapy can open a therapeutic window into the treatment of musculoskeletal conditions in which muscle fatigue and fatigue-related impairment are barriers to treating musculoskeletal injuries.

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EFFECT OF PHOTOTHERAPY (LOW-LEVEL LASER THERAPY AND LIGHT-EMITTING DIODE THERAPY) ON EXERCISE PERFORMANCE AND MARKERS OF EXERCISE RECOVERY: A SYSTEMATIC REVIEW WITH META-ANALYSIS

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ABSTRACT

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Recent studies have explored if phototherapy with low-level laser therapy (LLLT) or narrow-band light-emitting diode therapy (LEDT) can modulate activity-induced skeletal muscle fatigue or subsequently protect against muscle injury. We performed a systematic review with meta-analysis to investigate the effects of phototherapy applied before, during and after exercises. A literature search was performed in Pubmed/Medline database for randomized controlled trials (RCTs) published from 2000 through 2012. Trial quality was assessed with the ten-item PEDro scale. Main outcome measures were selected as: number of repetitions and time until exhaustion for muscle performance, and creatine kinase (CK) activity to evaluate risk for exercise-induced muscle damage. The literature search resulted in 16 RCTs, and three articles were excluded due to poor quality assessment scores. From 13 RCTs with acceptable methodological quality (≥6 of 10 items), 12 RCTs irradiated phototherapy before exercise, and 10 RCTs reported significant improvement for the main outcome measures related to performance. The time until exhaustion increased significantly compared to placebo by 4.12 s (95 % CI 1.21–7.02, $p<0.005$) and the number of repetitions increased by 5.47 (95 % CI 2.35–8.59, $p<0.0006$) after phototherapy. Heterogeneity in trial design and results precluded meta-analyses for biochemical markers, but a quantitative analysis showed positive results in 13 out of 16 comparisons. The most significant and consistent results were found with red or infrared wavelengths and phototherapy application before exercises, power outputs between 50 and 200 mW and doses of 5 and 6 J per point (spot). We conclude that phototherapy (with lasers and LEDs) improves muscular performance and accelerate recovery mainly when applied before exercise.

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CONTRIBUTORSHIP STATEMENT

ECPLJ participated in the literature search, development of inclusion and exclusion criteria, selection of trials for inclusion in the analysis, methodological assessment, data extraction and interpretation, writing of the report, and supervised writing of the report as a whole. AAV and EFM participated in the selection of trials for inclusion in the analysis, methodological assessment of RCTs, and data analysis. SDC and PTCC participated in data interpretation and analysis and critically reviewed the report. JMB participated in development of inclusion and exclusion criteria, data analysis and interpretation, writing of the results section of the report, and supervised writing of the report as a whole. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

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IS LIGHT-EMITTING DIODE PHOTOTHERAPY (LED-LLLT) REALLY EFFECTIVE?

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CASE REPORT

BACKGROUND:

Low level light therapy (LLLT) has attracted attention in many clinical fields with a new generation of light-emitting diodes (LEDs) which can irradiate large targets. To pain control, the first main application of LLLT, have been added LED-LLLT in the accelerated healing of wounds, both traumatic and iatrogenic, inflammatory acne and the patient-driven application of skin rejuvenation.

RATIONALE AND APPLICATIONS:

The rationale behind LED-LLLT is underpinned by the reported efficacy of LED-LLLT at a cellular and subcellular level, particularly for the 633 nm and 830 nm wavelengths, and evidence for this is presented. Improved blood flow and neovascularization are associated with 830 nm. A large variety of cytokines, chemokines and macromolecules can be induced by LED phototherapy. Among the clinical applications, non-healing wounds can be healed through restoring the collagenesis/collagenase imbalance in such examples, and 'normal' wounds heal faster and better. Pain, including postoperative pain, postoperative edema and many types of inflammation can be significantly reduced.

EXPERIMENTAL AND CLINICAL EVIDENCE:

Some personal examples of evidence are offered by the first author, including controlled animal models demonstrating the systemic effect of 830 nm LEDLLLT on wound healing and on induced inflammation. Human patients are presented to illustrate the efficacy of LED phototherapy on treatment-resistant inflammatory disorders.

CONCLUSIONS:

Provided an LED phototherapy system has the correct wavelength for the target cells, delivers an appropriate power density and an adequate energy density, then it will be at least partly, if not significantly, effective. The use of LED-LLLT as an adjunct to conventional surgical or nonsurgical indications is an even more exciting prospect. LED-LLLT is here to stay.

KEY WORDS:

Grotthus-Draper law, nonhealing wound, photochemical cascade, photophysical reaction, irritant contact dermatitis, dissecting cellulitis, acne rosacea

INTRODUCTION

High level laser treatment (HLLT) means that high levels of incident laser power are used to deliberately destroy a specific target through a light-heat transduction process to induce photothermal damage of varying degrees. HLLT is used in many surgical fields, but probably most commonly in dermatologic, aesthetic or plastic surgery. On the other hand, when a laser or other appropriate light source is used on tissue at low incident levels of photon energy, none of that energy is lost as heat but instead the energy from the absorbed photons is transferred directly to the absorbing cell or chromophore, causing photoactivation of the target cells and some kind of change in their associated activity. In clinical applications, this was termed 'low level laser therapy' (LLLT) by Ohshiro and Calderhead in 1988,[1] with 'photobiomodulation' or 'photoactivation' referring to the activity at a cellular and molecular level.

GENESIS OF LLLT

In the late 1960's, the early days of the clinical application of the laser, there was fear that laser energy could induce carcinogenesis as a side effect of the use of the laser in surgery and medicine. To assess this, in a paper published in 1968, the late Professor Endrè Mester, the recognized father of phototherapy from Semmelweis University, Budapest, applied daily doses of low incident levels of defocused ruby laser energy to the shaved dorsum of rats.[2] No carcinogenetic changes were noted at all, but Mester incidentally discovered that LLLT accelerated hair regrowth in the laser-irradiated animals. Furthermore, during this period, early adopters of the surgical laser were reporting interesting and beneficial effects of using the laser as a scalpel compared with the conventional cold steel instrument, such as reduced inflammation, less postoperative pain, and better wound healing. Mester's experiments helped to show that it was the 'L' of laser, namely light, that was associated with these effects due to the bioactivative levels of light energy which exist simultaneously at the periphery of the photosurgical destructive zone, as illustrated in **Figure 1.**

In the 1970's, many clinicians, inspired by Mester's major publication in 1969 on the significantly successful use of LLLT for the treatment of nonhealing or torpid crural ulcers, started to apply LLLT clinically, particularly in France and Russia, and this spread to Japan, Korea, and other Asian countries in the early 1980's. However, it was still looked on as 'black magic' by the mainstream medicoscientific world in the USA. The first Food and Drug Administration (FDA) approval for laser diode phototherapy was not granted till 2002, but even then the sceptics were not silenced.

LLLT WITH LASERS

LLLT was first completely limited to treatment with laser sources, such as the helium neon (HeNe) laser in the visible red at 632.8 nm, various semiconductor (diode) lasers (visible red to near infrared, most notable being the GaAlAs at 830 nm) or defocused beams of a surgical laser (Nd:YAG or CO2, for example).[3] There are several mechanisms which have been reported as to how LLLT can induce a biomodulative effect **(Table 1).** In the case of LLLT with laser sources, these effects were achieved athermally and atraumatically through the special properties associated with the 'coherence' of laser energy, namely monochromaticity, directionality or collimation, and the photons all in phase temporally and spatially. Another phenomenon associated only with laser energy is the so-called 'speckle' phenomenon. When the spot from a 670 nm laser pointer is closely examined over a period of time, for example, it appears to be composed of exceptionally brighter spots of light energy which are constantly in motion: these are laser speckles. Speckles have their own characteristics, including high energy and polarization, and these intense spots of polarized light were associated with specific reactions in the absorbing target or chromophore.

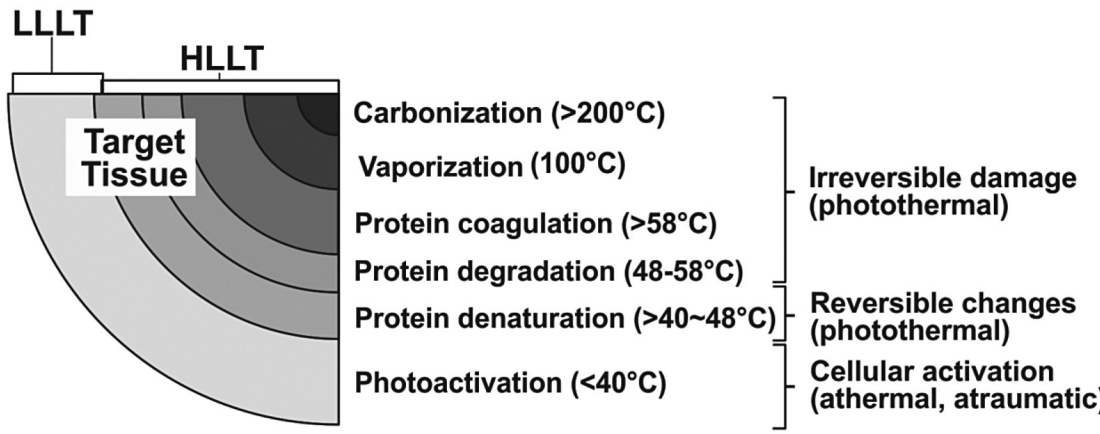

Fig. 1: Range of typical bioreactions associated with a surgical laser and their approximate temperature range. Note that some degree of photoactivation almost always occurs simultaneously with HLLT-mediated reactions. (Data adapted from Calderhead RG: Light/tissue interaction in photosurgery and phototherapy. In Calderhead RG. Photobiological Basics of Photosurgery and Phototherapy, 2011, Hanmi Medical Publishers, Seoul. pp 47–89)

Table 1: Major mechanisms associated with photobioactivation and LLLT

Mild thermal (<40°C)	Biochemical	Bioelectric	Bioenergetic
↑ Nerve conduction	(Mitochondrial events) ↑ ATP production ↑ Release of nitric oxide (NO) ↑ Very low levels of reactive oxygen species (ROS)	↑ Electromotive action on membrane bound ion transport mechanisms	↑ Rotational & vibrational changes to membrane molecule electrons
↑ Capillary dilatation	↑ Fibroblast proliferation → Collagen & elastin synthesis	↑ Intracellular extra-cellular ion gradient changes	↑ Stimulation of acupuncture meridian points
	↑ Mast cell degranulation: cytokine, chemokine and trophic factor release	↑ Depolarization of synaptic cleft → closure of synaptic gate	↑ Increased biophotonic activity
	↑ Macrophage activity (chemotaxis & internalization) → release of FGF	↑ Activation of the dorsal horn gate control mechanism → pain transmission slowed, pain control increased	
	↑ Keratinocyte activity cytokine release in epidermis and dermis		
	↑ Opiate and nonopiate pain control (endorphins, dynorphins and enkephalins)		
	↑ RNA/DNA synthesis		
	↑ Enzyme production		
	↑ Superoxide dismutase (SOD) production		

Up until the end of the 1990's, phototherapy was dominated by these laser sources, because although LEDs were cheap and cheerful, they were highly divergent with low and unstable output powers, and a wide waveband. With very few exceptions, old generation LEDs were incapable of producing really useful clinical reactions in tissue. It was easy to source a 'red' LED (output spread over approximately 600 – 700 nm) but it was more or less impossible to source LEDs at specific nominal wavelengths, for example 633 nm, similar to the HeNe laser.

LED PHOTOTHERAPY

Enter the NASA Light-Emitting Diode (LED)

All this changed in 1998 with the development of the so-called 'NASA LED' by Prof Harry Whelan and his group at the NASA Space Medicine Laboratory, which offered clinicians and researchers a useful phototherapy source having less divergence, much higher and more stable output powers, and quasimonochromaticity whereby nearly all of the photons were at the rated wavelength.[4] This new generation of LEDs also had its own phenomenon associated with photon intensity, namely photon interference, whereby intersecting beams of LED energy from individual LEDs produced photon interference, increasing the photon intensity dramatically and thus offering much higher photon intensities than the older generation. For LEDs emitting at visible red and near IR wavelengths, the greatest photon intensity was actually seen beneath the surface of the target tissue, due to the combination of the photon interference phenomenon and the excellent tissue scattering characteristics of light at these wavebands.[5] This phenomenon, together

with quasimonochromaticity, meant that the new generation of LEDs was a clinically viable source for phototherapy.[6] 'Low level laser therapy' was therefore renamed by the US photobiologist, Kendric C Smith, as 'low level light therapy', to encompass LED energy.[7] Accordingly, useful bioreactions could then be achieved with LEDs through cellular photoactivation without heat or damage, as shown by Whelan and colleagues in their early NASA LED wound healing studies.[8]

Although visible and near-infrared light energy induce the same tri-stage process in target cells, namely photon absorption, intracellular signal transduction and the final cellular photoresponse,[9] it should be noted that both wavebands have different primary targets and photoreactions in target cells. Visible light is principally a photochemical reaction, acting directly and mostly on cytochrome-c oxidase, the end terminal enzyme in the cellular mitochondrial respiratory chain,[10] and mainly responsible for inducing adenosine triphosphate (ATP) synthesis, the fuel of the cell and indeed the entire metabolism. Infrared light on the other hand induces a primary photophysical reaction in the cell membrane thereby

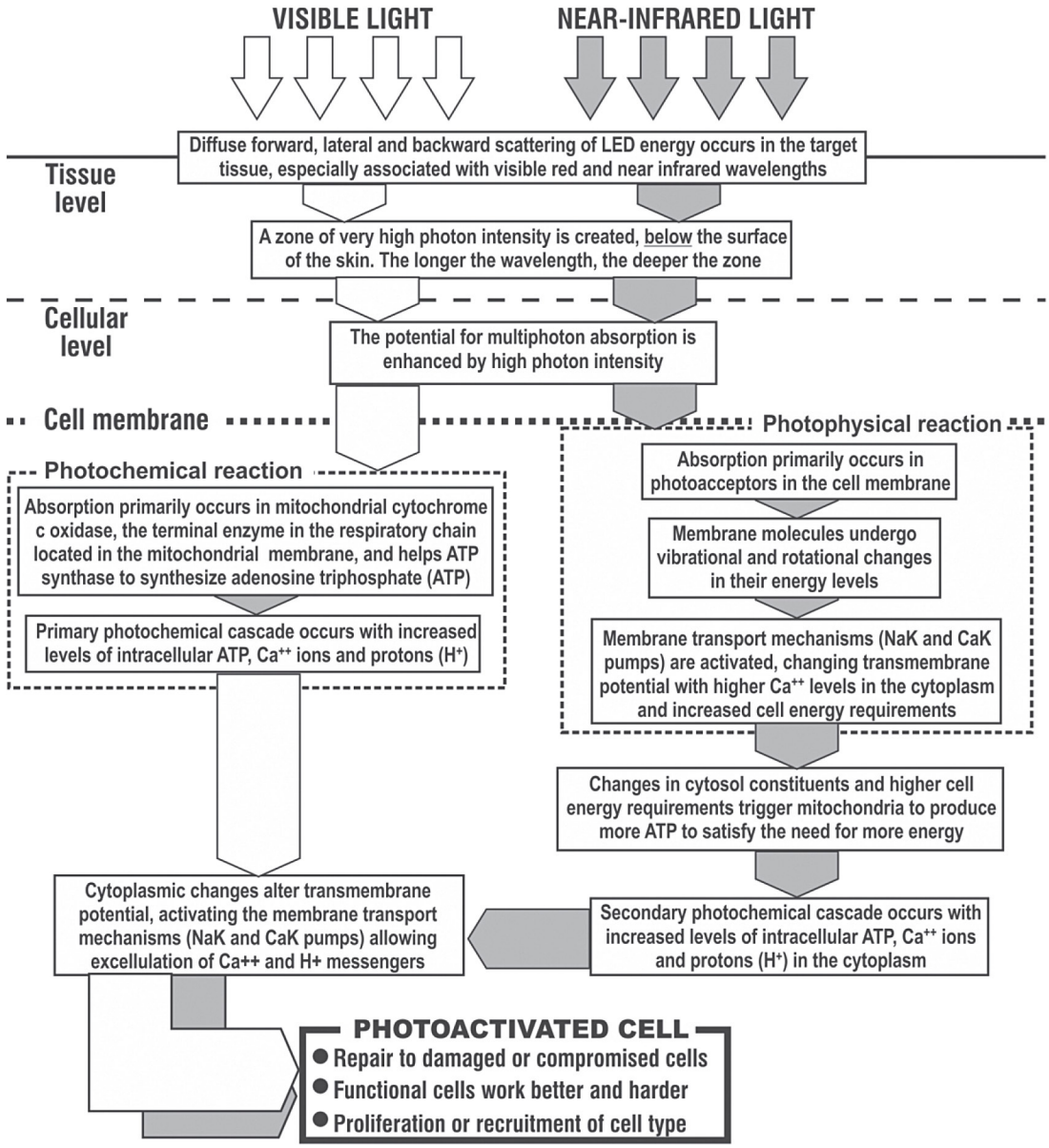

Fig. 2: The process of cellular photoactivation by low level light therapy (LLLT). Visible light induces a primary photochemical response particularly associated with mitochondrial cytochrome c-oxidase, whereas near IR induces a primary photophysical response in the cellular and organelle membranes. However the eventual photoresponse is the same. (Based on data from Karu & Smith, Refs 6 & 9)

kick-starting the cellular membrane transport mechanisms such as the Na++K++ pump,[6] and this in turn induces as a secondary reaction the same photochemical cascade as seen with visible light, so the end result is the same even though the target is different as illustrated schematically in **Figure 2.**

PHOTOTHERAPY IS BECOMING MAINSTREAM

The increasing number of papers on LLLT in the Photobiomodulation sessions presented at the 2010 and especially the 2011 meetings of the American Society for Lasers in Medicine and Surgery (ASLMS) bear witness to the fact that LLLT is no longer quite the bête noir it used to be in the USA, although there is still too much skepticism, and it has achieved a reliable status worldwide. LED phototherapy has now been well-proven to work, and is reported to be effective in a large variety of clinical indications such as pain attenuation, wound healing, skin rejuvenation, some viral diseases, allergic rhinitis, other allergy-related conditions and so on.

APPLICATIONS OF LLLT WITH LEDS

When we confirm in what fields LLLT phototherapy has been most used through a review of the literature, the main application is for pain control, with pain of almost all aetiologies responding well.[11] For example, 830 nm LED phototherapy significantly reduced both acute and chronic pain in professional athletes.[13] The first author has been using LED in the control of herpes zoster pain for some time, and also for intractable postherpetic neuralgia, corroborating previous studies with 830 nm LLLT for this indication.[14,15] This and other chronic pain entities have been historically very hard to control, but the good efficacy of LED phototherapy has been well recognized. From the large body of work from Rochkind and colleagues in Israel, LED phototherapy can help nerve regeneration, so it has been used for spinal cord injuries,[16] and many different types of neurogenic abnormality. In the case of the dental clinic and for the osseointegration of implants and prostheses in maxillofacial surgery it has been used for guided bone regeneration.[17] At present, the research into and development of new applications for LED phototherapy, especially in the processes of inflammatory cell regulation, are being assiduously studied in the dermatology field.

Fast taking over from pain attenuation, and particularly in the dermatology field, wound healing with LED phototherapy has attracted much attention. Reports have shown that, after making uniform burn wounds with a surgical laser, LED phototherapy of experimental wounds induces faster and better organized healing than in the control unirradiated wounds. This is due to the effect of 830 nm phototherapy on raising the action potential the wound-healing cells, at all three phases of the process, particularly mast cells,[18] macrophages[19] and neutrophils[20] in the inflammatory stage; fibroblasts in the proliferative phase (Personal Communication, Prof. Park, Seoul National University, Seoul, South Korea: unpublished data); and fibroblast-myofibroblast transformation in the remod-

Classification	Molecules	LLLT-Associated Biological Effects
Growth factors	BNF, GDNF, FGF, bFGF, IGF-1, KGF, PDGF, TGF-β, VEGF	Proliferation Differentiation Bone nodule formation
Interleukins	IL-1α, IL-2, IL-4, IL-6, IL-8	Proliferation Migration Immunological activation
Inflammatory cytokines	PGE2, COX2, IL1β, TNF-α	Acceleration/Inhibition of inflammation
Small molecules	ATP, cGMP, ROS, CA^{++}, NO, H^{+}	Normalization of cell function Pain relief Wound healing Mediation of cellular activities Migration Angiogenesis

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Table 2: Molecular level activation by LLLT with appropriate LEDs (From Ref 12)

eling phase.[21] As an additional mechanism, it has also been shown that 830 nm phototherapy increased the early vascular perfusion of axial pattern flaps in a controlled speckle flowmetry Doppler trial in the rat model, with actual flap survival significantly better in the irradiated than in the unirradiated control animals.[22]

In another very popular indication, studies have reported on the use of LED phototherapy for the rejuvenation of chronologically and photodamaged skin.[23,24] Lee and colleagues, in a randomized controlled study, showed that fibroblasts examined with transmission electron microscopy appeared more active, collagen and elastin synthesis was increased

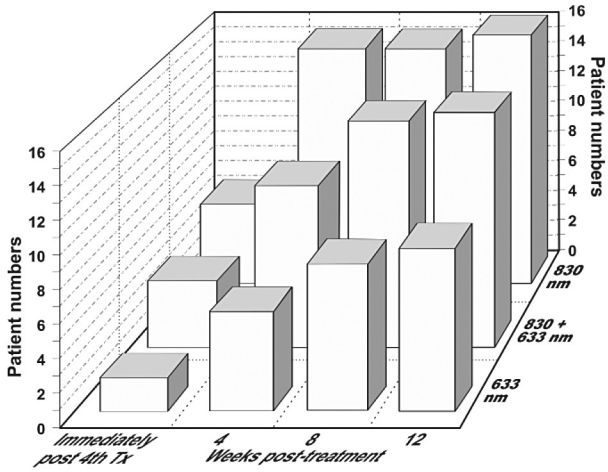

Fig. 3: Patient satisfaction curves compared for LED-mediated skin rejuvenation with 633 nm alone, 633 nm + 830 nm combined and 830 nm on its own, showing the numbers of patients who rated their improvement as excellent on a 5-scale rating. The first set of columns represents the findings immediately after the 8th of 8 weekly sessions, twice per week for 4 weeks. The 2nd, 3rd and 4th sets of columns are the findings at post-treatment weeks 4, 6 and 8 respectively. At all stages, LED phototherapy with 830 nm produced superior satisfaction. The increase over the post-treatment period is interesting, suggesting improved results through continued tissue remodeling as part of the LED-mediate wound healing process. (Data adapted from Ref 24)

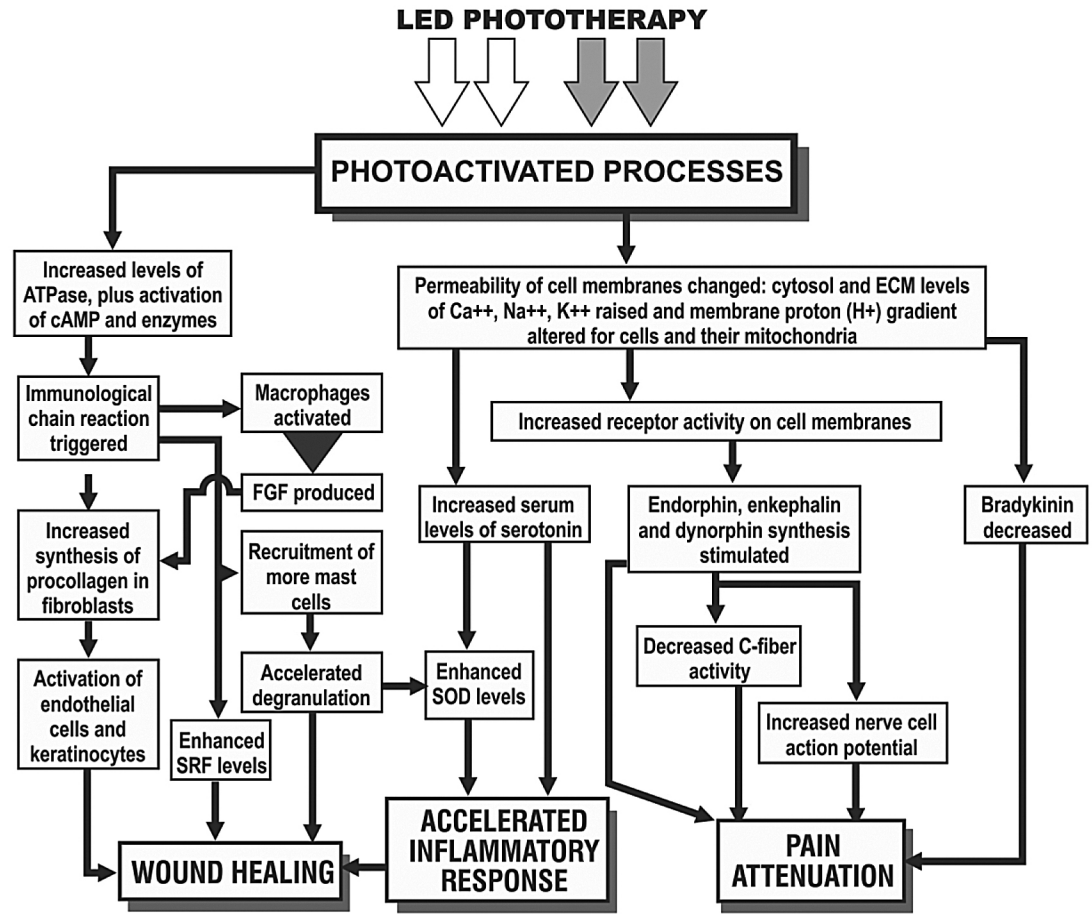

Fig. 4: Mechanisms underlying the three main LLLT endpoints, particularly associated with the wavelength of 830 nm, although 633 nm has beneficial effects as well.

and tissue inhibitors of matric metalloproteinases was increased, as a result of which, effective rejuvenation could be achieved which was maintained up to 12 weeks after the final treatment session. Patient satisfaction scores bore these histopathological findings out **(Figure 3).**[24] We must never forget that good skin rejuvenation is firmly based on the wound healing process, particularly neocollagenesis. LED phototherapy has also been reported as being very effective in the prophylaxis against scar formation, due amongst other factors to the response to photomediated interleukin-6 signaling.[12] Hair loss is another field where LED phototherapy may well have real efficacy, with red and infrared being the wavelengths of choice.[25–27] **Figure 4** illustrates schematically the mechanisms already confirmed underlying the three main endpoints of 830 nm LLLT, namely wound healing, the anti-inflammatory response through acceleration and quenching of the post-wound inflammatory phase and pain attenuation.

SYSTEMIC EFFECTS OF LED-LLLT

One of the advantages of LLLT with an LED system as compared with a laser source is that LED-based systems offer large planar arrays, so that they can irradiate a large area of the body in a hands-free manner, compared with the point-by-point application of a laser system. In addition, many different cell types can be simultaneously targeted. It may not even be necessary to irradiate every target area. The systemic effect of LED with an 830 nm system (HeaLite II, Lutronic Corp., Goyang, S. Korea, **Figure 5**) was studied by the first author.[28] The systemic effect associated with LLLT has already been suggested as far back as Mester's pivotal study on non-healing ulcers in 1969, whereby irradiation of one part of the body could induce effects in another unirradiated area.[29] To assess this, in the first author's study controlled wounds on the backs of rodents were created with an ablative fractional laser, and rather than irradiating the laser wounds with LED energy (HeaLite system as above), the animals' abdomens in the experimental group were irradiated, and sham irradiation was delivered to the control group. The results clearly indicated that the group which had LED treatment of the abdomen demonstrated significantly better healing than the control group **(Figure 6).** This means that LED phototherapy could very probably have a systemic effect on inflammatory or immune cells in nonadjacent tissues to the target area, as well as those cells in the irradiated tissues.

LED LLLT FOR SKIN INFLAMMATORY DISEASES

The anti-inflammatory effect of LED has been generally accepted, but up till now this has not been well shown well in inflammatory skin diseases such as allergic or irritant contact dermatitis, atopic

Fig. 5: HeaLite II LED phototherapy system, Lutronic Corp, Goyang, South Korea.

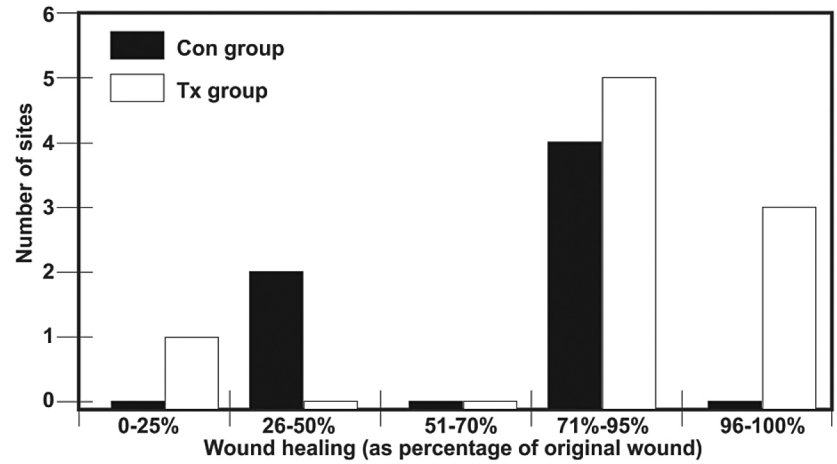

Fig. 6: The wound healing value compared between the group treated with 830 nm LED (Tx group) with LED and the unirradiated Con group without LED. Note that the 1 LED-irradiated animal in the 0–25% group had somehow removed the wound dressing very early in the experiment. (Adapted from Ref 28)

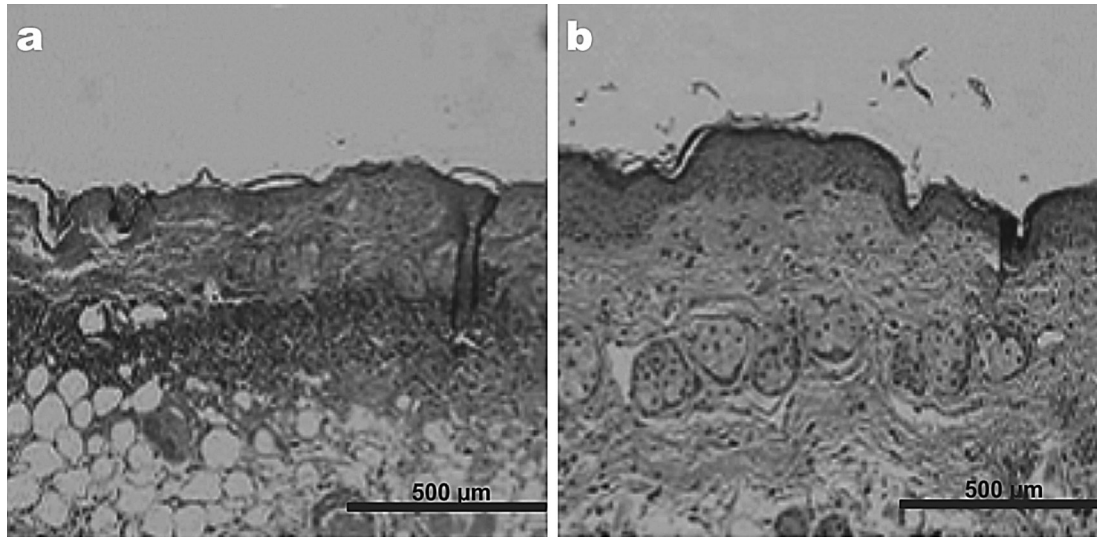

Fig. 7: The changes in dermatitis-associated inflammatory cells following 830 nm LED irradiation in the rat model (A: Control specimen, B: LED irradiated specimen). A marked reduction in inflammatory infiltration is evident.

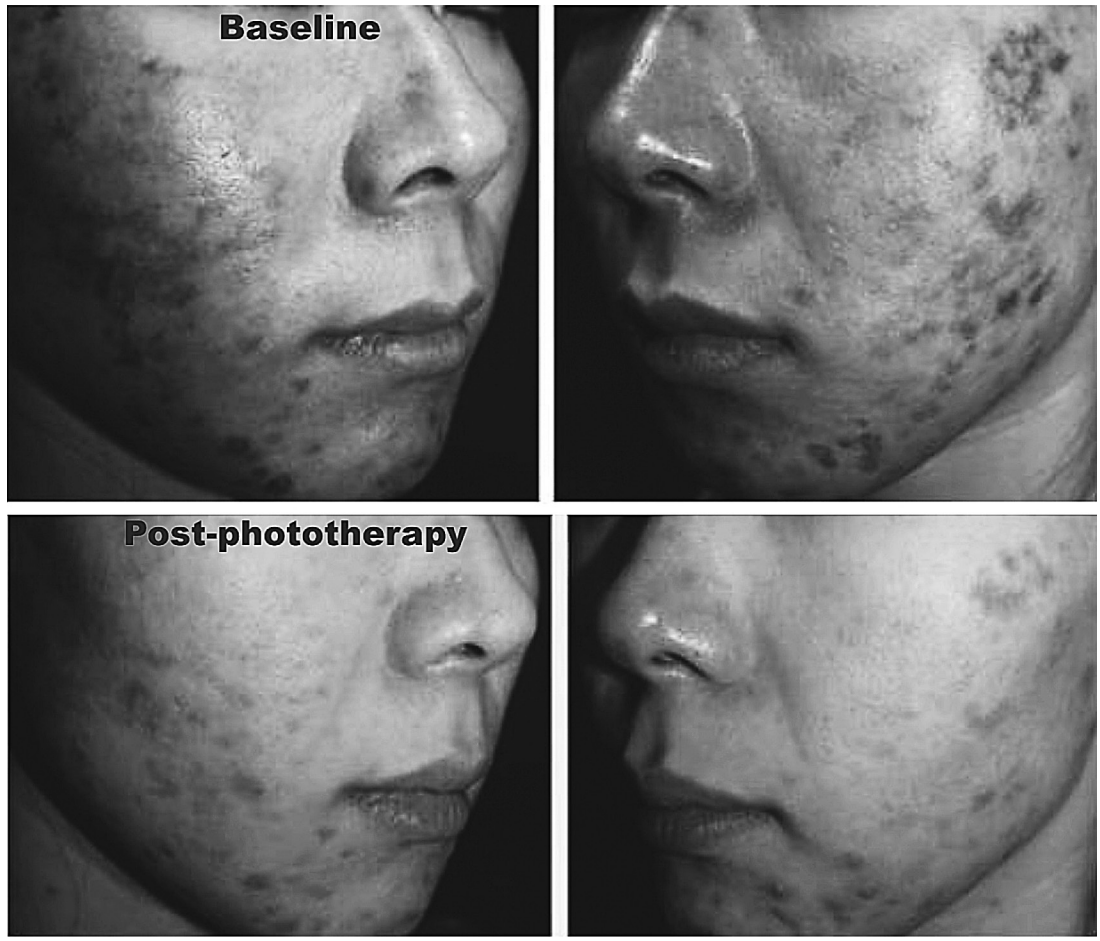

Fig. 8: Improvement in a patient (24-year-old female) with treatment-resistant post-chemical peel irritant contact dermatitis (AHA-related ICD) seen above at baseline, and below 10 days later following 3 830 nm LED treatment sessions, 3 days apart, 20 minutes per session (60 J/cm^2)

dermatitis or rosacea, although a significant degree of success has been demonstrated and reported for inflammatory acne and recalcitrant treatment-resistant psoriasis.[30,31] In an experimental animal model study the first author was able to demonstrate that when induced dermatitis in rats was treated with 830 nm LED phototherapy (HeaLite II system, Lutronic Corp, as above) at a dose of 60 J/cm^2 in continuous wave, compared with an untreated control group, the histopathological findings revealed significant decreased levels of inflammatory cells (**Figure 7).** Based on the success of that study, treatment-resistant inflammatory contact dermatitis due to a peel compound containing alpha-hydroxy acid (AHA) in a human subject also responded very well to 3 sessions of 830 nm LED therapy, 3 days apart, irradiance of 100 mW/cm^2, 10 min/session, dose of 60 J/cm^2, continuous wave **(Figure 8).**

CONCLUSIONS

In conclusion, based on the published data and the authors' own experience, LED phototherapy is proving to have more and more viable applications in many fields of medicine. However, it must always be remembered that not any old LED will do. In order to be effective, LED phototherapy must satisfy the following 3 criteria.

• The LED system being used must have first of all, and most importantly, the correct wavelength for the target cells or chromophores. At present, the published literature strongly suggests 830 nm for all aspects of wound healing, pain, anti-inflammatory treatment and skin rejuvenation, with a combination of 415 nm and 633 nm for light-only treatment of active inflammatory acne vulgaris. If the wavelength is incorrect, optimum absorption will not occur and

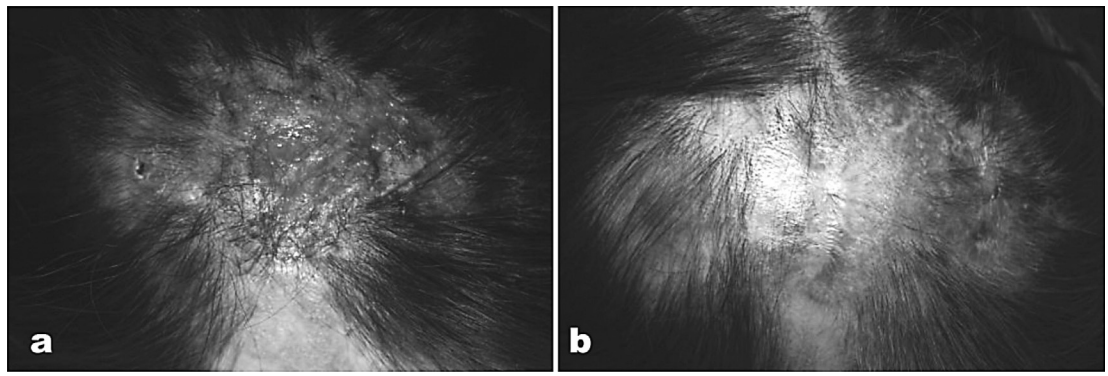

Fig. 9: Dramatic improvement in a case of dissecting cellulitis of the scalp (34-year-old male) (a) at baseline and (b) following 830 nm LED treatment (twice per week for 2 weeks, 20 min per session to give 60 J/cm^2)

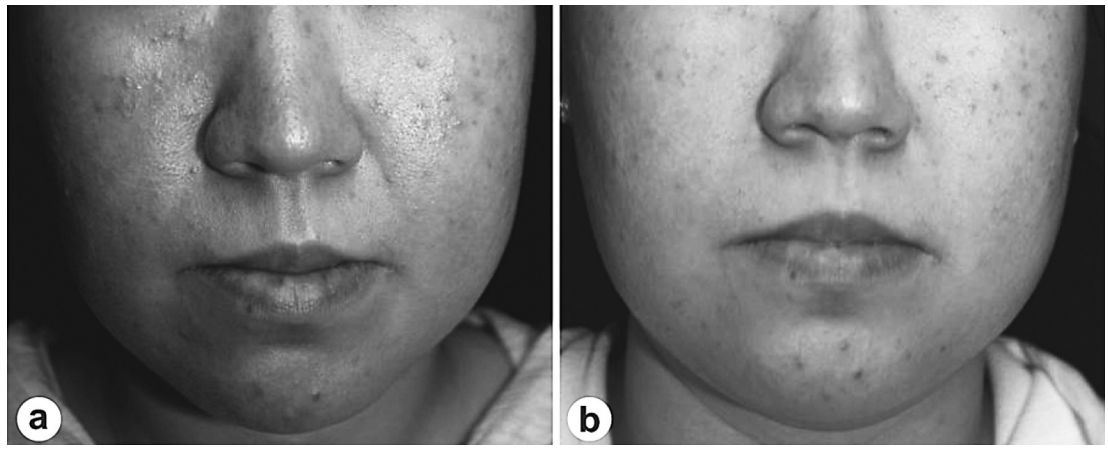

Fig. 10: Improvement of acne rosacea (33-year-old female) at baseline (a) and following LED treatments (once per every week for 6 weeks, 20 min and 60 J/cm^2 per session) (b). Although not very well noted in the grayscale illustrations, the small acneiform papules have disappeared, with a clear decrease seen in the redness on both cheeks.

as the first law of photobiology states, the Grotthus-Draper law, without absorption there can be no reaction.

- Secondly, the photon intensity, i.e., spectral irradiance or power density (W/cm^2), must be adequate, or once again absorption of the photons will not be sufficient to achieve the desired result. If the intensity is too high, however, the photon energy will be transformed to excessive heat in the target tissue, and that is undesirable.
- Finally, the dose or fluence must also be adequate (J/cm^2), but if the power density is too low, then prolonging the irradiation time to achieve the ideal energy density or dose will most likely not give an adequate final result, because the Bunsen-Roscoe law of reciprocity, the 2nd law of photobiology, does not hold true for low incident power densities.

Provided these three criteria are met, LED phototherapy does indeed work, and has many useful aspects in clinical practice for practitioners in many surgical specialities. As an exciting extension of the monotherapy approach with LED-LLLT, and even more importantly, the combination of appropriate LED phototherapy as an adjunct to many other surgical or nonsurgical approaches where the architecture of the patient's skin has been altered will almost certainly provide the clinician with even better results with less patient downtime, in a shorter healing period, and with excellent prophylaxis against obtrusive scar formation.

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LIGHT-EMITTING DIODE THERAPY AND LOW-LEVEL LIGHT THERAPY ARE PHOTOBIOMODULATION THERAPY

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GUEST EDITORIAL

In 2015, we published an editorial entitled *"Low-Level Light/Laser Therapy Versus Photobiomodulation Therapy."*[1] In that editorial, the major milestones that led to the development of the photonic specialty of photobiomodulation (PBM) and its medical application, PBM therapy (PBMT), were reviewed and the terms were defined. The current definitions for these terms are as follows: PBM is the mechanism by which nonionizing optical radiation in the visible and near-infrared spectral range is absorbed by endogenous chromophores to elicit photophysical and photochemical events at various biological scales without eliciting thermal damage: and PBMT is a photon therapy based on the principles of PBM. It involves the use of nonionizing forms of light from sources, including lasers, light-emitting diodes (LEDs), and broadband light, in the visible and near-infrared spectrum, to cause physiological changes and therapeutic benefits. These terms and their definitions are accurate and specific.

Also discussed was the excess of terms and "dismal lack of consistency and consensus on terminology"[1] for this field. Historically, the most frequently used term was low-level laser therapy (LLLT). However, we noted that this term is ambiguous since "low" and "level" are vague and not accurately definable, and the word "laser" is no longer appropriate, as other types or light devices such as LEDs and broadband light sources are currently used for this application.[2] We concluded that the universal use of PBM and PBMT would reduce confusion in the field and in the literature.

The editorial was well received and according to Scopus, has 66 citations since publication and a Citation Benchmark of 99th percentile.[3] Since its publication, several important milestones were reached regarding the use of the terms PBM and PBMT. The term PBMT was added in 2016 to the Medical Subject Headings (MeSH) terms contained in the National Library of Medicine's controlled thesaurus as an entry term to the existing record of laser therapy, low level.[4] The Optical Society (OA) added a Photobiomodulation Technical Group to their Bio-Medical Optics Division.[5] NAALT and WALT have officially changed their respective names from the North American or World Associations for Laser Therapy to the North American or World Association of Photobiomodulation Therapy. Several laser and LED societies adopted or included PBM and PBMT in the title of their recent annual conferences: the title of the 2018 NAALT conference was *Photobiomodulation-Drug Free Management of Pain:* the 2018 World Association of Laser Therapy (WALT) conference was titled *The World Convention for Photobiomodulation (Low Level Laser Therapy):* and the title or the European LED Academy's 2018 conference was *Advanced Age and Photobiomodulation.* In an editorial published in the August 2018 issue of *Photomedicine and Laser Surgery,* the new Editor-in-Chief, Dr. Hamblin, announced that the journal will be renamed *Photobiomodulation, Photomedicine, and Laser Surgery* and would be the "go to venue to publish PBM articles."[6]

Although much progress has been made in the universal acceptance or the terms PBM and PBMT, there has been a disturbing recent trend to introduce and use other terms such as LED therapy, LED light therapy,. laser PBMT, and LED PBMT with the implication that these are different and have different clinical effects or benefits.[7] The term LED therapy is often associated with dermatological applications, such as acne treatment and skin rejuvenation.[8,9] Manufacturers of these dermatological devices also imply that these are different from other PBMT devices. Such implications are not based on scientific facts. The definition of PBMT had been purposely developed to be inclusive for all nonionizing light sources, including lasers, LEDs, and broadband light. Therefore, LEDs, lasers, and broadband light sources that emit wavelengths in the near infrared (IR) and visible range have comparable and effective treatment dosages at the target tissue, and cause clinical effects by the same mechanistic basis as PBMT devices.

Recently, a guest editorial was published in *Photomedicine and Laser Surgery*[10] entitled *"Low Level Laser Therapy and World Association for Laser Therapy Dosage Recommendations in Musculoskeletal Disorders and Injuries,"* This editorial has caused confusion within the field of PBM given the persistent use or the term LLLT

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MEASUREMENT OF PHYSICAL PARAMETERS AND DEVELOPMENT OF A LIGHT EMITTING DIODES DEVICE FOR THERAPEUTIC USE

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ABSTRACT

ABSTRACT

Effectiveness of light-emitting diode (LED) in biological tissue is due to the correct application of physical parameters. However, most studies found do not provide complete information on the physical characteristics of the diodes. It is necessary to carefully evaluate the diode parameters so that the results of research with this feature can be reproduced. The objective of this study was to develop a light-emitting device using LED, with proper measurements for application in clinical research. It was used 267 LEDs, powered with 12-V voltage and fixed on a plate of ethylene-vinyl acetate (25 × 42 cm), equidistant at 1.0 cm. For the calculation of red and infrared irradiation, a spectrometer was used, and the data were processed in routines implemented in the OriginPro 8.5.0 SR1 Software. The irradiance was determined by the integration of the spectral irradiation in the LED emission region. The red LED has a wavelength of 620 ± 10 nm, a power density of 52.86 mW/cm^2, power of 6.6 mW, and total power of 1.76 W on the device. The infrared LED has a wavelength of 940 ± 10 nm, power density 33.7 mW/cm^2, power of 6 mW, and total power of 1.6 W on the device. The LED characterization enables the generation and application of energy with greater precision and reproducibility. Besides, it is a light source, a device capable of framing large areas, reducing the time and cost of the application in different clinical conditions related to neuromuscular performance or rehabilitation.

KEYWORDS:

Devices; Light-emitting diodes; Low-level light therapy; Radiation.

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MECHANISMS AND APPLICATIONS OF THE ANTI-INFLAMMATORY EFFECTS OF PHOTOBIOMODULATION

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CASE REPORT

ABSTRACT:

Photobiomodulation (PBM) also known as low-level level laser therapy is the use of red and near-infrared light to stimulate healing, relieve pain, and reduce inflammation. The primary chromophores have been identified as cytochrome c oxidase in mitochondria, and calcium ion channels (possibly mediated by light absorption by opsins). Secondary effects of photon absorption include increases in ATP, a brief burst of reactive oxygen species, an increase in nitric oxide, and modulation of calcium levels. Tertiary effects include activation of a wide range of transcription factors leading to improved cell survival, increased proliferation and migration, and new protein synthesis. There is a pronounced biphasic dose response whereby low levels of light have stimulating effects, while high levels of light have inhibitory effects. It has been found that PBM can produce ROS in normal cells, but when used in oxidatively stressed cells or in animal models of disease, ROS levels are lowered. PBM is able to up-regulate anti-oxidant defenses and reduce oxidative stress. It was shown that PBM can activate NF-kB in normal quiescent cells, however in activated inflammatory cells, inflammatory markers were decreased. One of the most reproducible effects of PBM is an overall reduction in inflammation, which is particularly important for disorders of the joints, traumatic injuries, lung disorders, and in the brain. PBM has been shown to reduce markers of M1 phenotype in activated macrophages. Many reports have shown reductions in reactive nitrogen species and prostaglandins in various animal models. PBM can reduce inflammation in the brain, abdominal fat, wounds, lungs, spinal cord.

KEYWORDS:

Photobiomodulation, low-level laser therapy, chromophores, inflammation, animal studies, clinical trials

1. INTRODUCTION

Photobiomodulation (PBM) was discovered almost 50 years ago by Endre Mester in Hungary [1]. For most of this time PBM was known as “low-level laser therapy” as ruby laser (694 nm) and HeNe lasers (633 nm) were the first devices used. Recently a consensus decision [2] was taken to use the terminology “PBM” since the term “low-level” was very subjective, and it is now known that actual lasers are not required, as non-coherent light-emitting diodes (LEDs) work equally well [3]. For much of this time the mechanism of action of PBM was unclear, but in recent years much progress has been made in elucidating chromophores and signaling pathways [4].

Most of the early work in this field was carried out with various kinds of lasers, and it was thought that laser light had some special characteristics not possessed by light from other light sources such as sunlight, fluorescent or incandescent lamps and now LEDs. However all the studies that have been done comparing lasers to equivalent light sources with similar wavelength and power density of their emission, have found essentially no difference between them.

Many wavelengths in the red (600–700 nm) and near-infrared (NIR, 770–1200 nm) spectral regions have shown positive results, however there is a region in between (700–770 nm) where broadly speaking, the results are likely to be disappointing. Recently blue and green wavelengths have also begun to be explored [5] but they have major problems with penetration depth. It is accepted that penetration of light into tissue is governed by both absorption and scattering by molecules and structures present in tissue. Both absorption and scattering become significantly less as the wavelength gets longer, so the penetration depth of NIR is maximal about 810 nm, and at longer wavelengths water becomes an important absorber and penetration depth gets shorter again [6].

The "biphasic dose response" describes a situation in which there is an optimum value of the "dose" of PBM most often defined by the energy density (J/cm^2) [7,8]. It has been consistently found that when the dose of PBM is increased a maximum response is reached at some value, and if the dose in increased beyond that maximal value, the response diminishes, disappears and it is even possible that negative or inhibitory effects are produced at very high fluences.

2. CHROMOPHORES RESPONSIBLE FOR PHOTOBIOMODULATION

2.1. Cytochrome c oxidase in mitochondria

Cytochrome c oxidase (CCO) is unit IV in the mitochondrial electron transport chain. It transfers one electron (from each of four cytochrome c molecules), to a single oxygen molecule, producing two molecules of water. At the same time the four protons required, are translocated across the mitochondrial membrane, producing a proton gradient that the ATP synthase enzyme needs to synthesize ATP. CCO has two heme centers (a and a3) and two copper centers (CuA and CuB). Each of these metal centers can exist in an oxidized or a reduced state, and these have different absorption spectra, meaning CCO can absorb light well into the NIR region (up to 950 nm) [9]. Tiina Karu from Russia was the first to suggest [10,11], that the action spectrum of PBM effects matched the absorption spectrum of CCO, and this observation was confirmed by Wong-Riley et al in Wisconsin [12]. The assumption that CCO is a main target of PBM also explains the wide use of red/NIR wavelengths as these longer wavelengths have much better tissue penetration than say blue or green light which are better absorbed by hemoglobin. The most popular theory to explain exactly why photon absorption by CCO could led to increase of the enzyme activity, increased oxygen consumption, and increased ATP production is based on photodissociation of inhibitory nitric oxide (NO) [13]. Since NO is non-covalently bound to the heme and Cu centers and competitively blocks oxygen at a ratio of 1:10, a relatively low energy photon can kick out the NO and allow a lot of respiration to take place [14].

2.2. Light gated ion channels and opsins

More recently it has become apparent that another class of photoreceptors, must be involved in transducing cellular signals, particularly responding to blue and green light. Thee photoreceptors have been proposed to be members of the family of light-sensitive G-protein coupled receptors known as opsins (OPN). Opsins function by photoisomerization of a cis-retinal co-factor leading to a conformational change in the protein. The most well known opsin is rhodopsin (OPN1), which is responsible for mediating vision in the rod and cone photoreceptor cells in the mammalian retina. There are other members of the opsin family (OPN2-5), which are expressed in many other tissues of the body including the brain [15]. One of the best-defined signaling events that occurs after light-activation of opsins, is the opening of light-gated ion channels such as members of the transient receptor potential (TRP) family of calcium channels [16]. TRP channels are now known to be pleiotropic cellular sensors mediating the response to a wide range of external stimuli (heat, cold, pressure, taste, smell), and involved in many different cellular processes [17]. Activation of TRP causes non-selective permeabilization (mainly of the plasma membrane) to calcium, sodium and magnesium [18]. It is now known that TRP channel proteins are conserved throughout evolution and are found in most organisms, tissues, and cell-types. The TRP channel superfamily is now classified into seven related subfamilies: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, and TRPN [19]. Light-sensitive ion channels are based on an opsin chromophore (isomerization of a cis-retinal molecule to the trans configuration) as illustrated in Drusophila photoreceptors [20].

We have shown that blue or green light (but not red or 810 nm NIR) increased intracellular calcium in adipose derived stem cells, that could be blocked by ion channel inhibitors [5].

2.3. Flavins and flavoproteins

There is another well-known family of biological chromophores called cryptochromes. These proteins have some sequence similarity to photolyases [21], which are blue light responsive enzymes that repair DNA damage in bacteria caused by UV exposure [22]. Cryptochromes rely on a flavin (flavin adenine dinucleotide, FAD) or a pterin (5,10-methenyltetrahydrofolic acid) to actually absorb the light (again usually blue or green). Cryptochromes have been studied mainly in plants and insects. Recent evidence has emerged that mammalian cryptochromes are important in regulation of the circadian clock. It is thought that human cryptochromes (CRY1 and CRY2) send signals via part of the optic nerve to the suprachiasmatic nucleus (SCN) in the brain, which is the master regulator of the CLOCK system to entrain biological responses to the light-dark cycle [23]. However the situation is complicated because retinal ganglion cells containing melanopsin (OPN4) are also involved in photoentrainment [24]. Studies are still ongoing to investigate this redundancy [25].

It should be emphasized that compared to CCO and mitochondria, evidence is still emerging concerning the extent to which opsins, cryptochomes and light-gated ion channels (which may be widely expressed in many different cell types) could be responsible for PBM effects. If their role is significant it is likely to be in the blue and green spectral regions. Further research will be necessary to explore their role in anti-inflammatory effects, wound healing and tissue regeneration.

2.4. Water as a chromophore and heat-gated ion channels

Since the biological effects of light continue to be observed, as the wavelength increases in the infra-red region (>1000 nm), beyond those known to be absorbed by CCO, it is now thought likely that an alternative chromophore must be responsible. The obvious candidate for this alternative chromophore is water molecules whose absorption spectrum has peaks at 980 nm, and also at most wavelengths longer than 1200 nm. Moreover, water is by the far the most prevalent molecule in biological tissue (particularly considering its low molecule weight = 18). At present the proposed mechanism involves selective absorption of IR photons by structured water layers (also known as interfacial water) [26] or water clusters [27], at power levels that are insufficient to cause any detectable bulk-heating of the tissue. A small increase in vibrational energy by a water cluster formed in or on a sensitive protein such as a heat-gated ion channel, could be sufficient

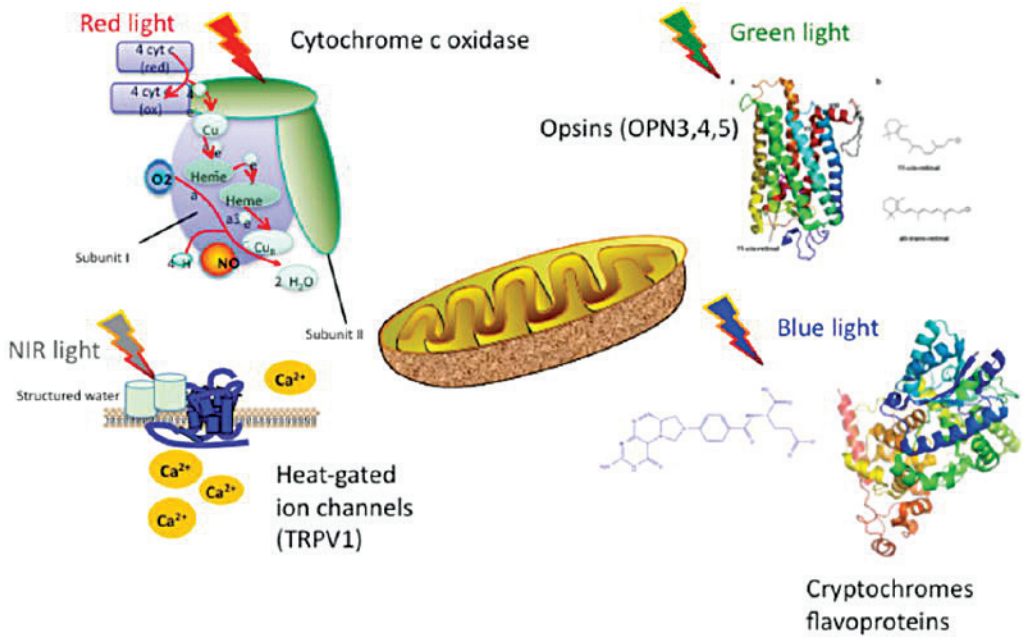

Figure 1. Chromophores in PBM. Cytochrome c oxidase in respiratory chain absorbs mainly red (and NIR) light by heme and copper; Heat-gated TRP ion channels absorb NIR (and blue light) via structured water; opsins absorb mainly blue/green light via cis-retinal; flavoproteins and cryptochromes absorb mainly blue light via pterin.

to perturb the tertiary protein structure thus opening the channel and allowing modulation of intracellular calcium levels [28]. Pollack has shown that interfacial water can undergo charge separation when it absorbs visible or NIR light [29]. This charge separation (equivalent to localized pH changes) could affect the conformation of proteins [30]. It has also been suggested that PBM could reduce the viscosity of interfacial water within the mitochondria, and allow the F0F1 ATP synthase, which rotates as a nanomotor to turn faster [31]. It should be noted here that the first regulatory approvals of PBM were gained as a 510 K device "equivalent to an non-heating IR lamp" [32]. While the involvement of water as a chromophore may still be considered hypothetical it is difficult to think of another explanation for the beneficial of PBM at wavelengths between 1000 nm all the way to 10,000 nm (carbon dioxide laser).

The molecular chromophores discussed above are graphically summarized in Figure 1.

3. EFFECTS OF PBM ON REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

3.1. PBM increases ROS in normal cells

When PBM stimulates CCO activity in normal healthy cells, the resulting increase in mitochondrial membrane potential (MMP) above normal baseline levels, leads to a brief and rather modest increase in generation of reactive oxygen species (ROS) [33]. However this brief burst of ROS caused by 3 J/cm^2 of 810 nm laser (Figure 2A) was shown to be sufficient to activate the redox-sensitive transcription factor, NF-kB in embryonic fibroblasts [34] (Figure 2B). Addition of the anti-oxidant N-acetyl-cysteine to the cells could block the NK-kB activation (Figure 2C), but not the increase in cellular ATP caused by the mitochondrial stimulation (Figure 2D). In primary cultured cortical neurons [35], 810 nm laser produced a biphasic dose response in ATP production (Figure 3A) and MMP (Figure 3B) with a maximum at 3 J/cm^2. At a high dose (30 J/cm^2) the MMP was actually lowered below baseline. Interestingly the dose-response curve between fluence (J/cm^2) and ROS production showed two different maxima (Figure 3C). One of these maxima occurred at 3 J/cm^2 where the MMP showed its maximum increase. The second maximum in ROS production occurred at 30 J/cm^2 where the MMP had been reduced below baseline. At a value between these two fluences (10 J/cm^2) a dose at which the MMP was approximately back to baseline, there was not much ROS generation. These data are very good examples of the "biphasic dose response" or "Arndt-Schulz curve" which is often discussed in the PBM literature [7,8].

Thus it appears that ROS can be generated within mitochondria when the MMP is increased above normal values and also when it is decreased below normal values. It remains to be seen whether these two kinds of PBM-generated ROS are identical or not. One intriguing possibility is that whether the ROS generated by PBM is beneficial or detrimental may depend on the rate at which it is generated. If superoxide is generated in mitochondria at a rate that allows superoxide dismutase (SOD) to detoxify it to hydrogen peroxide, then the uncharged H_2O_2 can diffuse out of the mitochondria to activate beneficial signaling pathways, while if superoxide is generated at a rate or at levels beyond the ability of SOD to deal with it, then the charged superoxide may build up inside mitochondria and damage them.

3.2. PBM reduces ROS in oxidative stressed cells and tissues

Notwithstanding, the ability of PBM to produce a burst of ROS in normal cells, it is well-accepted that PBM when as a treatment for tissue injury or muscle damage is able to reduce markers of oxidative stress [36,37,38]. How can these apparently contradictory findings be reconciled? A study attempted to answer this question [39]. Primary cultured cortical neurons were treated with one of three different interventions, all of which were chosen from literature methods of artificially inducing oxidative stress in cell culture. The first was cobalt chloride ($CoCl_2$), which is used as a mimetic for hypoxia and works by a Fenton reaction producing hydroxyl radicals [40]. The second was direct treatment with hydrogen peroxide. The third was treatment with the mitochondrial complex I inhibitor, rotenone [41]. All three of these different treatments increased the intracellular mitochondrial ROS as judged by Cell-Rox Red (Figure 4A), and at the same time lowered the MMP as measured by tetramethyl-rhodamine methyl ester (TMRM) (Figure 4B). PBM (3 J/cm^2 of 810 nm laser) raised the MMP back towards baseline, while simultaneously reducing the generation of ROS in oxidatively stressed cells (while slightly increasing ROS in normal cells). In control cells (no oxidative

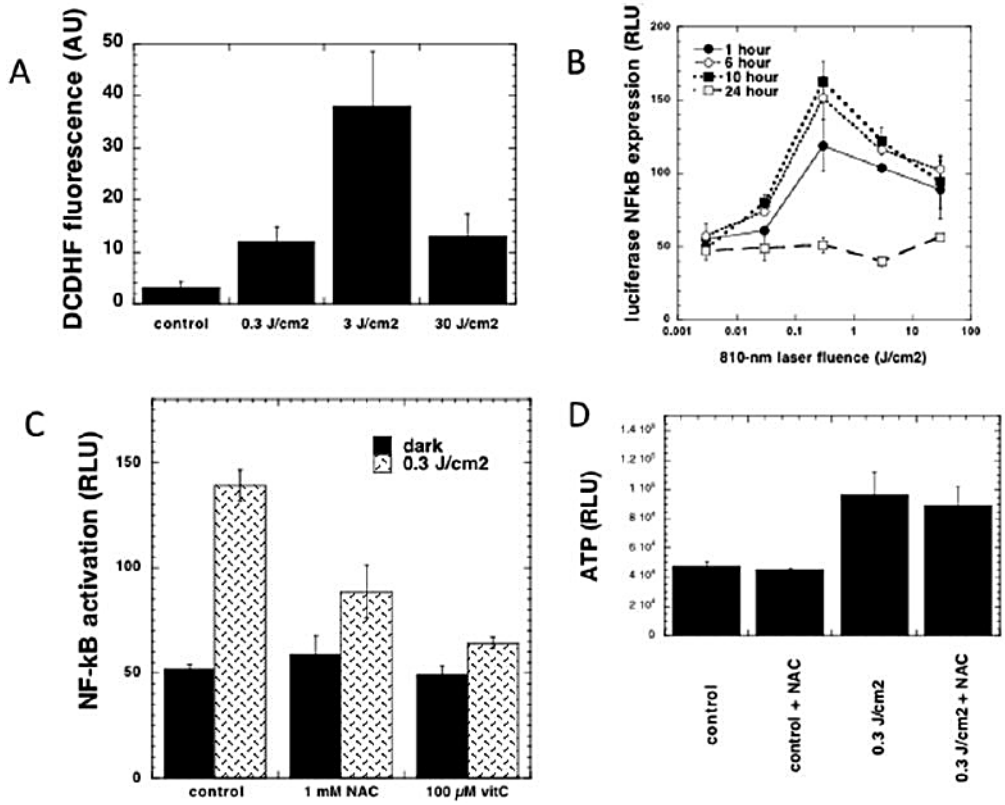

Figure 2. NFkB is activated by PBM induced ROS in embryonic fibroblasts. (A) Intracellular ROS measured by DCDHF fluorescence; (B) NF-kB activation measured by a luciferase assay; (C) NF-kB activation is inhibited by antioxidants; (D) ATP increase is not affected by antioxidants. Figure adapted from data in [34].

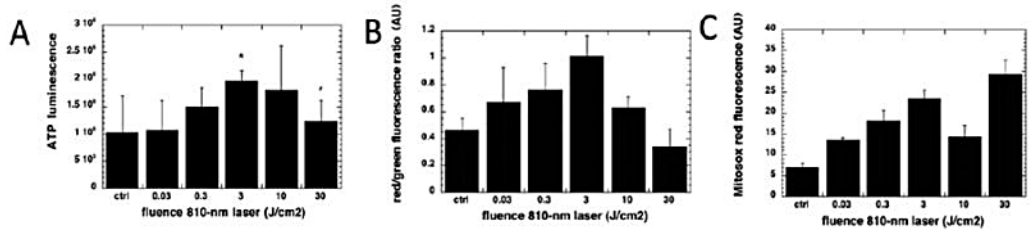

Figure 3. Dose response of 810 nm laser in cortical neurons. (A) ATP production as a function of fluence; (B) Mitochondrial membrane potential (JC1 red/green ratio); (C) Mitochondrial ROS. Figure adapted from data in [35].

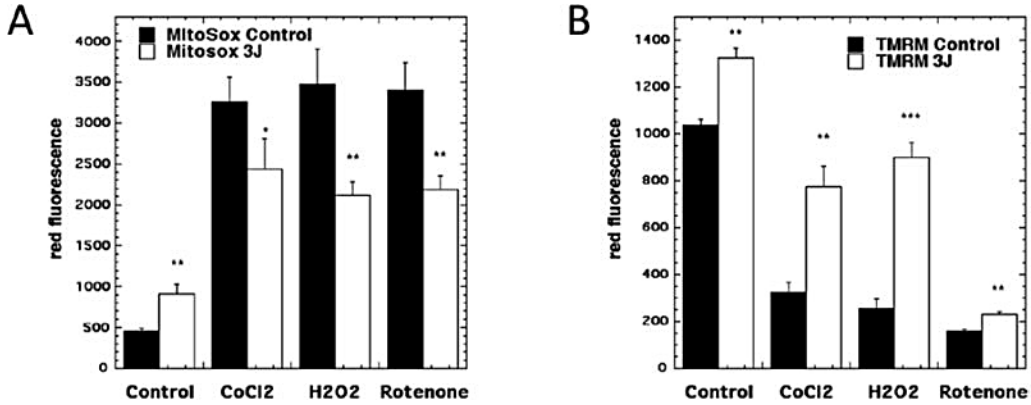

Figure 4. PBM reduces oxidative stress in cortical neurons. Oxidative stress was induced by three different treatments (cobalt chloride, hydrogen peroxide, rotenone) and cells were treated with 3 J/cm^2 810 nm. (A) Mitochondrial ROS, (B) Mitochondrial membrane potential (tetramethylrhodamine methyl ester). Figure adapted from data in [39].

stress), PBM increased MMP above baseline and still produced a modest increase in ROS.

Since most laboratory studies of PBM as a therapy have looked at various animal models of disease or injury, it is not surprising that most workers have measured reduction in tissue markers of oxidative stress (TBARS) after PBM [36,42]. There have been a lot of studies looking at muscles. In humans, especially in athletes, high-level exercise produces effects in muscles characterized by delayed-onset muscle soreness, markers of muscle damage (creatine kinase), inflammation and oxidative stress.

One cellular study by Macedo et al [43] used muscle cells isolated from muscular dystrophy mice (mdx LA 24) and found that 5 J/cm^2 of 830 nm increased the expression levels of myosin heavy chain, and intracellular [Ca^{2+}]i. PBM decreased H_2O_2 production and 4-HNE levels and also GSH levels and GR and SOD activities. The mdx cells showed significant increase in the TNF-α and NFκB levels, which were reduced by PBM.

While it is highly likely that the effects of PBM in modulating ROS are involved in the anti-inflammatory effects of PBM, it would be dangerous to conclude that that is the only explanation. Other signaling pathways (nitric oxide, cyclic AMP, calcium) are also likely to be involved in reduction of inflammation.

4. EFFECTS OF PBM ON NF-KB

4.1. PBM activates NF-kB in normal cells

As mentioned above we found [34] that PBM (3 J/cm^2 of 810 nm laser) activated NF-kB in embryonic fibroblasts isolated from mice that had been genetically engineered to express firefly luciferase under control of an NF-kB promoter. Although it is well-known that NF-kB functions as a pro-inflammatory transcription factor, but on the other hand it is also well known that in clinical practice or in laboratory animal studies) PBM has a profound anti-inflammatory effect in vivo. This gives rise to another apparent contradiction that must be satisfactorily resolved.

4.2. PBM reduces levels of pro-inflammatory cytokines in activated inflammatory cells

Part of the answer to the apparent contradiction highlighted above, was addressed in a subsequent paper [44]. We isolated primary bone marrow-derived dendritic cells (DCs) from the mouse femur and cultured them with GM-CSF. When these cells were activated with the classical toll-like receptor (TLR) agonists, LPS (TLR4) and CpG oligodeoxynucleotide (TLR9), they showed upregulation of cell-surface markers of activation and maturation such as MHC class II, CD86 and CD11c as measured by flow cytometry. Moreover IL12 was secreted by CpG-stimulated DCs. PBM (0.3 or 3 J/cm^2 of 810 nm laser) reduced all the markers of activation and also the IL12 secretion. Figure 5.

Yamaura et al [45] tested PBM (810 nm, 5 or 25 J/cm^2) on synoviocytes isolated from rheumatoid arthritis patients. They applied PBM before or after addition of tumor necrosis factor-α (TNF-α). mRNA and protein levels of TNF-α and interleukins (IL)-1beta, and IL-8 were reduced (especially by 25 J/cm^2).

Hwang et al [46] incubated human annulus fibrosus cells with conditioned medium obtained from macrophages (THP-1 cells) containing proinflammatory cytokines IL1β, IL6, IL8 and TNF-α. They compared 405, 532 and 650 nm at doses up to 1.6 J/cm^2. They found that all wavelengths reduced IL8 expression and 405 nm also reduced IL6.

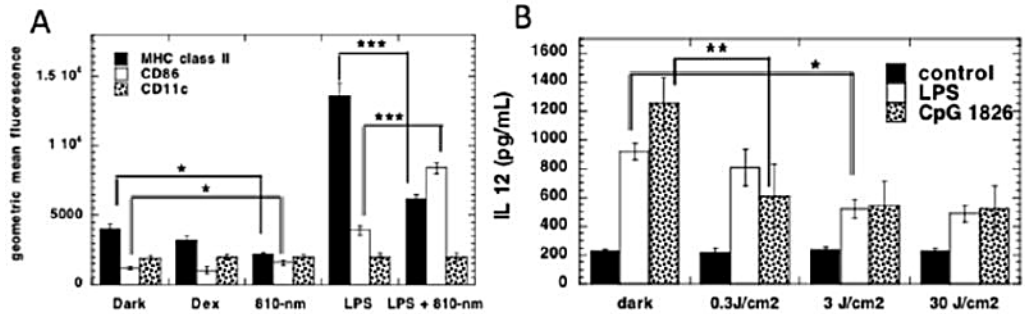

Figure 5. PBM reduces inflammatory markers in activated murine DCs in vitro. (A) Flow cytometry was used to measure MHC class II, CD86, CD11c (dexamethasone was used as positive control); (B) Secreted IL12 measured by ELISA. Figure adapted from data in [44].

The "Super-Lizer" is a Japanese device that emits linear polarized infrared light. Imaoka et al [47] tested it against a rat model of rheumatoid arthritis involving immunizing the rats with bovine type II collagen, after which they develop autoimmune inflammation in multiple joints. The found reductions in IL20 expression in histological sections taken from the PBM-treated joints and also in human rheumatoid fibroblast-like synoviocyte (MH7A) stimulated with IL1β.

Lim et al [48] studied human gingival fibroblasts (HGF) treated with lipopolysaccharides (LPS) isolated from Porphyromonas gingivalis. They used PBM mediated by a 635 nm LED and irradiated the cells + LPS directly or indirectly (transferring medium from PBM treated cells to other cells with LPS). Both direct and indirect protocols showed reductions in inflammatory markers (cyclooxygenase-2 (COX2), prostaglandin E2 (PGE2), granulocyte colony-stimulating factor (GCSF), regulated on activated normal T-cell expressed and secreted (RANTES), and CXCL11). In the indirect irradiation group, phosphorylation of C-Raf and Erk1/2 increased. In another study [49] the same group used a similar system (direct PBM on HGF + LPS) and showed that 635 nm PBM reduced IL6, IL8, p38 phosphorylation, and increased JNK phosphorylation. They explained the activation of JNK by the growth promoting effects of PBM. Sakurai et al reported [50] similar findings using HGF treated with Campylobacter rectus LPS and PBM (830 nm up to 6.3 J/cm^2) to reduce levels of COX2 and PGE2. In another study [51] the same group showed a reduction in IL1β in the same system.

4.3. Effects of PBM on macrophage phenotype

Another very interesting property of PBM is its ability to change the phenotype of activated cells of the monocyte or macrophage lineage. These cells can display two very different phenotypes depending on which pathological situation the cells are faced with. The M1 phenotype (classically activated) applies to macrophages that are faced with a situation in which bacteria or other pathogens need to be killed, or alternatively tumor cells need to be destroyed. Inducible nitric oxide synthase is a hallmark of the M1 phenotype and nitric oxide secretion is often measured. On the other hand the M2 phenotype (alternatively activated) applies to macrophages that are involved in disposal of cellular or protein debris and stimulation of healing by angiogenesis. The M2 phenotype produces arginase, an enzyme that inhibits NO production and allows them to produce ornithine, a precursor of hydroxyproline and polyamines [52]. The markers of these two phenotypes of activated macrophage have some aspects in common, but also show many aspects that are very different [53]. It should be noted that this concept of M1 and M2 activation states, applies to other specialized macrophage type cells that are resident in different tissues, such as microglia in brain [54], alveolar macrophages in lung [55], Kuppfer cells in liver [56], etc.

Fernandes et al used J774 macrophage-like cells activated with interferon-γ and LPS to produce a MI phenotype and compared 660 nm and 780 nm laser. They found that both wavelengths reduced TNF-α, COX-2 and iNOS expression, with the 780 nm being somewhat better [57]. Silva et al used RAW264.7 macrophages to test two wavelengths (660 nm and 808 nm) at a range of fluences (11-214 J/cm^2) [58]. They found increases in NO release with 660 nm at the higher fluences. von Leden et al carried out an interesting study looking at the effects of PBM on microglia and their interaction with cortical neurons [59]. They used both primary microglia isolated from mouse brains and the BV2 mouse microglial cell line and compared four fluences (0.2, 4, 10, and 30 J/cm^2, at 808 nm. Fluences between 4 and 30 J/cm^{2}induced expression of M1 markers in microglia. Markers of the M2 phenotype, including CD206 and TIMP1, were observed at lower energy densities of 0.2–10 J/cm^2. In addition, co-culture of PBM or control-treated microglia with primary neuronal cultures demonstrated a dose-dependent effect of PBM on microglial-induced neuronal growth and neurite extension. This suggests that the benefits of PBM on neuroinflammation may be more pronounced at lower overall doses. The same group went on to show that M1 activated macrophages receiving PBM (660 nm laser) showed significant decreases in CCL3, CXCL2 and TNFα mRNA expression 4 h after irradiation [60]. However, 24 h after irradiation, M1 macrophages showed increased expression of CXCL2 and TNFα genes. M1 activated macrophages irradiated with 780 nm showed a significant decrease in CCL3 gene expression 4h after irradiation. These data could explain the anti-inflammatory effects of LLLT in wound repair.

5. EFFECTS OF PBM ON INFLAMMATION IN ANIMAL MODELS OF DISEASE.

This section will cover some of the most important medical indications where PBM has been shown in laboratory studies to be effective (at least partly) by its pronounced anti-inflammatory effects. Figure 6 shows a graphical summary of the anti-inflammatory applications of PBM in experimental animal models.

5.1. Wound healing

Many papers have demonstrated the efficacy of PBM in stimulating wound healing. In animal models these studies have generally been on acute wounds [61], while in clinical trials they are often been concerned with chronic non-healing wounds such as diabetic ulcers [62]. Gupta et al [63] tested PBM using a superpulsed 904 nm laser on burn wounds in rats. They found faster healing, reduced inflammation (histology), decreased expression of TNF-α and NF-kB, and up-regulated expression of VEGF, FGFR-1, HSP-60, HSP-90, HIF-1α and matrix metalloproteinases-2 and 9 compared to controls. It is intriguing to speculate that the effects of PBM on wound healing (especially the use of for chronic non-healing wounds) could involve both pro-inflammatory effects and anti-inflammatory effects. This seemingly contradictory statement may be possible due to the recent discovery of resolvins and protectins, which are multifunctional lipid mediators derived from omega-3 polyunsaturated fatty acids [64]. If resolvins were produced as a result of the brief acute inflammation induced by application of PBM to chronic wounds, then it has been already shown that resolvins can hasten the healing of diabetic wounds in mice [65]. Resolvins have been shown to reduce tumor necrosis factor-α, interleukin-1β, and neutrophil platelet-endothelial cell adhesion molecule-1 in a mouse burn wound model [66].

5.2. Arthritis

In humans, arthritis is most often caused by a degenerative process occurring in osteoarthritis, or an autoimmune process occurring in rheumatoid arthritis. Both are characterized by pronounced inflammatory changes in the joint and even systemically. Different animal models are produced to mimic these diseases, but a common approach is to inject the sterile preparation of yeast cell walls known as zymosan into the knee joints of rats.

Castano et al [67] used this zymosan-induced arthritis model to study the effects of two different fluences of 810 nm laser (3 and 30 J/cm^2) delivered at two different power densities (5 and 50 mW/cm^2). PBM

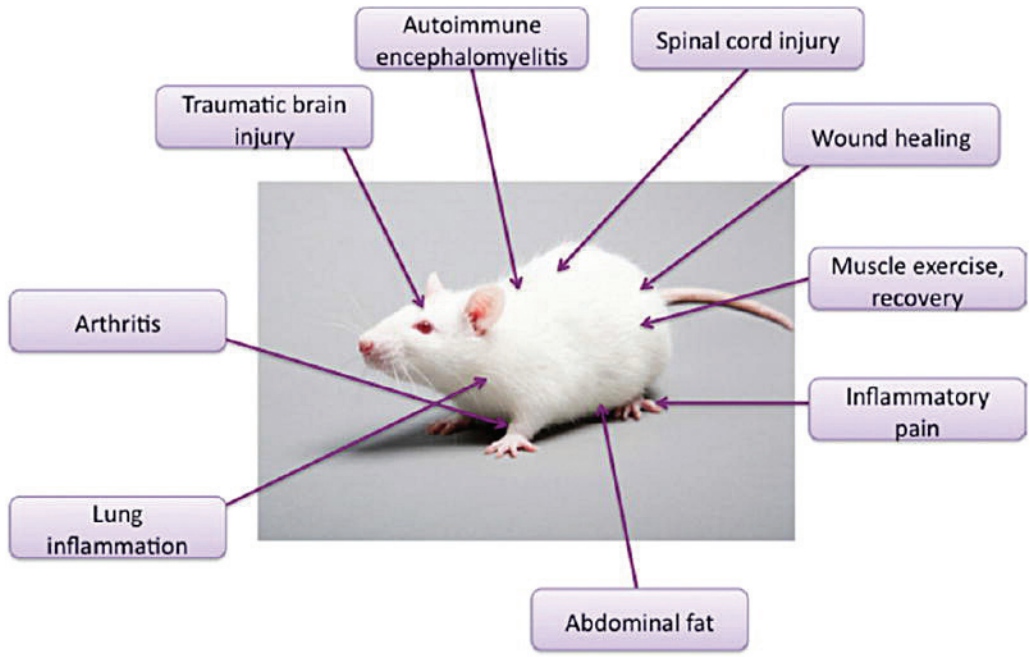

Figure 6. Animal models in which the anti-inflammatory effects of PBM have been shown. Acute traumatic brain injury; experimental autoimmune encephalomyelitis; spinal cord injury; wound healing; muscle exercise and recovery; inflammatory pain in paw; abdominal fat; lung inflammation; knee arthritis.

was delivered once a day for 5 days commencing after zymosan injection, and the swelling in the knee was measured daily. Prostagladin E2 (PGE2) was measured in the serum. They found that 3 out of the 4 sets of parameters were approximately equally effective in reducing swelling and PGE2, but the ineffective set of parameters was 3 J/cm^2 delivered at 50 mW/cm^2 which only took 1 min of illumination time. The conclusion was, that the illumination time was important in PBM, and if that time was too short, then the treatment could be ineffective.

Moriyama et al [68] used a transgenic mouse strain (FVB/N-Tg(iNOS-luc) that had been engineered to express luciferase under control of the inducible nitric oxide synthase promoter, to allow bioluminescence imaging of PBM of the zymosal-induced arthritis model in mice knees. They compared the same fluence of 635, 660, 690, and 905 nm (CW0 and 905 nm (short pulse). Animals younger than 15 weeks showed mostly reduction of iNOS expression, while older animals showed increased iNOS expression. Pulsed 905 nm also increased iNOS expression.

Pallotta et al [69] used a model where carageenan was injected into the rat knee and tested 810 nm laser at 1, 3, 6 or 10 J/cm^2. Rats were sacrificed after 6 or 12 hours and the joint tissue removed. PBM was able to significantly inhibit the total number of leukocytes, as well as the myeloperoxidase activity. Vascular extravasation was significantly inhibited at the higher dose of energy of 10 J. Gene expression of both COX-1 and 2 were significantly enhanced by laser irradiation while PGE2 production was inhibited. These apparently contradictory results require more study to fully explain.

5.3. Muscles

One of the most robust applications of PBM, is its effects on muscles [70,71]. PBM can potentiate muscular performance especially when applied to the muscles 3 hours before exercise [72]. PBM can also make exercise-training regimens more effective. It is not therefore surprising that PBM can also help to heal muscle injuries, not to mention reducing muscle pain and soreness after excessive exercise. Many of the animal studies that have been done have looked at markers of inflammation and oxidative stress in muscle tissue removed from sacrificed animals. For instance, Silveira et al [73] caused a traumatic muscle injury by a single blunt-impact to

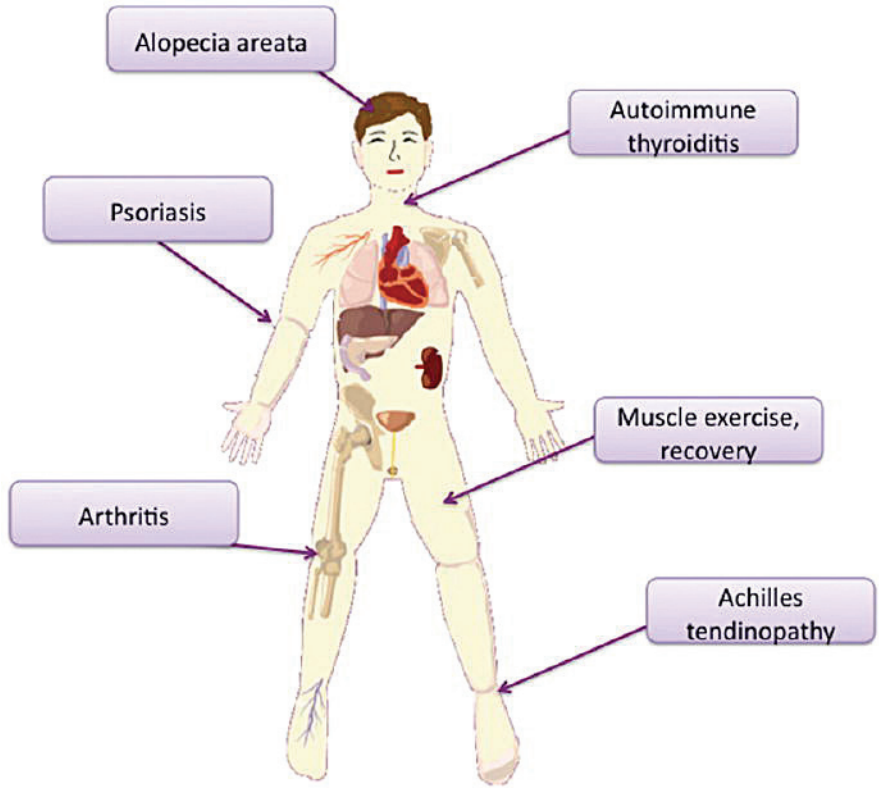

Figure 7. Human clinical indications concentrating on anti-inflammatory effects. Autoimmune thyroiditis; muscle exercise and recovery; Achilles tendinopathy; knee arthritis; psoriasis; alopecia areata.

the rat gastrocnemius muscle. PBM (850 nm, 3 or 5 J/cm^2) was initiated 2, 12, and 24 h after muscle trauma, and repeated for five days. The locomotion and muscle function was improved by PBM. TBARS, protein carbonyls, superoxide dismutase, glutathione peroxidase, and catalase, were increased after muscle injury, these increases were prevented by PBM. PBM prevented increases in IL-6 and IL-10 and reversed the trauma-induced reduction in BDNF and VEGF.

5.4. Inflammatory pain

There have been many studies that have looked at the effects of PBM on pain in animal models. Some studies have looked at sensitivity to pain [74] using the von Frey filaments (a graded set of fibers of increasing stiffness and when the animal feels the pressure it withdraws its foot [75]).

Some studies have looked at animal models of neuropathic pain such as the "spared nerve injury" [76]. This involves ligating two out of three branches of the sciatic nerve in rats and causes long lasting (>6 months) mechanical allodynia [77]. Kobelia Ketz et al found improvements in pain scores with PBM (980 nm applied to affected hind paw 1 W, 20 s, 41 cm above skin, power density 43.25 mW/cm^2, dose 20 J). They also found lower expression of the proinflammatory marker (Iba1) in microglia in the dorsal root ganglion, gracile nucleus, dorsal column and dorsal horn. The M1/M2 balance of the macrophage phenotype was switched from M1 to M2 by PBM, as judged by relative staining with anti-CD86 (M1) and anti-CD206 (M2).

Martins et al looked at the effect of PBM on a model of inflammatory pain [42]. This involved injecting complete Freund's adjuvant (CFA) into the mouse paw, and produces hyperalgesia and elevated cytokine levels (TNF-α, IL-1β, IL-10). They found that LEDT (950-nm, 80 mW/cm^2, 1, 2 or 4 J/cm^2) applied to the plantar aspect of the right hind limb, reduced pain, increased the levels of IL-10 prevented TBARS increase in both acute and chronic phases, reduced protein carbonyl levels and increased SOD and CAT activity in the acute phase only.

5.5. Lung inflammation

Aimbire and his laboratory in Brazil have carried out several studies on the use of PBM to reduce acute lung inflammation (ALI) in various animal models. In a mouse model of lung inflammation caused either by inhalation of lipolysaccharide or intranasal administration of TNFα they analyzed the bronchoalveolar lavage fluid (BALF). PBM (660 nm, 4.5 J/cm^2) was administered to the skin over the right upper bronchus 15 min after ALI induction. PBM attenuated the neutrophil influx and lowered TNFα in BALF. In alveolar macrophages, PBM increased cAMP and reduced TNFα mRNA.

They also studied a different model of ALI caused by intestinal ischemia and reperfusion (I/R), that produces an analogue of acute respiratory distress syndrome (ARDS) [78]. Rats were subjected to superior mesenteric artery occlusion (45 min) and received PBM (660 nm, 7.5 J/cm^2) carried out by irradiating the rats on the skin over the right upper bronchus for 15 and 30 min, and rats were euthanized 30 min, 2, or 4 h later. PBM reduced lung edema, myeloperoxisdase activity, TNF-α and iNOS, LLLT increased IL-10 in the lungs of animals subjected to I/R.

A third animal model was related to asthma [79]. Mice were sensitized to ovalbumin (OVA), and then challenged by a single 15-min exposure to aerosolized OVA. PBM was applied as above (660 nm, 30 mW, 5.4 J). Bronchial hyper-responsiveness (as measured by dose response curves to acetylcholine) was reduced by PBM as well as reductions in eosinophils and eotaxin. PBM also diminished expression of intracellular adhesion molecule and Th2 cytokines, as well as signal transducer and activator of transduction 6 (STAT6) levels in lungs from challenged mice. Recently Rigonato-Oliveira et al. presented a study that concluded that the reduced lung inflammation and the positive effects of PBM on the airways appear to be mediated by increased secretion of the anti-inflammatory cytokine IL-10, and reduction of mucus in the airway [80].

5.6. Traumatic brain injury

In recent years the use of PBM as a treatment for traumatic brain injury [81,82], and other brain disorders including stroke, neurodegenerative diseases and even psychiatric disorders has increased markedly [83]. It is thought that the actions of NIR light shone on the head and penetrating into the brain are multi-factorial, but one clear effect is the anti-inflammatory action of transcranial PBM. This was shown by a series of mouse experiments conducted by

Khuman et al [84]. They used the controlled cortical impact model of TBI and delivered PBM (800 nm) was applied directly to the contused parenchyma or transcranially in mice beginning 60–80 min after CCI. Injured mice treated with 60 J/cm^2 (500 mW/cm^2 × 2 min) had improved latency to the hidden platform and probe trial performance in the Morris water maze. PBM in open craniotomy mice reduced the number of activated microglia in the brain at 48 h (21.8 ± 2.3 versus 39.2 ± 4.2 IbA-1 + cells/field).

5.7. Spinal cord injury

Spinal cord injury (SCI) is another promising area of central nervous system injury that could be benefited by PBM. Veronez et al [85] used a rat model of SCI involving a contusion produced by a mechanical impactor (between the ninth and tenth thoracic vertebrae), with a pressure of 150 kdyn. Three different doses of PBM (808-nm laser) were tested: 500 J/cm^2, 750 J/cm^2 and 1000 J/cm^2 delivered daily for seven days. Functional preformance and tactile sensitivity were improved after PBM, at 1000 J/cm^2. PBM at 750 and 1000 J/cm^2 reduced the lesion volume and also reduced markers of inflammation (lower CD-68 protein expression).

5.8. Autoimmune diseases

Experimental autoimmune encephalomyelitis (EAE) is the most commonly studied animal model of multiple sclerosis (MS), a chronic autoimmune demyelinating disorder of the central nervous system. Immunomodulatory and immunosuppressive therapies currently approved for the treatment of MS slow disease progression, but do not prevent it. Lyons et al [86] studied a mouse model of EAE involving immunization with myelin oligodendrocyte glycoprotein (MOG35-55). They treated the female C57BL/6 mice with PBM (670 nm) for several days in different regimens. In addition to improved muscular function, they found down-regulation of inducible nitric oxide synthase (iNOS) gene expression in the spinal cords of mice as well as an up-regulation of the Bcl-2 anti-apoptosis gene, an increased Bcl-2:Bax ratio, and reduced apoptosis within the spinal cord of animals over the course of disease. 670 nm light therapy failed to ameliorate MOG-induced EAE in mice deficient in iNOS, confirming a role for remediation of nitrosative stress in the amelioration of MOG-induced EAE by 670 nm mediated photobiomodulation.

5.9. Abdominal fat

Yoshimura et al [87] looked at a mouse model of obesity and type 2 diabetes [87]. Four weeks old male adult C57BL/6 mice were fed a hypercaloric high-fat diet (40% calories derived from fat) for eight weeks to induce obesity and hyperglycemia. Over a period of four weeks mice were exposed to six irradiation sessions using an 843 nm LED (5.7 J cm^{-2}, 19 mW cm^{-2}). Non-irradiated control mice had areas of inflammation in their abdominal fat almost five times greater than the PBM group. The PBM group had significantly lower blood glucose levels 24 hours after the last session.

6. CLINICAL APPLICATIONS OF PBM FOR INFLAMMATION

Amongst the many hundreds of reports of clinical applications of PBMT, we will highlight a few here, which seem to be especially relevant to inflammation, and inflammatory disorders.

6.1. Achilles tendinopathy

Bjordal et al in Norway carried out a randomized, placebo controlled trial of PBM (904 nm, 5.4 J per point, 20 mW/cm^2) for activated Achilles tendinitis [88]. In addition to clinical assessment, they used microdialysis measurement of peritendinous prostaglandin E2 concentrations. Doppler ultrasonography measurements at baseline showed minor inflammation shown by increased intratendinous blood flow, and a measurable resistive index. PGE2 concentrations were significantly reduced with PBM vs placebo. The pressure pain threshold also increased significantly.

6.2. Thyroiditis

Chavantes and Chammas in Brazil have studied PBM for chronic autoimmune thyroiditis. An initial pilot trial [89] used 10 applications of PBM (830 nm, 50 mW, 38–108 J/cm^2), twice a week, using either the punctual technique (8 patients) or the sweep technique (7 patients). Patients required a lower dosage of levothyroxine, and showed an increased echogenicity by ultrasound. The next study [90] was a randomized, placebo-controlled trial of 43 patients with a 9-month follow-up. In addition to improved thyroid function they found reduced autoimmunity evidenced by lower thyroid peroxidase antibodies (TPOAb), and thyroglobulin antibodies (TgAb). A third study [91] used color Doppler ultrasound to

show improved normal vascualrization in the thyroid parenchyma. Finally [92] they showed a statistically significant increase in serum TGF-β1 levels 30 days post-intervention in the PBM group, thus confirming the anti-inflammatory effect. Recently a long-term follow up study of these thyroiditis patients (6 years later) was presented showing that PBM was safe in the long term and demonstrated lasting benefits [93].

6.3. Muscles

PBM for muscles aims to benefit athletic performance and training, to reduce delayed onset muscle soreness (DOMS), as well as to ameliorate signs of muscle damage (creatine kinase) after intense or prolonged exercise. Moreover PBM can also be used to treat frank muscle damage caused by muscle strains or trauma. The International Olympic Committee and the World Anti-Doping Agency cannot ban light therapy for athletes considering (1) the intensity is similar to sunlight, and (2) there is no forensic test for light exposure. There have been several clinical trials carried out in Brazil in athletes such as elite runners [94], volleyball players [95] and rugby players [96]. Ferraresi et al conducted a case-controlled study in a pair of identical twins [97]. They used a flexible LED array (850 nm, 75 J, 15 sec) applied to both quadriceps femoris muscles (real to one twin and sham to the other) immediately after each strength training session (3 times/wk for 12 weeks) consisting of leg press and leg extension exercises with load of 80% and 50% of the 1-repetition maximum test, respectively. PBM increased the maximal load in exercise and reduced fatigue, creatine kinase, and visual analog scale (DOMS) compared to sham. Muscle biopsies were taken before and after the training program and showed that PBM decreased inflammatory markers such as interleukin 1β and muscle atrophy (myostatin). Protein synthesis (mammalian target of rapamycin) and oxidative stress defense (SOD2, mitochondrial superoxide dismutase) were up-regulated.

6.4. Psoriasis

Psoriasis is a chronic autoimmune skin disease. Psoriasis is characterized by the abnormally excessive and rapid growth of keratinocytes (instead of being replaced every 28–30 days as in normal skin, in psoriatic skin they are replaced every 3–5 days). This hyperproliferation is caused by an inflammatory cascade in the dermis involving dendritic cells, macrophages, and T cells secreting TNF-α, IL-1β, IL-6, IL-17, IL-22, and IL-36γ [98]. PBM has been used for psoriasis because of its anti-inflammatory effects, which is a different approach from UV phototherapy which tends to kill circulating T-cells. Ablon [99] tested PBM using LEDs (830 nm, 60 J/cm^2 and 633 nm, 126 J/cm^2) in two 20-min sessions over 4 or 5 weeks, with 48 h between sessions in 9 patients with chronic treatment-resistant psoriasis. Clearance rates at the end of the follow-up period ranged from 60% to 100%. Satisfaction was universally very high.

Choi et al [100] tested PBM in case report of a patient with another inflammatory skin disease called acrodermatitis continua, who also had a 10-yr history of plaque psoriasis on her knees and elbows. As she was pregnant and not suited for pharmacological therapy, she received treatment with PBM (broad-band polarized light, 480–3,400 nm, 10 J/cm^2). In two weeks (after only 4 treatments), the clinical resolution was impressive and no pustules were found. Topical methylprednisolone aceponate steroid cream was switched to a moisturizer, and she was treated twice or once a week with PBM until a healthy baby was delivered.

6.5. Arthritis

As can be seen from the animal studies section, arthritis is one of the most important clinical indications for PBM [101,102]. The two most common forms of arthritis are osteoarthritis (degenerative joint disease that mostly affects the fingers, knees, and hips) and rheumatoid arthritis (autoimmune joint inflammation that often affects the hands and feet). Osteoarthritis (OA) affects more than 3.8% of the population while rheumatoid arthritis (RA) affects about 0.24%. Both types have been successfully treated with PBM. Cochrane systematic reviews found for good evidence for its effectiveness in RA [103], and some evidence in the case of OA [104]. Most clinical studies have used pain scales and range of movement scores to test the effectiveness, rather than measures of inflammation which are difficult to carry out in human subjects.

Barabas and coworkers [105] made an attempt by testing PBM on ex vivo samples of synovial tissue surgically removed from patients receiving knee joint replacement. Synovial membrane samples received exposure to PBM (810 nm, 448 mW, 25 J/cm^2, 1 cm^2 area). PBM caused an increase in mitochondrial heat shock protein 1 60 kD, and decreases in calpain small

subunit 1, tubulin alpha-1C, beta 2, vimentin variant 3, annexin A1, annexin A5, cofilin 1,transgelin, and collagen type VI alpha 2 chain precursor all significantly decreased compared to the control

6.6. Alopecia areata

Alopecia areata (AA) is one of the three common types of hair loss, the other two being androgenetic alopecia (AGA, male pattern baldness) and chemotherapy induced alopecia. AA is a common autoimmune disease resulting from damage caused to the hair follicles (HFs) by T cells. Evidence of autoantibodies to anagen stage HF structures is found in affected humans and experimental mouse models. Biopsy specimens from affected individuals demonstrate a characteristic peri- and intrafollicular inflammatory infiltrate around anagen-stage HFs consisting of activated CD4 and CD8 T lymphocytes [106]. PBM is an excellent treatment for hair loss in general and AGA in particular [107,108]. Yamazaki et al [109] reported the use of the "Super-Lizer" delivering linear-polarized light between 600–1600 nm at a power of 1.26 W to the areas of hair loss on the scalp (4-s pulses delivered at 1-s intervals for 3 min every 1 or 2 weeks until hair growth was observed). Regrowth of vellus hairs was achieved on more than 50% ofthe involved areas in all 15 cases. The frequency of irradiation until regrowth ranged from one to 14 times and the duration of SL treatment was 2 weeks to 5 months.

7. CONCLUSION AND FUTURE STUDIES.

The clinical applications of PBM have been increasing apace in recent years. The recent adoption of inexpensive large area LED arrays, that have replaced costly, small area laser beams with a risk of eye damage, has accelerated this increase in popularity. Advances in understanding of PBM mechanisms of action at a molecular and cellular level, have provided a scientific rationale for its use for multiple diseases. Many patients have become disillusioned with traditional pharmaceutical approaches to a range of chronic conditions, with their accompanying distressing side-effects and have turned to complementary and alternative medicine for more natural remedies. PBM has an almost complete lack of reported adverse effects, provided the parameters are understood at least at a basic level. The remarkable range of medical benefits provided by PBM, has led some to suggest that it may be "too good to be true". However one of the most general benefits of PBM that has recently emerged, is its pronounced anti-inflammatory effects. While the exact cellular signaling pathways responsible for this anti-inflammatory action are not yet completely understood, it is becoming clear that both local and systemic mechanisms are operating. The local reduction of edema, and reductions in markers of oxidative stress and pro-inflammatory cytokines are well established. However there also appears to be a systemic effect whereby light delivered to the body, can positively benefit distant tissues and organs.

There is a lot of scope for further work on PBM and inflammation. The intriguing benefits of PBM on some autoimmune diseases, suggests that this area may present a fertile area for researchers. There may be some overlap between the ability of PBM to activate and mobilize stem cells and progenitor cells, and its anti-inflammatory action, considering that one of the main benefits of exogenous stem cell therapy has been found to be its anti-inflammatory effect. The versatile benefits of PBM on the brain and the central nervous system, encourages further study of its ability to reduce neuroinflammation. Chronic diseases of the modern age involving systemic inflammation such as type II diabetes, obesity, Alzheimer's disease, cardiovascular disease and endothelial dysfunction are again worth investigating in the context of PBM.

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CONFLICT OF INTEREST

The author declares no conflict of interest in this paper.

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MECHANISMS AND MITOCHONDRIAL REDOX SIGNALING IN PHOTOBIOMODULATION

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CASE REPORT

ABSTRACT

Photobiomodulation (PBM) involves the use of red or near-infrared light at low power densities to produce a beneficial effect on cells or tissues. PBM therapy is used to reduce pain, inflammation, edema, and to regenerate damaged tissues such as wounds, bones and tendons. The primary site of light absorption in mammalian cells has been identified as the mitochondria, and more specifically, cytochrome c oxidase (CCO). It is hypothesized that inhibitory nitric oxide can be dissociated from CCO thus restoring electron transport and increasing mitochondrial membrane potential. Another mechanism involves activation of light or heat-gated ion channels. This review will cover the redox signaling that occurs in PBM and examine the difference between healthy and stressed cells, where PBM can have apparently opposite effects. PBM has a marked effect on stem cells, and this is proposed to operate via mitochondrial redox signaling. PBM can act as a pre-conditioning regimen, and can interact with exercise on muscles.

KEYWORDS:

photobiomodulation, low-level laser therapy, chromophores, mitochondria, reactive oxygen species, stem cells

INTRODUCTION

Photobiomodulation (PBM) also known as low-level laser (light) therapy (LLLT) is approaching its 50thanniversary (1). LLLT was originally discovered by Endre Mester working in Hungary, who was trying to repeat an experiment described by Paul McGuff in Boston. McGuff had used the newly discovered ruby laser to cure experimental tumors implanted in Syrian hamsters (2, 3). However, Mester's laser only had a small fraction of the power possessed by McGuff's laser and was insufficient to cure any tumors. Nevertheless, Mester observed that the skin wounds that had been made during implantation of the tumors healed better in laser treated animals (4, 5). Since those early days, LLLT has become gradually more accepted in scientific, medical and popular circles, especially as the number of peer-reviewed papers has grown.

For much of this time, lasers were thought to have special biological properties due to their coherence and monochromaticity (6), and the field was sometimes called "laser biostimulation" (7). However in recent years it has become clear that non-coherent light-emitting diodes (LEDs) perform equally to medical lasers, with the added advantage of being much less expensive and having fewer safety concerns (8). The first lasers to be used generally emitted red light. The ruby (694 nm) and HeNe (633 nm) lasers were popular, but after the introduction of diode lasers, many more wavelengths became available including several in the near-infrared region (780–940 nm).

In 2016 there was an international consensus to change the terminology away from LLLT and the old term "low-level", and instead use the new term "photobiomodulation" (9). The reasons for this decision were several-fold: (1) nobody had any idea exactly what "low-level" actually meant; (2) the term laser was inappropriate as LEDs are rapidly taking over; (3) due to the biphasic dose response, PBM can have inhibitory or stimulatory effects even at the same wavelength with just the use of a much higher energy density.

One feature of PBM that is becoming appreciated is the biphasic dose response (10, 11) (also known as the Arndt-Schulz law) (12). This principle states that a very low dose of light has no effect, a somewhat bigger dose has a positive effect until a plateau is reached. If the light dose is increased beyond that point the benefit progressively decreases, until the baseline (no effect) is reached, and further increases will actually start to have damaging effects on the tissue. This curve is well known in the field of toxicology, where the phenomenon is called "hormesis" (13). Part of the explanation of this "U" or "J" shaped curve is that small doses of a potentially toxic drug or harmful intervention can induce expression inside the cells of a range of protective factors such as anti-oxidant enzymes and anti-apoptotic proteins that will enhance normal function and protect against subsequent lethal challenges (14).

There have been over 1,000 papers published on experimental laboratory studies in PBM covering a vast range of different systems, including many different types of cultured cells looking at many different molecular markers, a large number of animal studies, and of course another large range (1000+) of clinical studies (both randomized controlled trials and case series). A recently published textbook serves as a starting point to further explore the literature of this field (1).

MITOCHONDRIAL CHROMOPHORES FOR RED AND NIR LIGHT

The first law of photobiology states that photons of light must be absorbed by some molecule (called a chromophore) located within the tissue to have any biological effect. Tiina Karu working in Russia and Salvatore Passarella in Italy were the first to suggest that one of the principal chromophores responsible for the beneficial effects of PBM was located inside mitochondria (15). Previously Britton Chance had observed that the mitochondrial fraction accounted for 50% of the optical absorption of blood-free rat liver (16) at 780 nm. Although hemoglobin and myoglobin have high absorption coefficients in the visible spectral regions (blue, green and red), their absorption in the NIR region, (where PBM is highly effective) is not very high. The purified enzyme, cytochrome c oxidase (CCO) was shown to be activated in vitro by red laser (633 nm) (17). CCO is unit IV of the mitochondrial respiratory chain and is a complex molecule with 13 separate protein subunits. CCO contains two different copper centers Cu_A and Cu_B and two heme centers, heme-a and heme-a3. All these centers can be in a reduced or an oxidized state giving a total of sixteen possibilities. CCO transfers four protons to molecular oxygen to form two water molecules using the electrons from reduced cytochrome c. The proton gradient so formed drives the

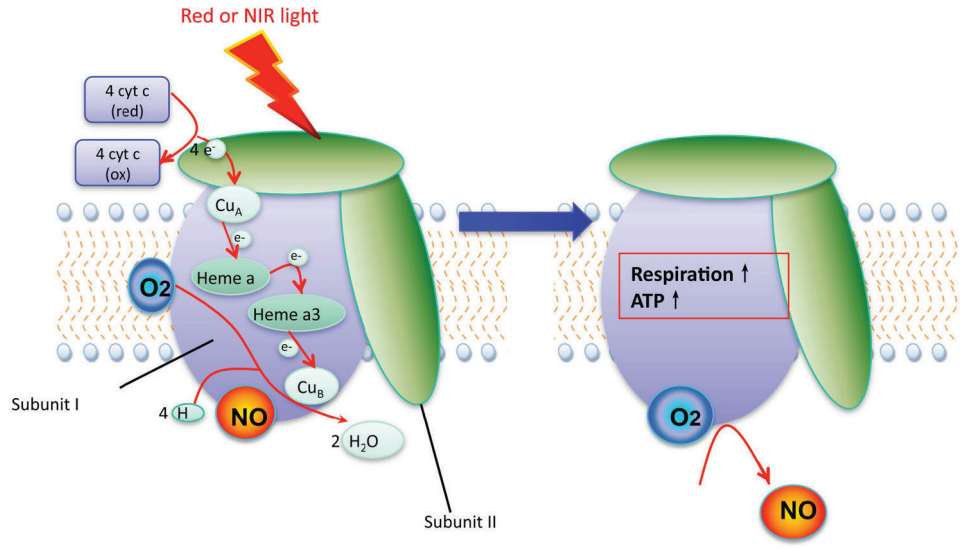

Figure 1. Proposed photodissociation of NO from cytochrome c oxidase (CCO)
CCO is a multi-subunit enzyme containing two heme co-factors and two copper centers that oxidizes four reduced cytochrome c molecules, while at the same time reducing oxygen to water and producing four protons that go on to form ATP via ATP synthase. Nitric oxide can inhibit this process by binding to Cu_B and it is proposed that red or NIR light can dissociate this non-covalently bound NO increasing the rate of respiration and ATP production.

activity of ATP synthase. Several investigators have reported that the action spectra (relative efficiency of different wavelengths for mediating aspects of the PBM process) correspond to the absorption spectrum of CCO (18, 19). The leading hypothesis to explain how exactly light increases CCO enzyme activity is that nitric oxide (a molecule that is known to inhibit CCO by non-covalently binding between heme-a3 and Cu_B (20), can be photodissociated by absorption of a photon of red or NIR light (21). One theory to explain why PBM appears to have greater effects in diseased or damaged cells and tissues, and to not dramatically affect healthy cells, is that unhealthy or hypoxic cells are more likely to have inhibitory concentrations of NO. This proposed mechanism is illustrated in Figure 1.

Since the principle chromophores for PBM are located inside the mitochondria, it follows that cells with a large number of mitochondria and a high metabolic activity are particularly responsive to light. This consideration applies to muscle cells (both skeletal and cardiac), neurons (especially CNS neurons), but also cells of the liver, kidney and other internal organs. It should be noted that these cells are not commonly exposed to light during normal living activity, while the skin, which has evolved to be constantly exposed to light does not have large numbers of mitochondria.

LIGHT/HEAT GATED ION CHANNELS AND BLUE-LIGHT CHROMOPHORES

An important discovery was made by Hardie & Minke working with the fruitfly Drosophila melanogaster in 1992 (22). A spontaneous mutation (later found to be in the trp gene) led to a blind mutant, even though the flies were exposed to intense light. A combination of electrophysiological, biochemical, calcium measurements, combined with genetic studies in these flies, and eventually in other invertebrates finally showed that TRP was a novel phosphoinositide-regulated calcium permeable ion channel (23). The underlying mechanism of vision is quite different in insects (relying on TRP channels) and mammalian organisms (relying on rhodopsin photoreceptors) (24).

Transient receptor potential (TRP) channels are now known to be pleiotropic cellular sensors mediating the response to a wide range of external stimuli (heat, cold, pressure, taste, smell), and involved in many different cellular processes (25). Activation of TRP causes non-selective permeabilization (mainly of the plasma membrane) to calcium, sodium and magnesium (26). Interestingly it was recently reported that TRP channels were involved in sensing the "redox status" (27).

It is now known that TRP channel proteins are conserved throughout evolution and are found in most organisms, tissues, and cell-types. The TRP channel superfamily is now classified into seven related subfamilies: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, and TRPN (28). Light-sensitive ion channels are based on an opsin chromophore (isomerization of a cis-retinal molecule to the trans configuration) as illustrated in Drosophila photoreceptors (29).

It is possible that blue light interacts with mitochondrial chromophores in the same way as red/NIR light since heme centers that are widespread in cytochromes have a significant absorption peak that coincides with the Soret band of porphyrins. However, there are several other plausible chromophores for blue light (and to a lesser extent green light). It should be noted that the term "blue light" can refer to a relatively wide range of wavelengths from violet (390–425 nm), indigo (425–450 nm), royal blue (450–475 nm), blue green (475–500 nm). Because of the width of a typical absorption band (30 nm full width half maximum), it is theoretically possible that blue light could be absorbed by several distinct chromophores. For blue light these potential chromophores are in order of increasing wavelength: (A) tryptophan that can be photo-oxidized to form 6-formylindolo[3,2-b] carbazole (FICZ) that acts as an endogenous ligand of the aryl-hydrocarbon receptor (AhR) (30, 31). The shortest wavelength blue light (380–400nm) would be optimal here, as in general UV wavelengths are thought to be responsible for trytptophan photodegradation. (B) Next is the Soret band of heme groups (400 nm) where presumably similar processes are initiated as have been proposed for red/NIR light. Cytochromes b and a/a(3) were found to be responsible for the inhibitory effects of blue light on yeast (32). (C) Wavelengths in the 440 nm range have been found to be optimal for activation of cryptochromes (33). Cryptochromes are blue-light sensitive flavoproteins that have wide applications in plants and lower life-forms, mediating such functions as photomorphogenesis (34). Cryptochromes are thought to play a role in entraining circadian rhythms (35) and may even be involved in sensing of magnetic

fields in fruitflies (36). Cryptochromes have recently been found to be expressed in some mammalian cells and tissues (37) and also to have activity in regulating circadian rhythms (38). (D) The family of opsins are light-sensitive G-protein coupled receptors that rely on isomerization of cis-retinal. The wavelength maximum can range from UVA all the way to the green and red, but melanopsin (OPN4) has a λ_{max} of 479 nm (39). The signaling pathways differ between different opsins. Opsins signal via two main pathways depending on the type of G-protein they are coupled with (40, 41). Those opsins (OPN1, OPN2, OPN3, OPN5) that are coupled with G_o, G_i, G_t, G_s proteins, signal via a pathway involving cyclic nucleotides (cAMP and cGMP). On the other hand OPN4 (melanopsin) is coupled to Gq and signals via the phospholipase C pathway leading to production of inositol triphosphate and di-acylglycerol. These signaling pathways are shown in Figure 2. It is known that activation of retinal opsins by blue light can generate ROS, which is partly responsible for ocular phototoxicity caused by violet and blue light (42). Osborne et al. discussed the phototoxicity caused to retinal ganglion cells (RGCs) and photoreceptors by short wavelength light (SWL) (43). The threat of damage to photoreceptor mitochondria may be less than to RGCs, since macular carotenoid, located chiefly in Henle's layer of the photoreceptor inner segment absorbs SWL. They proposed that SWL contributes to RGC death when these neurons are not in an optimum homoeostatic state as is likely to occur in conditions such as glaucoma and aging.

REDOX EFFECTS INDUCED BY PBM

One of the most frequently observed changes when PBM experiments are conducted in vitro, has been modulation of levels of reactive oxygen species (ROS) (44). ROS have particularly been reported to be produced by large doses of light, and even more particularly by blue light. The production of modest amounts of ROS by red/NIR light being absorbed in the mitochondria is reasonably well established (45). It is known that mitochondria are one of the most important sources of ROS in mammalian cells

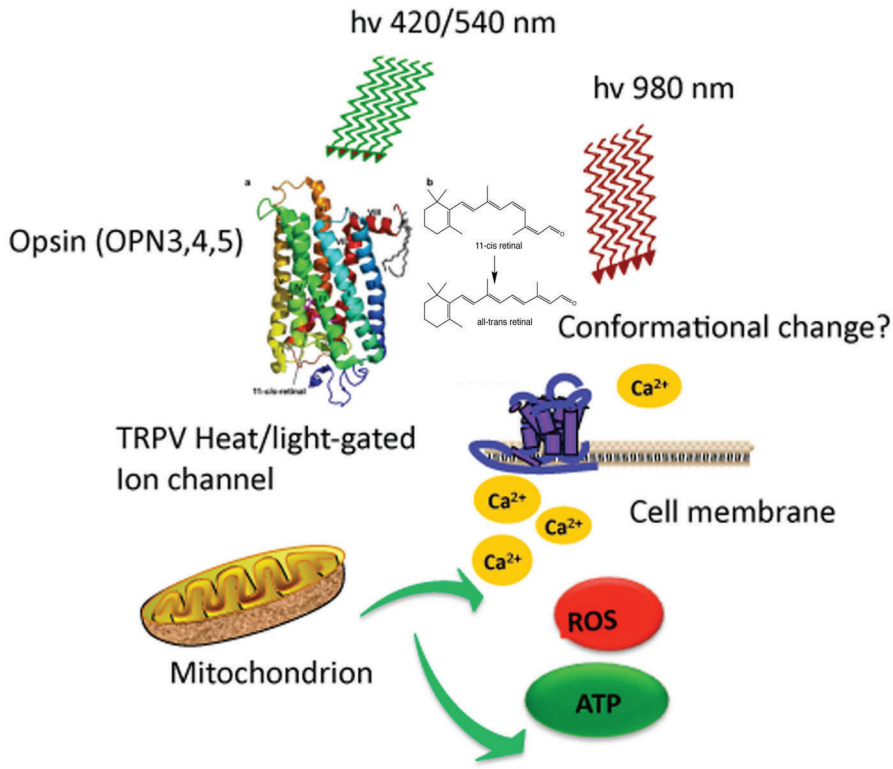

Figure 2. **Proposed activation of TRP (transient receptor potential) ion channels by blue/green light or 980 nm NIR**
It is proposed that blue light (in the region of 420 nm) or green light in the region of 540 nm can activate opsins such as melanopsin (OPN5) by a cis-trans retinal isomerization. Activation of OPN5 can in turn, open TRPV calcium ion channels via $G\alpha_o$, phospholipase C and phosphoinositide signaling. Alternatively, NIR light in the region of 980 nm may directly perturb the conformational structure of TRPV channels via absorption by nanostructured water.

(46). Leakage of electrons leads to production of superoxide anion that is then removed by manganese-dependent superoxide dismutase (MnSOD) (47).

The mitochondrial membrane potential (MMP) is increased by PBM, leading to increased electron transport. Classically it is believed that increased MMP will produce increased ROS (48). However, it is also well known that dysfunctional mitochondria also produce ROS. This process is characterized by a self-amplifying feedback loop called "ROS-induced ROS release" (RIRR) (49). Under conditions such as exposure to excessive or prolonged oxidative stress, the increase in ROS may reach a threshold level that triggers the opening of a mitochondrial channel such as the mitochondrial permeability transition (MPT) pore, or the mitochondrial inner membrane anion channel (IMAC). Activation of these channels in turn leads to the simultaneous collapse of MMP and increased ROS generation by the electron transport chain (50). Production of a large enough burst of ROS to flood the cytosol could potentially function as a "second messenger" to activate RIRR in neighboring mitochondria, which could then act as another damaging feedback loop to increase cellular damage (51).

We first showed that PBM with 810 nm laser (3 J/cm^2) could activate NF-kB in embryonic fibroblasts isolated from NF-kB luciferase reporter mice (44). We showed that ROS were generated inside the cells, and ATP production was increased. Although we did not conclusively show that these ROS originated from the mitochondria in this particular study, in subsequent studies using cortical neurons the light-induced ROS were shown to come from mitochondria. Interestingly, addition of the anti-oxidant N-acetylcysteine abrogated the activation of NF-kB by quenching the ROS, but had no effect on the increase in ATP (Figure 3). The explanation for these observations is that PBM raised MMP leading to more ATP production, and at the same time produced a burst of ROS that activated NF-kB, probably by activation of protein kinase D (52).

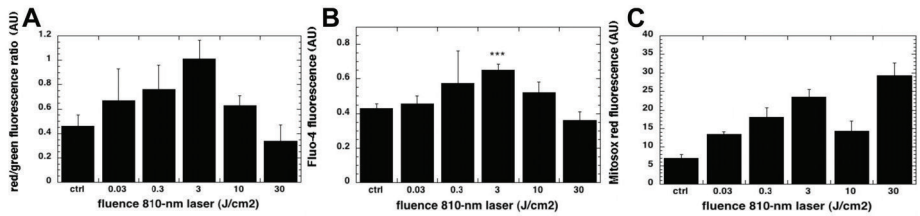

Figure 4. Dose response of PBM in primary cultured cortical neurons
Primary cultured mouse cortical neurons were treated with a wide range of doses of 810 nm laser from 0.03 to 30 J/cm^2. (A) Mitochondrial membrane potential measured by red/green ratio of fluorescence from JC1 probe. (B) Intracellular calcium measured by fluorescence from fluo-4 calcium probe. (C) Intracellular ROS mesured by fluorescence from mitosox red probe. Adapted from data contained in (53).

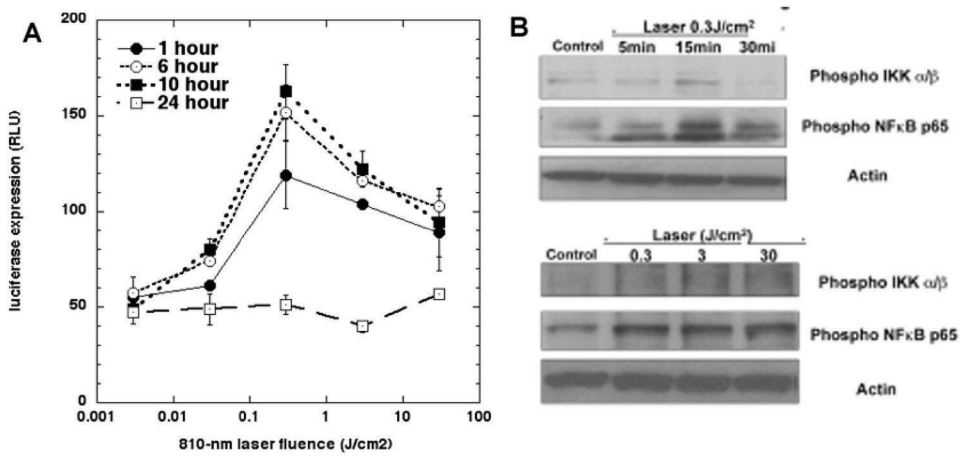

Figure 3. **Activation of NF-kB (nuclear factor kappaB) in mouse embryonic fibroblasts**
Cells were isolated from NF-kB luciferase reporter mice. (A) Biphasic dose response of NF-kB activation (0.003 to 30 J/cm^2 of 810 nm laser) measured by bioluminescence signal production at 1, 6, 10 and 24 hours post-PBM. (B) Western blot showing phosphorylation of NF-kB with different doses and times. Adapted from data contained in (45).

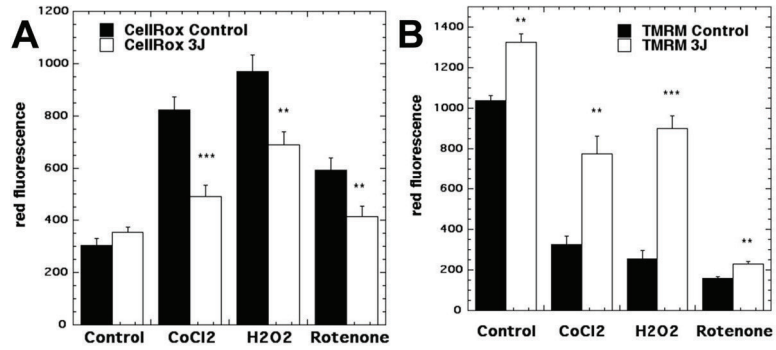

Figure 5. Effects of PBM on cells under oxidative stress
Primary cortical neurons were treated with one of three different agents (cobalt chloride, hydrogen peroxide, rotenone) each of which produced oxidative stress. They were treated either with no PBM or with 3 J/cm^2 of 810 nm laser. (A) Intracellular ROS (measured by CellRox red fluorescent probe) were modestly increased in control cells, but significantly reduced in all three types of oxidative stress. (B) In every case the mitochondrial membrane potential (measured by tetramethyl-rhodamine methyl ester fluorescent probe, TMRM) was significantly increased. Adapted from data contained in (54).

We next went on to show that PBM (810 nm laser) had biphasic dose response effects in primary cultured cortical neurons from embryonic mouse brains (53). Using a wide range of energy densities (0.03 to 30 J/cm^2) we found a maximum effect at 3 J/cm^2 on MMP, and intracellular calcium. Higher doses of light (10 or 30 J/cm^2) had lower effects and 30 J/cm^2 actually lowered the MMP below baseline. When we looked at intracellular ROS production we found a double peak. The first peak was at 3 J/cm^2 while there was a second peak at 30 J/cm^2 (Figure 4). We interpret these data to mean that ROS were produced from mitochondria by raising MMP above baseline at 3 J/cm^2, and also by lowering the MMP below baseline at 30 J/cm^2.

Next we asked what would happen when PBM was delivered to cells that had already been subjected to oxidative stress (54). Using the same cultured cortical neurons, we applied three different chemical treatments that were all designed to cause oxidative stress as shown by increases in intracellular ROS. These were cobalt chloride (activation of hypoxia-inducible factor 1), rotenone (complex 1 inhibitor), or hydrogen peroxide. All these chemicals led to reductions in MMP and ATP production, as well as increased intracellular ROS. PBM (810 nm laser, 3 J/cm^2) led to increased MMP and ATP and reduced ROS, while by contrast control cells (no oxidative stress) had a small increase in ROS accompanied by increases in MMP and ATP above baseline levels (Figure 5). We interpret these results to mean that PBM tends to increase MMP back towards baseline thereby reducing ROS production.

In contrast to NIR light, the mechanisms for production of ROS by blue light are less well established. Using adipose-derived stem cells, we showed that blue light (415 nm) produced a linear dose-dependent increase in ROS accompanied by reductions in ATP and MMP and inhibition of proliferation (submitted for publication). However, 810 nm (and 660 nm) gave a biphasic dose-dependent increase in proliferation and ATP accompanied by a rise in MMP and a rise in ROS that was less than that found with blue light. Interestingly many of the effects of blue light (including ROS generation) could be at least partly blocked by the TRPV ion channel inhibitor capsazepine (Figure 6).

One intriguing question is how PBM generated ROS can apparently be both beneficial and detrimental. If superoxide is produced inside mitochondria and is then dismutated to hydrogen peroxide by MnSOD then the uncharged H_2O_2 is free to diffuse outside the mitochondria where it can take part in many signaling pathways (55). This manageable level of H_2O_2 has recently been termed "oxidative eustress" (56). However if the levels of superoxide within the mitochondria exceed the capacity of MnSOD to detoxify it then the charged $O_2^{-\bullet}$ may accumulate within the mitochondrial matrix and cause damage (57). The ability of MnSOD to detoxify superoxide may depend on the rate at which $O_2^{-\bullet}$ is produced, which in turn may depend on the rate at which light is delivered i.e. the power density. This consideration may explain some observations that have been made where the biological effects of PBM depended on the power density of the light (mW/cm^2), and not on the total dose (J/cm^2) (58–60).

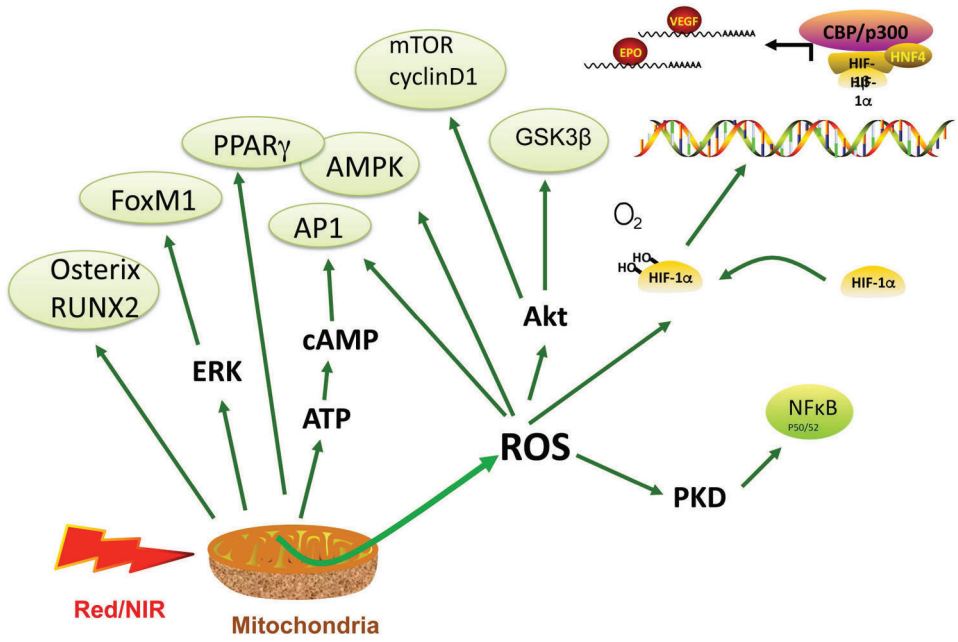

Figure 6. Activation of transcription factors and signaling pathways after PBM
Akt, protein kinase B; AMPK, 5′ adenosine monophosphate-activated protein kinase; AP1, activator protein 1; ATP, adenosine triphosphate; camp, cyclic adenosine monophosphate; CBP, CREB-binding protein; cyclin D1, cyclin-rependent kinase co-regulator; EPO, erythropoietin; ERK, extracellular regulated kinase; FoxM1, forkhead box protein M1; GSK3β, glycogen synthase kinase 3 beta; HIF1α, hypoxia-inducible factor 1alpha; mTOR, mechanistic target of rapamycin; osterix, osteoblast-specific transcription factor; p300, CBP co-activator; PPARγ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; RUNX2, runt-related transcription factor 2; VEGF, vascular endothelial growth factor.

It should be noted that there is evidence that another mechanism has been detected that relies on light generated ROS (61). This involves the ROS activation of latent transforming growth factor beta (TGF-β). The ROS generated by PBM can activate TGF-β in an extracellular manner by destroying the disulfide bond linking the latency associated peptide to the TGF-β.

LIGHT-INDUCED ACTIVATION OF TRANSCRIPTION FACTORS AND SIGNALING PATHWAYS

Many of the secondary mediators of PBM (ROS, NO, cAMP) are able to activate transcription factors and signaling pathways. This activation of transcription factors is proposed to explain why a relatively brief exposure to light can have long-lasting results. See Figure 6 for a graphical depiction of some of these transcription factors.

As mentioned above, the redox-sensitive NF-kB was activated in embryonic fibroblasts (45). Many transcription factors related to osteogenesis have been reported to be activated by light. Receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane protein member of the TNF superfamily, involved in bone regeneration and remodeling (acting on osteoclast differentiation and activation). It is also a ligand for osteoprotegerin (OPG). The RANKL/OPG ratio determines whether bone is removed or formed during the remodeling process (62). Parenti et al. investigated the RANKL/OPG ratio in osteoblast-like cells that were irradiated with GaAlAs laser (915 nm) using doses ranging from 1 to 50 J cm^{-2}. It seems that this ratio after PBM depends on the tissue and on the parameters used, since there was an increase in RANKL/OPG ratio in human alveolar bone-derived cells irradiated with 780 nm light, while in rat calvarial cells irradiated with 650 nm light the results were the opposite (63). Runt-related transcription factor 2 (RUNX-2) is related to osteoblastic differentiation and skeletal morphogenesis. Its cross-talk between the wnt (wingless-tail)/β-catenin signaling pathway regulates the expression of genes related to extracellular matrix components during bone cell proliferation (64). PBM can increase the expression of RUNX-2, contributing to a better tissue organization, even in diabetic animals as seen by Patrocínio-Silva (65). Osterix is another transcription factor related to osteogenesis (66). Wang et al. found that PBM

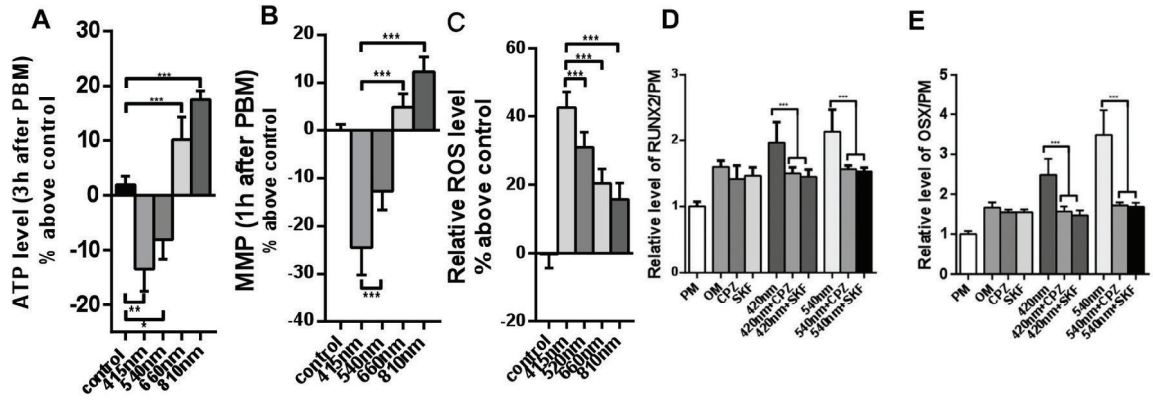

Figure 7. Effect of PBM with four different wavelengths on human adipose-derived stem cells (hADSC)
hADSCs in prolioferation medium (PM) were exposed to 3 J/cm^2 of 415, 540, 660, or 810 nm light. (A) ATP measured 3 h post PBM for luciferase assay. (B) MMP measured by TMRM 1 h post PBM. (C) intracellular ROS measured by CM-H2DCFDA fluorescence probe 30 min post-PBM. (D) Expression of RUNX2 and (E) expression of osterix (OSX), both measured by RT-PCR after cells were cultured in osteogenic differentiation medium and received PBM as above every 2 days for 3 weeks. Both 415 nm and 540 nm gave significant increases in osteogenic markers that could be blocked by TRP ion channel inhibitors capsazepine, CPZ and SKF96365. 660 nm and 810 nm were less effective at osteogenic differentiation and ion channel blockers had no effect (data not shown). Partly adapted from data contained in (67).

with blue and green light increased expression of osterix and RUNX-2 in adipose derived stem cells cultured in differentiation medium (67) (Figure 7). Interestingly the TRP inhibitors capsazepine and SKF96365 (Figure 8) abrogated the upregulation of the differentiation markers in the case of blue and red light but not in the case of red and NIR light.

Hypoxia-inducible factor (HIF-1α) acts as a transcription factor to govern tissue responses to hypoxia. HIF-1α is stabilized at low oxygen tensions, but in the presence of higher oxygen concentrations it is rapidly degraded by prolyl hydroxylase enzymes, which are oxygen-dependent (68). HIF-1α activates

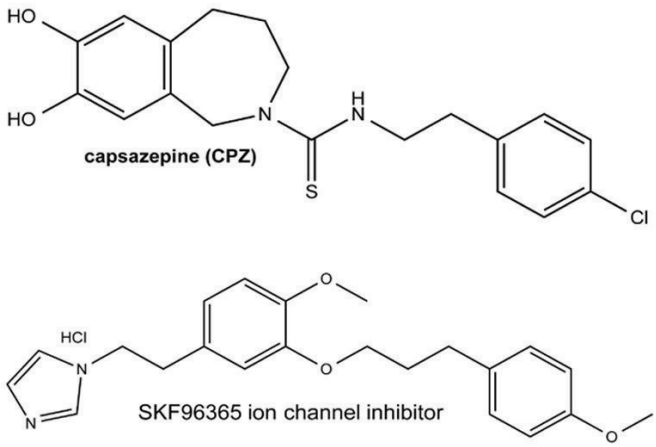

Figure 8. Chemical structures of TRP ion channel inhibitors
Capsazepine (CPZ) and broad-spectrum ion channel blocker, SKF96365.

genes that are important to the cellular response to hypoxic conditions, such as vascular endothelial growth factor (VEGF), VEGF-receptor, glucose carrier (GLUT-1) and phosphoglycerate kinase (PGK) genes. Since the tissue oxygen concentration does not undergo a sharp drop during PBM, HIF-1α activation may be mediated by activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (69). Another possible explanation is that the sudden boost in cellular respiration caused by light depletes the low amount of oxygen that is present in hypoxic tissues but which is not being rapidly consumed because of inhibited respiration. The sudden oxygen depletion seen after the inhibition of respiration is lifted, rapidly activates HIF-1α.

Cury et al. showed that PBM could have a pro-angiogenic effect using 660 nm and 780 nm light in skin flaps in rats. They observed that angiogenesis was induced by an increase in HIF-1α and VEGF expression, as well as by a decrease in matrix metalloproteinase 2 (MMP-2) activity (69). Cury observed that only 660 nm light was able to increase HIF-1α expression, and although VEGF induction occurred in all light doses used, only 40 J/cm^2 was able to induce angiogenesis, as well as increase MMP-2 activity.

PBM may exert a pro-survival effect on cells via the activation of AKT/GSK3β/β-catenin pathway. Basically, protein kinase B (also known as AKT) can

be activated by PBM, and then interact with glycogen synthase kinase 3β (GSK3β), inhibiting its activity. GSK3β is a serine-threonine kinase involved in cell death and in oncogenesis. There is evidence that GSK3β is involved in the pathogenesis of Alzheimer's disease, since it promotes hyperphosphorylation of tau protein and causes the formation of neurofibrillary tangles (NTFs) (70).

PBM-activates Akt, which phosphorylates the Ser9 residue in GSK3β, rendering the enzyme inactive. β-catenin is an important component of Wnt signaling pathway but GSK3β-mediated phosphorylation of β-catenin or the tau protein seems to enhance neuronal cell death, and conversely phosphorylated GSK3β leads to neuronal survival (71).

Zhang et al. (72) proposed that PBM activation of Akt could inhibit the activation of GSK3β, thus inhibiting Bax translocation. Using inhibitor compounds such as wortmannin and lithium chloride combined, there was a significant inhibition of the anti-apoptotic effect observed after PBM, suggesting that PI3K/Akt pathway (inhibited by wortmannin) and GSK3β translocation (inhibited by lithium chloride) play a key role in the protection against apoptosis caused by PBM. LiCl, however, was not able to reduce Bax translocation and apoptosis, so there must be other upstream regulators of Bax translocation during apoptosis.

Another pathway that can be activated by PBM-mediated activation of Akt is Akt/mTOR/cyclinD1. Sperandio et al. showed that oral dysplastic cells, considered pre-malignant, had their viability increased after PBM (660 or 780 nm, 2 to 6 J/cm^2) (73). There was higher expression of proteins related to cancer progression and invasion, i.e. Akt, HSP90, $pS6_{ser240/244}$, and Cyclin D1. The data suggested that Akt/mTOR/Cyclin D1 pathway was important for this phenotype differentiation, since the tested oral cancer cells showed higher levels of the signaling mediators that are part of this pathway (73). It is not yet clear precisely how PBM activates Akt, but it is well-known that Akt and ROS generation are closely intertwined (74).

Forkhead box protein M1 (FOXM1) is a transcription factor involved in the regulation of the transition from G1 to S phase of the cell cycle leading to mitotic division (75). FOXM1 is activated by epidermal growth factor via extracellular signal-regulated kinase (ERK) and allows implantation of the trophoblast (76). Ling et al. investigated the protective effect of PBM (632.8 nm) against senescence caused by UV light, and reported an activation of the ERK/FOXM1 pathway that caused a reduction in the expression of p21 protein and G1 phase arrest. Senescence was attenuated by over-expression of FOXM1c with or without PBM, and if FOXM1 was inhibited by shRNA, the effect of PBM in reducing cell senescence was abrogated. PBM promoted the nuclear translocation of, increasing FOXM1 accumulation in the nucleus and the transactivation of c-Myc and p21 expression.

Inhibition of the mitogen-activated kinase (MEK)/ERK pathway with an MEK inhibitor PD98059 prevented the nuclear translocation of FOXM1 after PBM, suggesting that Raf/MEK/MAPK/ERK signaling is crucial for the anti-cell senescence effect of PBM mediated by FOXM1 [89].

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that govern lipid and glucose metabolism and are involved in a variety of diseases, from metabolic disorders to cancer (77). PPARs play a role in the regulation of mitochondrial metabolism (78). They are present in airway epithelial cells, but also in smooth muscle cells, myofibroblasts, endothelial cells of the pulmonary vasculature and in inflammatory cells such as alveolar macrophages, neutrophils, eosinophils, lymphocytes and mast cells (79). They are nuclear receptors that regulate gene expression. PPAR-γ is involved in the generation of heat shock protein 70 (HSP-70), which is anti-inflammatory (80). Lima and co-authors reported a study in which rats were irradiated with 660 nm light (5.4 J) on the skin over the bronchus (chest). They observed a marked rise in the expression of PPAR mRNA after PBM, as well as increased PPAR-y activity in bronchoalveolar lavage (BALF) cells from animals subjected to laser treatment. In conclusion, Lima proposed that PBM can work as a homeostatic facilitator, increasing the expression of a transcription factor that is signaling the synthesis of HSP70 and other anti-inflammatory proteins (81).

One mechanism has been recently shown to be involved in the toxicity that follows an excessive exposure to PBM (82). This involves Activation Transcription Factor-4 (ATF-4) that can cause endoplasmic reticulum stress and autophagy. If the heat or ROS produced by excessive PBM were neutralized (cooling or antioxidants) or if ATF-4 was overexpressed the cells were rescued from phototoxicity.

PROTECTIVE MECHANISMS INDUCED BY PBM

Preconditioning describes the application of various interventions to animals or tissues, which has no major effects immediately, but can prevent or mitigate subsequent damage after a stressful event or hypoxic insult. Ischemic preconditioning (IPC) was the classical example of this approach, where repeated tissue ischemia produced by for instance by application of a tourniquet to a limb, led to protection against cardiac damage caused by a heart attack (83) or brain damage caused by a stroke (84). This concept was originally discovered using a rabbit heart attack model, when an initial episode of mild ischemia followed by reperfusion made the heart more resistant to a subsequent lethal ischemic insult (85). Locally released agonists such as adenosine, bradykinin, catecholamines and opioids activate the protective response through various G-protein coupled receptors which, when stimulated, increase activity of phospholipases C and D (86). Kinases such as protein kinase C tyrosine kinase (p38MAP kinase) contribute in the signaling pathway. Ytrehus et al. demonstrated inhibition of the protection obtained after IPC by administration of PKC inhibitors in the rabbit heart model (87). Agrawal et al. attempted to draw parallels between IPC and PBM (14). It is clear from the literature that PBM can carry out preconditioning both in vitro and in vivo.

In vitro application of light has been shown to protect cells (often neuronal cells) against a subsequent challenge with toxic substances. For instance, the voltage-dependent sodium channel-blocker (tetrodotoxin, TTX) functions by impeding neuronal impulse activity, decreasing ATP demand, and down-regulating cytochrome c oxidase activity. Treatment with PBM using 670 nm LEDs restored cytochrome c oxidase activity back to control levels or even higher (88). PBM treatments improved the retinal function in rats that had been administered toxic doses of methanol (methanol is metabolized to formic acid that inhibits cytochrome c oxidase (89)). Based on these findings, Wong-Riley et al. showed that PBM could reduce the toxic effect of the cytochrome c oxidase inhibitor, potassium cyanide (KCN) on primary cultured neurons (19). Pre-conditioning of the primary neuronal cells enhanced the protective action of PBM during KCN exposure (10–100 μM).

Liang et al. investigated the effect of NIR-LED preconditioning on primary neuronal cultures to test if it could inhibit apoptotic cell death induced by KCN (90). The primary neuronal cells were cultured from postnatal rat visual cortex and were pre-treated with LED for 10 min at a total energy density of 30 J/cm2 and were then exposed to potassium cyanide (100–300 μM) for 28 h. PBM pre-conditioning significantly reduced apoptosis, expression of caspase-3, reversed the increased expression of Bax and decreased expression of Bcl-2 to control levels.

Hearing damage caused by excessive noise exposure is due to levels of oxidative stress in auditory cells going beyond the intracellular threshold levels that can be repaired by natural defenses. PBM using 810 nm LEDs was applied to cultured HEI-OC1 cochlear hair cells that were subsequently challenged with gentamicin or lipopolysaccharide (91). These challenges produce oxidative stress in a similar manner to excessive noise. 1 or 3 J/cm^2 altered mitochondrial metabolism and oxidative stress response for up to 24 hours post treatment and decreased inflammatory cytokines, ROS and NO caused by gentamicin or lipopolysaccharide. Other workers have used PBM to treat or prevent hearing loss in various experimental models (92–94).

Application of light in vivo before the actual insult or injury occurs has been shown to be protective against muscle damage occurring after exercise in both animals (95, 96) and in humans (97, 98), cardiac damage occurring after heart attack (99), brain damage occurring after transient cerebral ischemia with bilateral common carotid artery occlusion (100). improved wound healing and protects against scarring after surgery (101), sunburn occurring after UV exposure (102).

STEM CELLS AND PBM

Stem cells are undifferentiated cells that can differentiate into more specialized cells (called progenitor cells) and can divide (through mitosis) to produce a continuous supply of stem cells. The stem-cell niche is a specific anatomic location that regulates how stem cells behave. The niche protects stem cells from dying, while also protecting the host by regulating excessive stem-cell proliferation (103). The niche has both anatomical and functional attributes and integrates the signals that ensure that stem cells can be made available on demand to repair damaged tissue or replenish short-lived somatic cells. This control-mechanism is carried out by a complicated range of factors including adhesion molecules, extracellular

matrix components, ATP, growth factors, cytokines, and physical factors such as oxygen tension, pH and Ca^{2+} concentration of the environment.

Due to the intense interest in possible therapeutic applications of stem cells, many investigators have asked how PBM and various light therapy interventions affect stem cell proliferation and differentiation (104, 105). Although the exact mechanisms by which PBM can activate stem cells is not known, there are some theories. The first theory is that stimulation of the mitochondria leads to a switch from anaerobic glycolysis to oxidative phosphorylation. This switch acutely increases the demand for oxygen by the newly activated mitochondria. However in the hypoxic niche (that is characteristic of stem cells) the oxygen supply is strictly limited (106). When the cells suddenly require more oxygen they must leave their niche and go in search of a higher pO_2 level (107). Whether they then proliferate or differentiate is governed by the specific cues they encounter in their new environment (108). Another somewhat related consideration concerns induction of ROS by PBM. It is thought that the reason that the stem cell niche is hypoxic is that very long-lived stem cells need to avoid any oxidative damage that may cause undesirable mutations in their DNA (109). When they encounter ROS their differentiation program is activated (110). Perhaps whether proliferation or differentiation is the primary outcome of PBM of stem cells may depend on the level of oxidative stress that the PBM parameters cause. In agreement with this theory we found that blue and green light that produced higher levels of ROS also produced more osteogenic differentiation of ADSCs cultured in osteogenic medium (67).

PBM has been widely studied in connection with stem cells (111). A large number of in vitro studies have been carried out using adipose-derived stem cells (112, 113), dental pulp stem cells (61, 104) or mesenchymal stem cells derived from bone marrow (114). While many different applications have been investigated, two of the most often studied appear to have been the use of PBM stimulated stem cells for formation of osteoblasts for bone repair (115), and for vascularization for wound healing (116). It has been shown that shining light on the legs (for instance) in order to irradiate the bone marrow can have remarkable effects. Damage sustained after a heart attack (117), or ischemic kidney injury (118), and defects in memory and spatial learning in Alzheimer's disease (119) all in experimental animal models, can be ameliorated by PBM delivered to the bone marrow. PBM delivered to the bone marrow can improve thrombocytopenia caused by gamma-irradiation (120) or by immune-mediated platelet destruction (121) in mouse models.

IN VIVO APPLICATIONS AND BIOMARKERS FOR PBM

Many of the experimental assays in tissues that have been taken from laboratory animals treated with PBM have looked at markers of oxidative stress and nitrosative stress. One of the most often studied markers is known as TBARS (thiobarbituric acid reacting substances) that is largely equivalent to malondialdehyde (produced by oxidation of unsaturated lipids) (122). TBARS have been shown to be lower in tissue removed from PBM treated wounded diabetic mice after five daily irradiations with a superpulsed 904 nm laser, 40 mW, 60 s (123). In addition to TBARS, reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities were significantly higher in muscle tissue from rats that underwent daily swimming training for 6 weeks with or without additional PBM mediated by an 808 nm laser at 4 J (124). Another paper showed that administration of PBM (660 nm and 780 nm, 10 J/cm^2, 40 mW, 3.2 J) both prior to a tibialis anterior muscle injury in rats by a cryoprobe, and after the muscle injury, had more pronounced benefits than PBM either before or after the injury alone. At day 7, TBARS were lower and CAT and SOD activity as well as glutathione peroxidase were higher in the double treated group (125).

De Marchi et al. showed that this remarkable reduction in markers of oxidative stress also applied in humans (126). A group of untrained male volunteers in a clinical trial received PBM (810 nm, 200 mW, 30 J at each site, 30 sec) using a multi-diode cluster at 12 sites on each lower limb (quadriceps, hamstrings, and gastrocnemius muscles) 5 min before a standardized progressive-intensity running protocol on a motor-driven treadmill until exhaustion. Levels of oxidative damage to lipids and proteins, SOD and CAT activities measured in blood samples, were significantly improved by PBM.

One of the more commonly reported actions of PBM is the induction of angiogenesis as observed during wound healing studies (127). One possible explanation for why PBM is effective at inducing

angiogenesis is the following. If cells have moderate levels of hypoxia as judged by the actual oxygen availability to the tissue, but the mitochondrial CCO enzyme is inhibited by bound NO, then the actual depletion of oxygen by respiration will be less pronounced that it otherwise would have been if CCO had not been inhibited. However, if PBM displaces the NO allowing respiration to resume, then the levels of oxygen will suddenly drop to very low levels causing the transcription factor HIF1α to be stabilized and VEGF and other pro-angiogenic mediators to be produced (128).

FUTURE DIRECTIONS

One of the intriguing questions that has never been satisfactorily answered is the following. If the initial increase in ROS produced in normal cells as a result of PBM, is indeed important in the physiological response, then what would be the effect of concomitant administration of anti-oxidants? Many individuals (especially those who are likely to be interested in alternative and complementary medicine) take large amounts of health supplements including a range of anti-oxidants. Would this consumption of anti-oxidants be counter-productive for the effects of PBM?

There are several similarities between the systemic effects of PBM and those of physical exercise. Both appear to cause a brief increase in ROS, but in the long-term can increase anti-oxidant defenses. A study (129) reported that administration of vitamins C and E abrogated the health-promoting effects of exercise training in healthy young men, as measured by insulin sensitivity and antioxidant defenses. On the other hand, PBM appears to combine well with exercise. One report showed that TBARS were reduced, while glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) were increased in muscle tissue from rats that underwent daily swimming training for 6 weeks combined with PBM, compared to either exercise or PBM alone (124).

Mitochondria are generated by the expression of genes on both nuclear and mitochondrial genomes. Mitochondrial biogenesis is highly responsive to cellular demands for energy and environmental stimuli (130). The mechanistic target of rapamycin (mTOR) pathway regulates mitochondrial biogenesis to co-ordinate energy homeostasis with cell growth (131). It is well known that exercise induces the proliferation of mitochondria within the cells, particularly mitochondrial biogenesis within muscle cells (132). A recent paper (133) reviewed the new subject of "exercise mimetics" in other words, pharmacological substances (aminoimidazole carboxamide ribonucleotide (AICAR), endurobol, irisin, resveratrol, (−)epicatechin) that can duplicate many of the physiological effects of exercise without actually doing any. PGC-1α (peroxisome proliferator activated receptor γ coactivator 1α) is a master transcriptional coactivator regulating oxidative metabolism in skeletal muscle (134, 135). Many of these "exercise mimetics" can activate PGC-1α. Moreover research is progressing into dietary modifications that can stimulate PGC-1α (136). One paper showed that PGC-1α mRNA was increased in rat gastrocnemius muscle using PBM (3.75 J/cm^2 of 810 nm). It is highly likely that in some circumstances, PBM can be considered to act like an "exercise mimetic", but further work is needed to fully corroborate this hypothesis.

AICAR can activate adenosine monophosphate (AMP)-activated kinase (AMPK) and was shown to improve exercise performance (running endurance of untrained mice) by 45% (137). AMPK acts as an 'energy sensor' and constitutes an important regulator of cellular metabolism. It is activated in states of ATP depletion such as excessive training, hypoxia/ischemia, heat stress and starvation (138). Activation of AMPK leads to processes that inhibit ATP consumption, such as gluconeogenesis in the liver and fatty acid release in liver and adipose cells. A recent study (139) found that AMPK activation can promote mitochondrial biogenesis by phosphorylating the epigenetic factors DNMT1, RBBP7, and HAT1 leading to increased expression of PGC-1α, transcription factor A (Tfam), and uncoupling proteins 2 and 3 (UCP2 and UCP3). Zhang et al. showed that PBM (810 nm, 3 J/cm^2) could promote mitochondrial biogenesis in megakaryocytes (MKs) (120). Four hours after PBM there was upregulation of PGC-1α followed by increases in Tfam, dynamin-related protein (Drp1), mitochondrial fission 1 protein (Fis1), and mitochondrial fission factor (Mff). The role of PGC-1α was confirmed by the finding that PBM on MKs sorted from PGC-1α +/− heterozygous transgenic mice produced only a 10% rise in mitochondrial mass, while wild-type MKs showed an 87% increase in mitochondrial mass.

Interestingly, another compound that has been shown to act (at least partly) by activation of AMPK and

PGC-1α is the TRPV1 ion channel agonist, capsaicin (CAP) (140). CAP has been shown to increase mitochondrial biogenesis (141), and, in addition, dietary supplementation reduces physical fatigue and improves exercise performance in mice (142). We have previously shown that PBM can activate TRPV1 channels in a similar manner to capsaicin (67, 143). See Figure 9 for a summary of the similarities between PBM and exercise.

Certainly animal experiments have shown that exercise and PBM combine very well together. Aquino and colleagues reported that PBM combined with swimming exercise improved the lipid profiles in rats fed a high-fat high-cholesterol diet better than either PBM or exercise alone (144).

PBM can clearly function as a performance-enhancing intervention in athletic activity and to enhance response to sports training regimens (145, 146). This has been shown in individual athletes (for instance an elite runner (97)) and in sports teams such as a volleyball team in a National Championship in Brazil (98). However, since at present there is no conceivable biochemical assay for having exposed oneself to light, it cannot be outlawed by the World Anti-Doping Agency (WADA).

Another important question that remains to be settled is the degree to which PBMT is a localized therapy, and to what extent it has systemic effects? In other words, does the principal therapeutic response happen in the tissue that receives the light? However, multiple publications report substantial systemic effects of PBMT, both in experimental animals and in humans. One example is from the Mitrofanis laboratory where they studied transcranial PBM for Parkinson's disease in mouse models (147). Having established the effectiveness of shining light on the head, they proceeded to cover up the head with aluminum foil and shine light on the rest of the mouse body (148). There was still a significant benefit on the brain (although not as pronounced as transcranial light delivery). They called this phenomenon an "abscopal neuroprotective effect". Another example is from a human clinical trial of wound healing (149). These workers carried out a randomized, triple-blind, placebo-controlled trial with 22 healthy subjects who received a partial thickness abrasion on each forearm. They received either real or sham PBM to one of the two wounds. At days 6, 8, and 10 the real PBM group had smaller wounds than the sham group for both the treated and the untreated wounds ($P < 0.05$). A third example is the growing popularity of intravenous laser therapy (135, 150, 151). Conditions treated include type 2 diabetes, fibromyalgia/chronic pain, and shoulder pain.

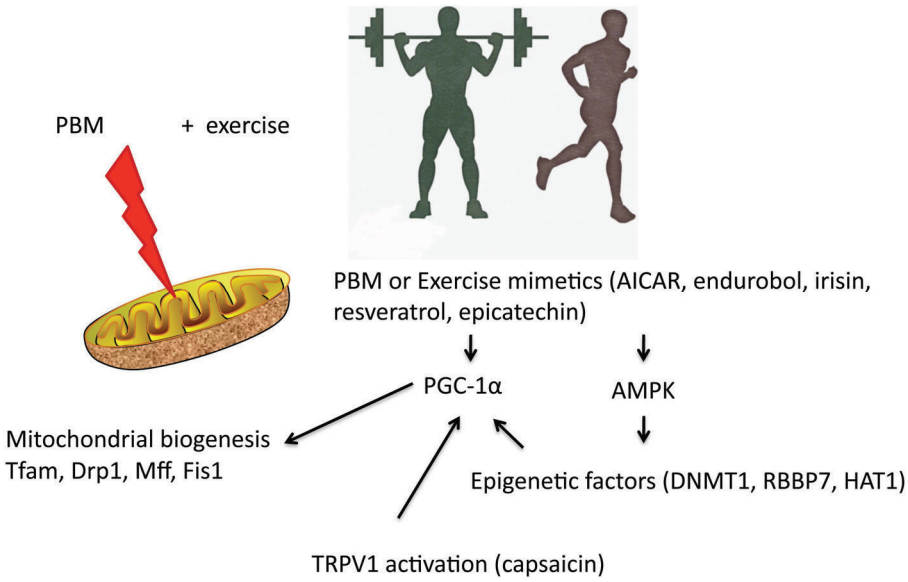

Figure 9. **Future directions. Similarities between PBM and exercise and synergistic combinations**
AICAR, aminoimidazole carboxamide ribonucleotide; PCG-1α, peroxisome proliferator activated receptor γ coactivator 1α; AMPK, adenosine monophosphate (AMP)-activated kinase; DNMT1, DNA methyltransferase 1; RBBP7, RB binding protein 7; HAT1, histone deacetylase 1; TRPV1, transient receptor potential vanilloid 1; Tfam, mitochondrial transcription factor A; Drp1, dynamin-related protein; Fis1, mitochondrial fission 1 protein; Mff, mitochondrial fission factor.

In conclusion, it has emerged that many of the mechanistic pathways for mediating the biological effects of PBM do in fact involve ROS. This came as somewhat of a surprise originally, as many people believed that ROS and oxidative stress were entirely harmful. However, the prevailing view on the merits of limited levels of ROS and brief bouts of oxidative stress has changed away from a black and white dogma (152, 153). Now it is accepted that ROS can have both good and bad sides depending on the magnitude and duration (56).

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PHOTOBIOMODULATION IN HUMAN MUSCLE TISSUE: AN ADVANTAGE IN SPORTS PERFORMANCE?

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REVIEW

ABSTRACT

Photobiomodulation (PBM) describes the use of red or near-infrared (NIR) light to stimulate, heal, and regenerate damaged tissue. Both preconditioning (light delivered to muscles before exercise) and PBM applied after exercise can increase sports performance in athletes. This review covers the effects of PBM on human muscle tissue in clinical trials in volunteers related to sports performance and in athletes. The parameters used were categorized into those with positive effects or no effects on muscle performance and recovery. Randomized controlled trials and case-control studies in both healthy trained and untrained participants, and elite athletes were retrieved from MEDLINE up to 2016. Performance metrics included fatigue, number of repetitions, torque, hypertrophy; measures of muscle damage and recovery such as creatine kinase and delayed onset muscle soreness. Searches retrieved 533 studies, of which 46 were included in the review (n = 1045 participants). Studies used single laser probes, cluster of laser diodes, LED clusters, mixed clusters (lasers and LEDs), and flexible LED arrays. Both red, NIR, and red/NIR mixtures were used. PBM can increase muscle mass gained after training, and decrease inflammation and oxidative stress in muscle biopsies. We raise the question of whether PBM should be permitted in athletic competition by international regulatory authorities.

Keywords: LEDT; LLLT; creatine kinase; delayed onset muscle soreness; fatigue; photobiomodulation.

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PROPOSED MECHANISMS OF PHOTOBIOMODULATION OR LOW-LEVEL LIGHT THERAPY

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CASE REPORT

ABSTRACT

Photobiomodulation (PBM) also known as low-level laser (or light) therapy (LLLT), has been known for almost 50 years but still has not gained widespread acceptance, largely due to uncertainty about the molecular, cellular, and tissular mechanisms of action. However, in recent years, much knowledge has been gained in this area, which will be summarized in this review. One of the most important chromophores is cytochrome c oxidase (unit IV in the mitochondrial respiratory chain), which contains both heme and copper centers and absorbs light into the near-infra-red region. The leading hypothesis is that the photons dissociate inhibitory nitric oxide from the enzyme, leading to an increase in electron transport, mitochondrial membrane potential and ATP production. Another hypothesis concerns light-sensitive ion channels that can be activated allowing calcium to enter the cell. After the initial photon absorption events, numerous signaling pathways are activated via reactive oxygen species, cyclic AMP, NO and Ca2+, leading to activation of transcription factors. These transcription factors can lead to increased expression of genes related to protein synthesis, cell migration and proliferation, anti-inflammatory signaling, anti-apoptotic proteins, antioxidant enzymes. Stem cells and progenitor cells appear to be particularly susceptible to LLLT.

KEYWORDS

Low Level Light Therapy; Mechanism; Mitochondria; Cytochrome c oxidase; Photobiomodulation; Light sensitive ion channels

HISTORICAL INTRODUCTION

The first evidence of the action of low-level laser irradiation came from the experiments of Dr. Endre Mester, at the Semmelweis Medical University (Hungary) in 1967. The experiment consisted of shaving the back of mice and implanting a tumor via an incision in the skin. Mester applied light from a ruby laser (694 nm) in an attempt to repeat one of the experiments described by McGuff in Boston [1]. McGuff had used the newly discovered ruby laser to cure malignant tumors both in rats and also tested it in human patients. Unfortunately (or perhaps fortunately for scientific discovery), Mester's laser had only a small fraction of the power possessed by McGuff's laser. Therefore Mester failed to cure any tumors, but did observe a faster rate of hair growth in the treated mice compared to the controls [2], calling this effect "laser biostimulation". He later used a HeNe laser (632.8 nm) to stimulate wound healing in animals, as well as in clinical studies [3]. For several decades, the profession believed that coherent laser light was necessary, but as of today, non-coherent light sources such as light emitting diodes (LED) have proved to be just as efficient as lasers in promoting photobiomodulation (PBM) [4].

Low-level light therapy (LLLT) or PBM consists of the application of light with the purpose of promoting tissue repair, decreasing inflammation, and producing analgesia, usually using a low-power light source (laser or LED) [5]. Because of the low power, (usually below 500 mW depending on the target tissue) the treatment causes no evident temperature rise in the treated tissue and, therefore, no significant

change in the gross tissue structure [6]. PBM/LLLT differs from other light-based treatments because it does not ablate and is not based on heating. It also differs from photodynamic therapy (PDT), which is based on the effect of light to excite exogenously delivered chromophores to produce toxic reactive oxygen species (ROS) [7].

With the advantage of being non-invasive, the applications of PBM are broad, going from pain relief to promoting the recovery of tendinopathies, nerve injuries, osteoarthritis and wound healing. The complete mechanism of action is still elusive, but the knowledge that has been gained so far is the subject of the present review. The importance of parameters in PBM will be discussed, together with the possible chromophores or photoacceptors, signaling molecules produced after photon absorption, transcription factors that may be activated to account for the lasting effects of a brief light exposure, downstream effector molecules that follow on, and specific mechanisms that may be applicable to the different cells and tissues being treated with PBM.

PARAMETERS OF PBM

The light parameters and the doses applied are fundamental in PBM. The most important parameters regarding the light source and the light doses are described on the following tables (Table 1 and Table 2, respectively):

Low level light therapy refers to the use of light in the red or near-infrared region, with wavelengths usually in the range of 600 to 700nm and 780 to 1100 nm, and the laser or LEDs typically having an irradiance or power density between 5 mW cm^{-2} to 5 W cm^{-2}. This type of irradiation can be a continuous wave or a pulsed light consisting of a relatively low-density beam (0.04 to 50 J cm^{-2}), but the output power can vary widely from 1 mW up to 500 mW in order not to allow thermal effects [8]. The wavelength range between 700 and 780 nm has been found to be rather ineffective as it coincides with a trough in the absorption spectrum of cytochrome c oxidase (see later). Moreover red/NIR light is chosen because its penetration through tissue is maximal in this wavelength range, due to lower scattering and absorption by tissue chromophores. Although for many years it was thought that the monochromatic nature and coherence of laser light provided some sort of added benefit over non-coherent LED light, this view is no longer widely held. Continuous or pulsed light sources have both been used. The studies performed for PBM on acute pain and pre-operative analgesia show that a single treatment (usually only 30–60 seconds) is enough to cause analgesia, while for chronic pain and some degenerative conditions, more sessions are required [5].

It is known that if the incorrect parameters are applied, the treatment is likely to be ineffective. There is a

Table 1: Description of the irradiation parameters.

IRRADIATION PARAMETERS		
Irradiation Parameter	Measurement unit	Description
Wavelength	nm	Light is an electromagnetic form of energy with a wave-like behavior. Its wavelength is measured in nanometers (nm), and it is visible within the 400–700 nm range.
Irradiance	W cm^{-2}	It can also be called Power Density or Intensity, and corresponds to the power (in W) divided by the area (in cm^{-2}).
Pulse Structure	Peak Power (W) Pulse frequency (Hz) Pulse width (s) Duty cycle (%)	If the beam is pulsed, the Power should be called Average Power, which is calculated as follows: Average Power (W) = Peak Power (W) x pulse width (s) x pulse frequency (Hz)
Coherence	Coherence length depends on spectral bandwidth	Coherent light produces laser speckle, which is believed to play an important role on photobiomodulation interaction with cells and organelles.
Polarization	Linear polarized or circular polarized	Polarized light is known to lose its polarity in highly scattering media such as biological tissues, therefore this property is not considered very often on the effects of PBM.

biphasic dose response curve (or the phenomenon known as hormesis) in which when too low or too high doses (fluence (J/cm^2), irradiance (mW/cm^2), delivery time, or number of repetitions) can lead to no significant effect or, sometimes, excessive light delivery can lead to unwanted inhibitory effects [8], [9]. This biphasic response follows the "Arndt-Schulz Law" (which states that weak stimuli slightly accelerate vi¯tal activity, stronger stimuli raise it further until a peak is reached, whereas even stronger stimuli suppress it until a negative response is achieved), and has been demonstrated several times in low level light works [10]–[16].

For instance, Bolton irradiated macrophages with the same energy density (in J cm^{-2}) but with different irradiances (W cm^{-2}), and observed different results between the two conditions [17]; Karu and Kolyakov, in 2005, found that the stimulation of DNA synthesis rate is dependent on light intensity at a constant energy density of 0.1 J cm^{-2} with a clear maximum at 0.8 mW cm^{-2} [18]; Orion and co-workers worked with a constant energy density and different irradiances on an infarct model in rats after induced heart attack, and found that the beneficial effects were obtained at 5 mW cm^{-2}, while with irradiances as low as 2.5 mW cm^{-2} or as high as 25 mW cm^{-2} there were significantly less effects [11]; finally, Lanzafame and collaborators used a fixed energy density of 5 J cm^{-2} and variable irradiances, ranging from 0.7 to 40 mW cm^{-2}, observing that only with 8 mW cm^{-2} there were improvements on pressure ulcers in the treated mice [10].

There were some studies with constant irradiance and varying fluences. al-Watban and Andres, for instance, observed the effects of He-Ne laser on the proliferation of Chinese hamster ovary and human fibroblast. The light was delivered at a constant irradiance of 1.25 mW cm^{-2}, and a biphasic dose response was found with a peak at 0.18 J cm^{-2} [19]. Zhang and collaborators also found a biphasic dose response when they observed a maximum increase in human fibroblast cells after irradiation of light at 628 nm with fluence of 0.88 J cm^{-2}, while there was a marked reduction in the proliferation rate at 9 J cm^{-2} [20].

Regarding the time interval between treatments, Brondon and colleagues found that the best results for human HEP-2 and murine L-929 cells proliferation rates were achieved with two treatments per day, in comparison with one or four treatments per day. They used an LED with light at 670 nm and irradiance fixed at 10 mW cm^{-2}, and each treatment consisted on the delivery of 5 J cm^{-2} (the course was stopped after 50 J cm^{-2} had been delivered) [21].

There are also some systematic reviews and meta analyses of randomized, double-blind, placebo-controlled, clinical trials (RCTs) available in the literature. We can give as an example the review from Bjordal, who identified 14 RCTs of suitable methodological quality. 4 of them failed to report significant effects because the irradiance was either too low or too high, or because there was an insufficient delivery of energy [22]. Another

Table 2: Description of the light dose parameters.

LIGHT DOSE PARAMETERS		
Irradiation Parameter	Measurement unit	Description
Energy	Joules (J)	It cannot be mistaken as dose, as it assumes reciprocity (the inverse relationship between power and time). It is calculated as:Energy (J) = Power (W) x Time (s)
Energy Density	J cm^{-2}	This is an important descriptor of dose, but it could be unreliable when we consider that it assumes a reciprocity relationship between irradiance and time.
Irradiation Time	s	Possibly the best way to prescribe and to record PBM would be to define the four parameters of Table 1 and then define the irradiation time as thereal "dose."
Treatment Interval	Hours, days or weeks	Different time intervals may result in different outcomes, but more data need to be gathered in order to define the extent of the differences between them.

review was performed by Tumilty with 25 RCTs of tendinopathies, 55% of which failed to produce positive outcomes because of an excessive irradiance delivery in comparison with the guidelines set by the World Association for Laser Therapy [23].

As we have seen, at low doses (up to 2 J cm^{-2}), PBM stimulates proliferation, whereas at higher doses (16 J cm^{-2} or higher) PBM is suppressive, pointing to the dose dependence of biological responses after light exposure [24]. Other authors, however, have observed stimulating effects outside the cited range [25], [26]. A number of different laser light sources, including helium-neon, ruby, and galliumaluminum-arsenide, have been used to deliver PBM in different treatments and on different schedules.

Many researchers fail to consider the importance of selecting the optimum parameters, or they do not have the necessary instrumentation or trained personnel to measure them accurately, resulting in treatment failures. Another cause of failure occurs whenever the terms are misused or wrongly reported. For instance, energy (J) or energy density (J cm^{-2}) are both usually referred to as "dose", but they are, in fact, different calculations, as demonstrated in table 2 [27].

MOLECULAR MECHANISMS OF PBM

Chromophores

Cytochrome c oxidase – Cytochrome C oxidase (Cox) is the terminal enzyme of the electron transport chain, mediating the electron transfer from cytochrome c to molecular oxygen. Several lines of evidence show that Cox acts as a photoacceptor and transducer of photosignals in the red and near-infrared regions of the light spectrum [28]. It seems that PBM increases the availability of electrons for the reduction of molecular oxygen in the catalytic center of Cox, increasing the mitochondrial membrane potential (MMP) and the levels of ATP, cAMP and ROS as well [29].

PBM increases the activity of complexes I, II, III, IV and succinate dehydrogenase in the electron transfer chain. Cox is known as complex IV and, as mentioned before, appears to be the primary photoacceptor. This assumption is supported by the increased oxygen consumption during low-level light irradiation (the majority of the oxygen consumption of a cell occurs at complex IV in the mitochondria), and by the fact that sodium azide, a Cox inhibitor, prevents the beneficial effect of PBM. Besides ATP and cAMP, nitric oxide (NO) level is increased, either by release from metal complexes in Cox (Cox has two heme and two copper centers) or by up-regulation of Cox activity as a nitrite reductase [30].

In fact, it was proposed that PBM might work through the photodissociation of NO from Cox, thereby reversing the mitochondrial inhibition of cellular respiration due to excessive NO binding [31]. NO is photodissociated from its binding sites on the heme iron and copper centers from Cox, where it competes with oxygen and reduces the necessary enzymatic activity. This allows an immediate influx of oxygen and, thus, the resumption of respiration and generation of reactive oxygen species. NO can also be photo-released from other intracellular sites, such as nitrosylated hemoglobin and myoglobin [32].

Retrograde mitochondrial signaling – One of the most accepted mechanisms for light-cell interaction was proposed by Karu[33], referring to the retrograde mitochondrial signaling that occurs with light activation in the visible and infrared range (Figure 1). According to Karu, the first step is the absorption of a photon with energy hv by the chromophore Cox. This interaction increases mitochondrial membrane potential ($\Delta\psi m$), causing an increase in the synthesis of ATP and changes in the concentrations of reactive oxygen species (ROS), Ca^{2+} and NO. Furthermore, there is a communication between mitochondria and the nucleus, driven by changes in the mitochondria ultrastructure, i.e. changes in the fission-fusion

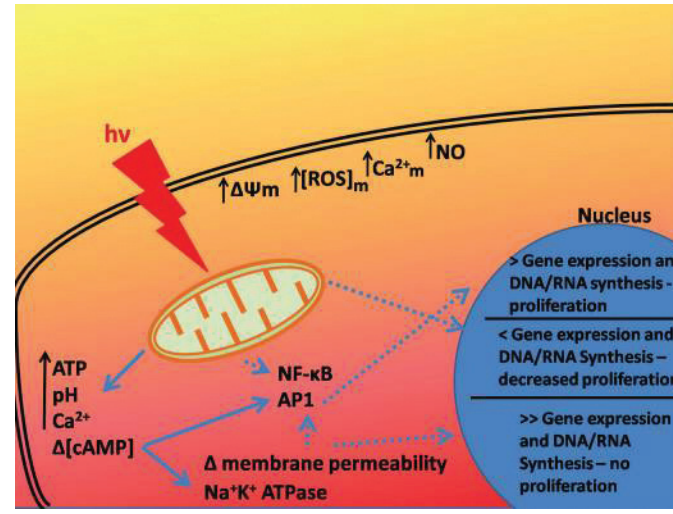


Figure 1: Scheme of mitochondrial retrograde signaling pathways as proposed by Karu. The main pathway is represented by continuous arrows, and the complementary ones are represented by segmented arrows.

homeostasis in a dynamic mitochondrial network. The alteration in the mitochondrial ultrastructure induces changes in ATP synthesis, in the intracellular redox potential, in the pH and in cyclic adenosine monophosphate (cAMP) levels. Activator protein-1 (AP1) and NF-κB have their activities altered by changes in membrane permeability and ion flux at the cell membrane. Some complementary routes were also suggested by Karu, such as the direct up-regulation of some genes [34].

Light sensitive ion channels – The most well-known ion channels that can be directly gated by light are the channelrhodopsins (ChRs), which are seven-transmembrane-domain proteins that can be naturally found in algae providing them with light perception. Once activated by light, these cation channels open and depolarize the membrane. They are currently being applied in neuroscientific research in the new discipline of optogenetics [35].

However, members of another broad group of ion-channels are now known to be light sensitive [36]. These channels are called "transient receptor potential" (TRP) channels as they were first discovered in a Drosophila mutant [36] and are responsible for vision in insects. There are now at least 50 different known TRP isoforms distributed amongst seven subfamilies [37], namely the TRPC ('Canonical') subfamily, the TRPV ('Vanilloid'), the TRPM ('Melastatin'), the TRPP ('Polycystin'), the TRPML ('Mucolipin'), the TRPA ('Ankyrin') and the TRPN ('NOMPC') subfamilies (see Figure 2). A wide range of stimuli modulate the activity of different TRP such as light, heat, cold, sound, noxious chemicals, mechanical forces, hormones, neurotransmitters, spices, and voltage. TRP are calcium channels modulated by phosphoinositides [38].

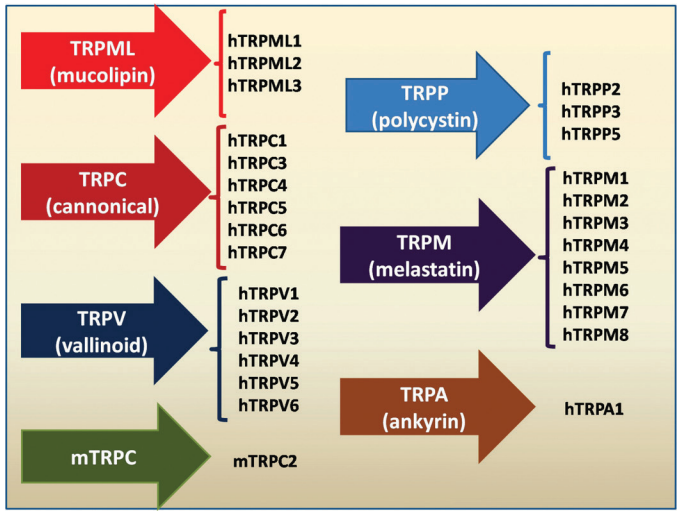


Figure 2: All the seven subfamilies of Transient Receptor Potential Channels (TRP).

The evidence that light mediated activation of TRP is responsible for some of the mechanisms of action of PBM is somewhat sparse at present, but is slowly mounting. Mast cells are known to accumulate at the site of skin wounds, and there is some degree of evidence suggesting that these cells play a role in the biological effects of laser irradiation on promoting wound healing. Yang and co-workers demonstrated that after laser irradiation (532 nm), the intracellular [Ca^{2+}] was increased and, as a consequence, there was a release of histamine. If the TRPV4 inhibitor, ruthenium red, was used, the histamine release was blocked, indicating the central role of these channels in promoting histamine-dependent wound healing after laser irradiation [39].

It seems that TRPV1 ion channels are involved in the degranulation of mast cells and laser-induced mast cell activation. It was demonstrated that capsaicin, temperatures above 42°C and acidic pH could induce the expression of TRPV1 in oocytes, and these ion channels can be activated by green light (532 nm) in a power-dependent manner, although blue and red light were not able to activate them [40]. Infrared light (2,780 nm) attenuates TRVP1 activation by capsaicin in cultured neurons, decreasing the generation of pain stimuli. TRPV4 is also attenuated by laser light, but the antinociceptive effect was less intense, therefore the antinociception in this model is mainly dependent on TRPV1 inhibition [41] The stimulation of neurons with pulsed infrared light (1,875 nm) is able to generate laser-evoked neuronal voltage variations and, in this case, TRPV4 channels were demonstrated to be the primary effectors of the chain reaction activated by the laser [42]. However, these effects after exposure to light above 1,500 nm might occur due to thermal effects, since water is the main absorber in this region of infrared spectrum. If it turns out that green light is primarily needed to activate ion channels then clinical applications may be limited due to lack of penetration into tissue.

Direct cell-free light-mediated effects on molecules– There have been some scattered reports that light can exert effects on some important

molecules in cell free systems (in addition to the established effect on Cox). The latent form of transforming growth factor beta has been reported to be activated by light exposure [43]. Copper/Zinc Superoxide dismutase (Cu-Zn-SOD) from bovine erythrocytes that had been inactivated by exposure to pH 5.9 was reactivated by exposure to He-Ne laser light (632.7 nm) [44]. The same treatment also reactivated the heme-containing catalase. Amat et al. showed that irradiation of ATP in solution by 655 nm or 830 nm light appeared to produce changes in its enzyme reactivity, fluorescence and Mg2+ binding capacity [45]. However other workers were unable to repeat this somewhat surprising result [46].

Signaling Molecules

Adenosine triphosphate (ATP) – An increase in intracellular ATP is one of the most frequent and significant findings after PBM both in vitro and in vivo [47]. The stimulated synthesis of ATP is caused by an increased activity of Cox when activated by light. According to Ferraresi et al. [48], increased Cox activity is the mechanism of enhanced muscle performance when PBM is carried out before various types of exercises, for example. The authors found an increased ATP synthesis after LED (850±20 nm and 630±10 nm) therapy in different muscles (one with a predominantly aerobic metabolism, and other with mixed aerobic and glycolytic metabolism), just like previous data from Ferraresi et al. [49].

Extracellular ATP participates in a wide array of signaling pathways, known as purinergic signaling [50]. Originally discovered by Burnstock [51] as a non-adrenergic, non-cholinergic neurotransmitter, ATP purinergic signaling is mediated by P2Y G-protein-coupled receptors, and P2X ligand-gated ion channels [52]. ATP can be hydrolyzed to adenosine that carries out signals via the P1 G-protein-coupled receptor [53]. Up to the present date we are not aware of any studies that specifically show that extracellular (as opposed to intracellular) ATP or adenosine can be stimulated by PBM.

Cyclic AMP (cAMP) – Several workers have shown an increase in adenosine-3',5'-cyclic-monophosphate (cAMP) after PBM [54], [55]. Although it is tempting to suppose that this increase in cAMP is a direct consequence of the rise in ATP caused by light, firm evidence for this connection is lacking. It has been reported that cAMP-elevating agents, i.e. prostaglandin E_2, inhibit the synthesis of TNF and, therefore, down-regulate the inflammatory process. Lima and co-authors investigated the signaling pathways responsible for the anti-inflammatory action of PBM (660 nm, 4.5 J cm^{-2}) in lung and airways. They found reduced TNF levels in the treated tissue, probably because of an increase in cAMP levels. Furthermore, the authors demonstrated that the inflammation caused by LPS or by TNF in mice lungs was inhibited by cAMP-elevating agents. Rolipram, a cAMP-elevating agent, acts through inhibition of the enzyme phosphodiesterase, but it does not share this mechanism with low level light [54].

cAMP exerts its cellular effects via activation of three different kinds of sensors: cAMP-dependent protein kinase A (PKA) which phosphorylates and activates cAMP response element-binding protein (CREB), which then binds to CRE domain on DNA and in turn activates genes [56];cyclic nucleotide-gated channels (CNGC) [57] and exchange proteins directly activated by cAMP (Epac) [58].

Reactive oxygen species (ROS) – It was shown that PBM can produce mitochondrial ROS leading to activation of the transcription factor nuclear factor kappa B (NF-κB), which can act as a redox-sensor. The fact that the addition of antioxidants inhibits the activation of NF-κB by 810 nm light reinforces this assumption [59].

ROS are one of the classic "Janus face" mediators; beneficial in low concentrations and harmful at high concentrations; beneficial at brief exposures and harmful at chronic long-term exposures [60]. ROS are produced at a low level by normal mitochondrial metabolism [61]. The concept of mitohormesis was introduced to describe the beneficial of low controlled amounts of oxidative stress in the mitochondria [62]. However when the mitochondrial membrane potential is altered either upwards or downwards, the amount of ROS is increased. In normal cells, absorption of light by Cox leads to an increase in mitochondrial membrane potential and a short burst of ROS is produced. However when the mitochondrial membrane potential is low because of pre-existing oxidative stress [63], excitotoxicity [64], or inhibition of electron transport [63], light absorption leads to an increase in mitochondrial membrane potential towards normal levels and the production of ROS is lowered.

There are many different cellular systems that are

designed by evolution to detect excessive levels of ROS and activate transcription factors to produce extra levels of antioxidant defenses [65]. Hydrogen peroxide and lipid hydroperoxides [66] are thought to be the ROS most likely to carry out beneficial redox signaling by reversible oxidation of cysteine thiols in the sensor protein.

Calcium (Ca2+) – Changes in the mitochondrial ultrastructure may lead to alterations in Ca^{2+} concentration. The increment might be a result of Ca^{2+} influx from the extracellular environment and gated by the Ca^{2+}channel TRPV. There is evidence that cytosolic alkalinization can facilitate the opening of TRPV channels and, since laser irradiation can induce cellular alkalinization, PBM could induce TRPV opening and a consequent Ca^{2+} influx. In mast cells, this Ca^{2+} influx can mediate histamine release [67]. However it is also possible that light can directly activate TRPV channels as discussed above. It should be noted that PBM usually leads to an increase in intracellular Ca^{2+} as shown by fluorescent probes [68]. However when intracellular Ca^{2+} levels have been artificially raised (for instance by causing excitotoxicity with excess glutamate), then PBM can produce a drop in intracellular calcium and protect the neurons from dying [64]. The increase in calcium seen after PBM could also be a result of the release of Ca^{2+} from intracellular stores [69].

Calcium-sensitive signaling pathways are too numerous to cover in detail here, but include calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) and calcineurin (CaCN) [70], the extracellular calcium–sensing receptor (CaSR) [71], mitochondrial calcium signaling [72], calcium-sensitive adenylyl cyclase [73], and many others.

Nitric oxide (NO) – As mentioned above, NO is often found to be produced after PBM [74]. NO is a well-known vasodilator acting via stimulation of soluble guanylate cyclase to form cyclic-GMP (cGMP). cGMP activates protein kinase G, which causes reuptake of Ca^{2+} and opening of calcium-activated potassium channels. The fall in concentration of Ca^{2+} prevents myosin light-chain kinase (MLCK) from phosphorylating the myosin molecule, leading to relaxation of the smooth muscle cells in the lining of blood vessels and lymphatic vessels [75]. There are several other mechanisms by which NO could carry out signaling pathways, including activation of iron-regulatory factor in macrophages [76], modulation of proteins such as ribonucleotide reductase [77] and aconitase [78], stimulating ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase [79], and protein sulfhydryl group nitrosylation [80].

Activation of transcription factors

Nuclear factor kappa B (NFkB) – NF-κB is a transcription factor that regulates the expression of various genes related to many cellular functions, i.e. inflammatory and stress-induced responses and survival. Its activity is regulated by a negative feedback mediated by an inhibitor called IκB, which binds to NF-κB to inactivate it, or can undergo ubiquitination and go to proteasomal degradation in order to release NF-κB. The transcription factor, then, can be translocated to the nucleus and promote gene transcription. Several lines of evidence reveal that NF-κB is redox-sensitive, since ROS can directly activate it, or alternatively ROS could be involved in indirect activation of NF-κB via TNF, interleukin-1 (IL-1) and phorbol esters. PBM can boost ROS generation, and it was shown that light irradiation can induce NF-κB activation [59].

The increased NF-κB production after PBM stimuli leads to enhanced gene transcription that leads to reduced cell death, to cell proliferation, to cell migration [81] and enhanced neurological function. Figure 3 shows an overview of the different groups of genes that have NF-kB response elements.

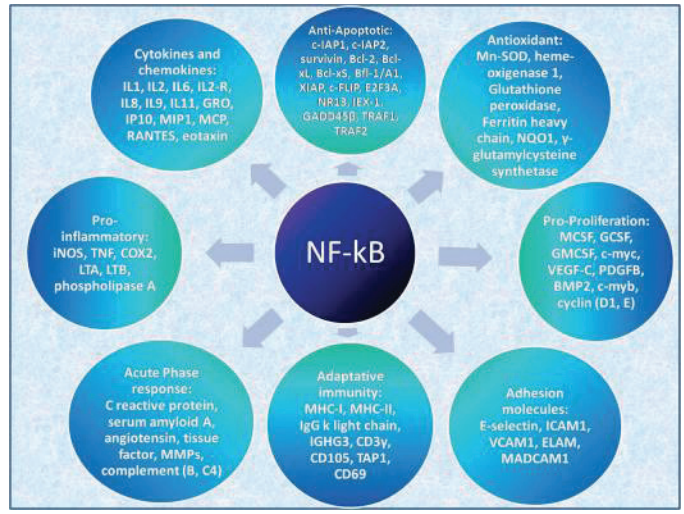


Figure 3: Overview of the different groups of genes and molecules that have NF-kB response elements. In principle these could be activated by NK-kB signaling pathway triggered by the ROS produced during LLLT

If the total energy density delivered is too high, however, the injury paradoxically tends to be exacerbated by increased oxidative stress, and an over-abundant activation of NF-κB. The biphasic dose effects of PBM are thought to occur due to an excessive generation of ROS, excessive production of NO, to the activation of some cytotoxic pathways, and to excessive NF-κB activation [82]. In addition, if the tissue is stressed or ischemic, mitochondria can synthesize NO that can displace oxygen from binding to Cox, but this leads to a reduced ATP synthesis and to an increased oxidative stress that can lead to inflammation when NF-κB is activated [83].

Classical mitochondrial inhibitors such as rotenone are known to decrease mitochondrial ATP levels, produce ROS and activate NF-κB. Low-level light still produces ROS and activates NF-κB, but in this case increases ATP levels. Antioxidants do not inhibit this ATP increase, suggesting that light augments the electron transport and potentially causes electron leakage (in the absence of antioxidants) and superoxide production [59].

RANKL – Receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane protein member of the TNF superfamily, involved in bone regeneration and remodeling (acting on osteoclast differentiation and activation). It is also a ligand for osteoprotegerin (OPG). The RANKL/OPG ratio determines whether bone is removed or formed during the remodeling process. The remodeling cycle consists in the increase in the expression of RANKL by osteoblasts, and subsequent binding to RANK receptor, which is highly expressed on osteoclastic membrane. This causes an expansion of the osteoclast progenitor pool, differentiation into mononucleated progenitor cells, increased survival, fusion into multinucleated osteoclasts and, finally, their activation. Osteoblasts can modulate this process by expressing OPG, which is a secretory soluble receptor and inhibitor of RANK receptor.

Parenti et al. investigated the RANKL/OPG ratio in osteoblast-like cells that were irradiated with GaAlAs laser (915 nm) using doses ranging from 1 to 50 J cm^{-2}. Although the differences were not statistically significant, there was a trend for a rapid and transitory increase in the RANKL/OPG ratio for all the tested doses. It seems that this ratio after PBM depends on the tissue and on the parameters used, since there is evidence of an increase in RANKL/OPG ratio in human alveolar bone-derived cells irradiated with 780 nm light, while in rat calvarial cells irradiated with 650 nm light the results were the opposite [84].

Hypoxia inducible factor (HIF-1α) – HIF-1α is a protein involved in cellular adaptation to hypoxia. It is stabilized at low oxygen tensions, but in the presence of higher oxygen concentrations it is rapidly degraded by prolyl hydroxylase enzymes, which are oxygen-dependent. HIF-1α activates genes that are important to the cellular response to hypoxic conditions, such as vascular endothelial growth factor (VEGF), VEGF-receptor, glucose carrier (GLUT-1) and phosphoglycerate kinase (PGK) genes. Since there is no significant changes in gross tissue oxygen concentration during PBM, HIF-1α activation may be mediated by the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, by growth factors or cytokines [85]. Another possible explanation is that the sudden boost in cellular respiration caused by light activation of Cox depletes the low amount of oxygen that is present in hypoxic tissues but which is not being rapidly consumed because of inhibited electron transport. This sudden oxygen depletion then rapidly activates HIF-1α.

Cury demonstrated the pro-angiogenic effect of PBM using 660 nm and 780 nm light on skin flaps in rats. He observed that angiogenesis was induced by an increase in HIF-1α and VEGF expression, as well as by a decrease in matrix metalloproteinase 2 (MMP-2) activity [85]. Cury observed that only 660 nm light was able to increase HIF-1α expression, and although VEGF induction occurred in all light doses used, only 40 J cm^{-2} was able to induce angiogenesis, as well as an increase MMP-2 activity.

Akt/GSK3β/β-catenin pathway – Low-level light may exert a prosurvival effect on cells via the activation of AKT/GSK3β/β-catenin pathway. Basically, protein kinase B (also known as AKT) can be activated by LLL irradiation, and then interact with glycogen synthase kinase 3β (GSK3β), inhibiting its activity. GSK3B is a serine-threoninekinase which mediates various cellular signaling pathways, exerts metabolic control, influences embryogenesis, and is involved in cell death and in oncogenesis. There is evidence that this kinase is involved in the pathogenesis of Alzheimer's disease, since it promotes hyperphosphorylation of tau protein

and causes the formation of neurofibrillary tangles (NTFs), both classic hallmarks of this disease.

The decreased activity of GSK3β is due to the fact that PBM-activated AKT increases the phosphorylation level of its Ser9 residue, which allows the N-terminus of GSK3β to bind with its own binding site. This leads to an accumulation of β-catenin and its translocation into the nucleus, where it can exert its prosurvival action. β-catenin is an important component of Wnt signalling pathway, responsible for the inhibition of axin-mediated β-catenin phosphorylation by GSK3β. This helps to stabilize the under-phosphorylated form of β-catenin, and ensure that it is no longer marked for proteasome degradation, so it can accumulate and travel to the nucleus. Once there, the prosurvival action of β-catenin relies on the increased TCF/LEF-dependent transcriptional activity. This prosurvival effect can be useful in the treatment of neurodegenerative diseases, such as Alzheimer's [86].

One of the most important regulators of apoptosis is Bax, a member of Bcl-2 family. It is translocated from the cytosol to the mitochondria when a pro-apoptotic stimulus is present, and this translocation is inhibited by PBM, according to Zhang et al. The authors hypothesized that GSK3β is the mediator between Akt and Bax during the PBM anti-apoptotic process. The authors found that GSK3β interacts with Bax and activates it, promoting its translocation directly, but PBM activates Akt which inhibits the activation of GSK3β, thus inhibiting Bax translocation. Using inhibitor compounds such as wortmannin and lithium chloride, there was a significant inhibition of the anti-apoptotic effect observed after PBM, suggesting that PI3K/Akt pathway (inhibited by wortmannin) and GSK3β translocation (inhibited by lithium chloride) play a key role in the protection against apoptosis caused by low level light. LiCl, however, was not able to reduce Bax translocation and apoptosis like PBM, so there must be other upstream regulators of Bax translocation during apoptosis. In conclusion, PBM exerted a pro-survival action through selectively activating the PI3K/Akt pathway and suppressing GSK3β/Bax pathway [87].

Akt/mTOR/CyclinD1 pathway – PBM has been demonstrated to be useful for stimulating proliferation of normal cells, but for dysplastic and malignant cells it could be dangerous. Sperandio et al. provided an example of this situation, observing that oral dysplastic cells, considered pre-malignant, had their viability increased after PBM (660 or 780 nm, 2 to 6 J cm^{-2}). Moreover, these workers showed higher expression of proteins related to cancer progression and invasion, i.e. Akt, HSP90, $pS6_{ser240/244}$, and Cyclin D1. The data suggest that Akt/mTOR/Cyclin D1 pathway was important for this phenotype differentiation, since the tested oral cancer cells showed higher levels of the signaling mediators that are part of this pathway [88].

ERK/FOXM1 – Forkhead box protein M1 (FOXM1) is a protein involved in the regulation of the transition from G1 to S phase of the cell cycle and progression to mitotic division. Ling et al. investigated the protective effect of PBM using red light at 632.8 nm against senescence caused by UV light, and reported an activation of the ERK/FOXM1 pathway that caused a reduction in the expression of p21 protein and G1 phase arrest. Senescence was attenuated by over-expression of FOXM1c with or without PBM, and if FOXM1 was inhibited by shRNA, the effect of PBM in reducing cell senescence was abrogated. PBM promoted the nuclear translocation of extracellular signal-regulated kinase (ERK), increasing FOXM1 accumulation in the nucleus and the transactivation of c-Myc and p21 expression.

Inhibition of the mitogen-activated kinase (MEK)/ERK pathway with an MEK inhibitor PD98059 prevented the nuclear translocation of FOXM1 after PBM, suggesting that Raf/MEK/MAPK/ERK signaling is crucial for the anti-cell senescence effect of PBM mediated by FOXM1 [89]. Figure 4 summarizes these findings.

PPARγ – Peroxisome proliferator-activated receptors (PPAR) are mostly present in airway epithelial cells, but also in smooth muscle cells, myofibroblasts,

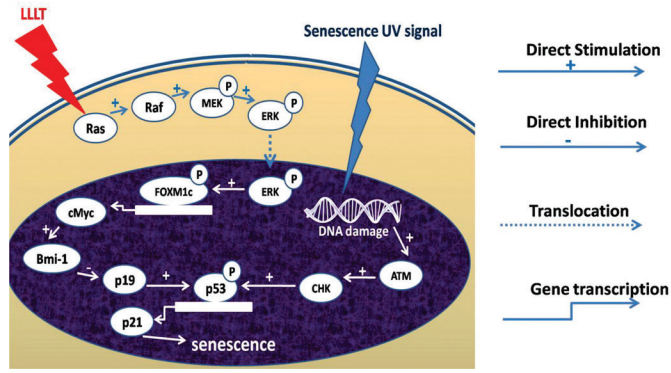


Figure 4: A model of the signaling pathways for LLLT protecting cell from UVB-induced senescence.

endothelial cells of the pulmonary vasculature and in inflammatory cells such as alveolar macrophages, neutrophils, eosinophils, lymphocytes and mast cells. They are nuclear receptors with transcription factors that regulate gene expression. PPAR-y is involved in the generation of heat shock protein 70 (HSP-70), which is anti-inflammatory, while PPAR-c expression occurs due to an inflammatory response and are associated with massive lung injury and neutrophil infiltration in lungs of mice subjected to endotoxic shock [90]. Lima and co-authors reported a study in which rats were irradiated with 660-nm light (5.4 J) on the skin over the bronchus (chest). They observed a marked rise in the expression of PPAR mRNA after PBM, as well as increased PPAR-y activity in bronchoalveolar lavage (BALF) cells from animals subjected to laser treatment. In conclusion, Lima proposed that PBM can work as a homeostatic facilitator, increasing the expression of a transcription factor that is signaling the synthesis of HSP70 and other anti-inflammatory proteins[90].

RUNX2 – Runt-related transcription factor 2 (RUNX-2) is related to osteoblastic differentiation and skeletal morphogenesis, acting as a scaffold for nucleic acids and regulatory factors that are involved in the expression of skeletal-related genes. It regulates the expression of genes related to extracellular matrix components during bone cell proliferation. PBM can increase the expression of RUNX-2, contributing to a better tissue organization, even in diabetic animals as seen by Patrocínio-Silva [91].

Effector molecules

Transforming growth factor (TGF-β) – TGF-β is a strong stimulator of collagen production, inducing the expression of extracellular matrix components and inhibiting its degradation by inhibiting matrix metalloproteinases (MMPs). TGF-β expression is elevated during the initial phase of inflammation after an injury, and stimulates cellular migration, proliferation and interactions within the repair zone [92].

Dang and co-workers suggested that TGF-β/SMAD signaling pathway might play a role in PBM used for non ablative rejuvenation [93]. They found that 800 nm diode laser irradiation was able to induce collagen synthesis through the activation of TGF-β/SMAD pathway in a light dose-dependent manner. 40 J $cm^{-2}$was the most effective light dose in enhancing the gene expression of procollagen type I and IV, compared to 20 and 60 J cm^{-2}. The dermal thickness followed the results for the synthesis of collagen, demonstrating that this process was indeed dose-dependent [93].

Aliodoust et al. treated rats with 632.8 nm light and observed increased expression of TGF-β1 (one of the three isoforms of TGF-β) mRNA. TGF-β1 is responsible for the initial scar tissue formed at the wound site. It enhances tendon repair during the fibrosis period via the stimulation of cell proliferation and migration, as well as the synthesis of collagen and proteoglycans [92].

Oxidative stress – The inflammatory process involves an increase in ROS and RNS production, accompanied by a reduction in the activity of antioxidant defenses. This oxidative stress situation can activate NF-κB, as mentioned before, leading to modifications in the expression of genes for pro-inflammatory cytokines, growth factors, chemokines and adhesion molecules.

Assis et al. investigated the effects of PBM on muscle injury using 808 nm light (1.4 J), and observed reduced lipid peroxidation accompanied by a decreased COX-2 mRNA expression and an increased SOD mRNA expression after irradiation. There was a reduced formation of nitrotyrosine, indicating that iNOS activity was lower and, consequently, NO and peroxynitrite production was decreased. In conclusion, the inhibition of oxidative and nitrosative stress contributed to a decrease in the deleterious effects observed after muscle injury [94].

Pro- and anti-inflammatory cytokines – Many cytokines and inflammatory mediators have their levels altered by low-level light irradiation, regardless if they have pro- or anti-inflammatory actions, i.e. TNF, various interleukins, histamine, TGF-β, prostaglandins and eicosanoids. It seems that when inflammation is present, PBM exerts an anti-inflammatory action, but in the absence of inflammation, PBM provide pro-inflammatory mediators that could help in tissue remodeling and to mediate cell function. Wu and co-workers investigated the photoacceptor role of Cox and found that the excitation of Cox initiates a photoreaction that results in histamine release in vitro. The induced signals from mitochondria to cytosol cause alkalinization of the cytosol, which leads to the opening of TRPV channels. This results in an increment of [Ca^{2+}] and, consequently, in an enhanced histamine release [67]. Chen demonstrated

in 2014 that an increased calcium influx occurred in mast cells after laser irradiation, and this caused histamine release that could help promoting wound healing. Furthermore, he found that during short-term muscle remodeling after cryoinjury, cytokines expression is also modulated by PBM, leading to a decreased expression of TNF and TGF-β[95].

Although NF-κB activation is known to be pro-inflammatory, PBM has a pronounced anti-inflammatory activity even with NF-κB activation. In fact, the anti-inflammatory effects of PBM could be abrogated if a NF-κB inhibitor is used. This probably occurs because the initial response to cell stress typical of NF-κB activation triggers another response to lower NF-κB activation after PBM had its therapeutic effect. Another possibility is that the initial pro-inflammatory response induced by PBM leads to the expression of eicosanoids that are able to decrease and to end inflammation [95].

Brain-derived neurotrophic factor (BDNF) – BDNF is part of the family of neurotrophins, molecules that exert actions on nerve cells. BDNF, specifically, seems to modulate dendritic structure and to potentiate synaptic transmission in the central nervous system. In order to investigate the effects of low-level light on BDNF levels, Meng et al. treated nerve cells with 632.8 nm light (doses from 0.5 to 4 J cm^{-2}). There was a regulatory role of PBM in neuroprotection and dendritic morphogenesis. PBM attenuated the decrease of BDNF, apparently by the ERK/CREB pathway, and this could be useful in the treatment of neurodegenerative disorders [96].

Vascular endothelial growth factor (VEGF) – Angiogenesis is a complex mechanism, requiring several cell types, mediators and signaling pathways. It is initiated by cell migration and invasion of endothelial cells, subsequent lumen formation and connection of the new vascular segments with preexisting ones, and finally, remodeling of extracellular matrix. This remodeling is dependent on an adequate matrix metalloproteinases (MMPs) activity. VEGF and HIF-1α are critical to the angiogenic process.

PBM has been reported to induce angiogenesis in several experimental models. For example, Cury et al. observed a marked increase in the number of vessels in the skin flap of animals treated with 660 and 780 nm PBM, alongside with a marked increase in VEGF mRNA expression [85].

Hepatocyte growth factor (HGF) – HGF is a cytokine that regulates cell proliferation, motility, morphogenesis and exerts anti-apoptotic and anti-inflammatory activity during hepatic regeneration. The activation of its transmembrane tyrosine kinase receptor, called Met receptor, leads to autophosphorylation of tyrosine residues and phosphorylation of downstream signaling molecules, such as PI3K and MAPK pathway proteins. Araújo and co-workers observed that, after 632.8 nm PBM, hepatectomized animals showed an increase in the expression of HGF followed by increased phosphorylation of Met and its downstream signaling molecules Akt and ERK. This indicates that PBM could enhance liver regeneration after hepatectomy [97].

Basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF) – Growth factors play a key role in the wound healing process, mediating the transfer of signals between the epithelium and the connective tissue, especially bFGF and KGF. bFGF is known to be a potent mitogen and chemoattractant for endothelial cells and fibroblasts, as well as accelerating the formation of granulation tissue and to induce re-epithelization. KGF is produced by fibroblasts and exerts a paracrine action on keratinocytes, therefore, it is responsible for the proliferation and migration of epithelial cells, as well as for the maintenance of the epithelium normal structure.

When gingival fibroblasts from a primary culture were irradiated twice with 660 or 780 nm low-level light in a study from Damante et al., production of KGF and bFGF was increased. Red light was more effective in stimulating KGF production, but no significant change in bFGF production was seen with red light. Near-infrared light, however, was capable of inducing bFGF release [98]. These results could explain how PBM can help the wound healing process.

Heat Shock Proteins (HSP) – Heat shock protein 27 (HSP27) is an important member of the small HSP family, with an ATP-independent chaperone activity that is produced in response to oxidative stress in order to modulate inflammation and to regulate the dynamics of the actin cytoskeleton. When HSP27 is activated, it facilitates the phosphorylation of IκB, causing it to be degraded in the proteasome and increasing NF-κB activity. It also contributes to the

regulation of NO and ROS production, iNOS expression and TNF secretion. However HSP27 plays a negative role in TNF-mediated IκB kinase (IKK) activation. The results of a study performed by Lim and co-workers with HSP27-silenced cells showed that 635 nm light irradiation was not able to decrease ROS generation if HSP27 was not present, indicating that this chaperone plays an important role in ROS decreasing during inflammation and PBM [99].

HSP70 is part of the normal wound healing process, alongside IL-6 and TGF-β1. Visible (532 nm) and NIR (815 nm) light have been demonstrated to induce HSP70 expression in treated skin cells, and this is important for skin rejuvenation interventions, since there is a consequent effect consisting on the assistance of the correct folding and transport of newly synthesized collagen [93].

HSP90 is another chaperone, which assists the maturation of Akt enabling it to perform its downstream actions. Increased activity of chaperones is certainly not desired in cancer, but it could be useful in healing processes. Sperandio et al. found higher levels of HSP90 in laser-treated cells, and an isoform of this chaperone, HSP90N, which has an oncogenic potential, was found in the experimental groups. This isoform is commonly overexpressed in tumor tissues and is secreted by advantage stages of melanoma [88].

Cellular mechanisms

Inflammation – Lim and co-workers found that 635 nm light irradiation at low power can lead to an anti-inflammatory effect by inhibiting prostaglandin E2 (PGE2) production and cyclooxygenase 1 and 2 (COX-1 and COX-2) mRNA expression. The light irradiation was able to decrease intracellular ROS, which mediate the expression of calcium-dependent phospholipase A2 (cPLA2), secretory phospholipase A2 (sPLA2), and COX-2, and also inhibit the release of PGE2 [99].

PGE2 synthesis is dependent on NF-κB modulation of the cellular signaling mechanism. NF-κB is found in the cytosol in its dimeric form of NF-κB/IκB (the latter is an inhibitory protein). Pro-inflammatory stimuli, such as LPS, are able to activate the NF-κB upstream signaling regulator IκB kinase (IKK), responsible for the phosphorylation and degradation of IκB. The free NF-κB is translocated to the nucleus and induces the expression of pro-inflammatory genes [8]. Lim demonstrated that 635 nm light irradiation suppressed the release of PGE2, possibly through a mechanism related to the inhibition of NF-κB pathway. It did not affect the phosphorylation of IκB, IKK and NF-κB in HSP27-silenced human gingival fibroblasts (hGFs), suggesting that NF-κB modulation by 635 nm light through HSP27 is required for the down-regulation of pro-inflammatory gene expression in these fibroblasts [99].

Macrophages are important antigen-presenting cells, and are involved in the induction of primary immunologic response. Interferon gamma (IFN-γ) polarization (either via classical or M1 activation) programs monocytes for increased phagocytic activity, as well as for anti-tumor activity and allergy suppression. Recently, Chen reported that 660 nm PBM could promote M1 polarization of monocytes, and influence the expression of cytokines and chemokines at the level of mRNA and protein expression. The effect was dose-dependent, since the optimal light dose found was that of 1 J cm^{-2}, compared to 2 and 3 J cm^{-2}. Furthermore, the author could also clarify the mechanisms of epigenetic regulation by PBM in immune cells. Modifications on histones, usually carried out by histone acetyl- or methyltransferases, could be induced by PBM: histone H3 and H4 acetylation and H3K4 trimethylation in the TNF gene promoter area, and histone H3 acetylation in the IP-10 gene promoter region. M1-related immunoregulation is important for antiviral immunity, antitumor immunity, and for the pathogenesis of infammation in autoimmune conditions, therefore PBM could help promoting anti-viral and anti-tumor immunity, but could enhance autoimmune and rheumatoid diseases [95].

Cytoprotection – Studies have shown that PBM in vitro protects cells at risk from dying due to treatment with various different toxins. Methanol, for instance, generates a toxic metabolite (formic acid) that inhibits cytochrome c oxidase. Since PBM enhances mitochondrial activity via stimulation of cytochrome c oxidase, it also promotes cell survival during formic acid toxicity. This was demonstrated by Eells, who used red light (670 nm) in a rodent model of methanol toxicity and found that the light irradiation induced a significant recovery of cone- and rod-mediated function in the retina of rats after methanol intoxication, as well as a protection against histological damage resulting from formic acid [100].

Cyanide is another toxic compound that can have its effects attenuated by PBM. Potassium cyanide-induced apoptosis of neurons was decreased with a pretreatment with 670 nm light. This is explained by the fact that PBM decreased the expression of caspase-3 (commonly increased by cyanide) and reversed the cyanide-induced increased expression of Bax, while decreasing the expression of Bcl-2 and inhibiting ROS generation [101]. Wong-Riley and co-workers show that LED pretreatment was not able to restore enzymatic activity in cells to control levels after cyanide toxicity, but it successfully reversed the toxic effect of tetrodotoxin, especially with 670 and 830 nm light. These wavelengths correspond to the peaks in the absorption spectrum of cytochrome c oxidase, suggesting that this photobiomodulation is dependent of the up-regulation of Cytochrome c oxidase [102].

PBM can be useful in the treatment of Alzheimer's disease, since low-power laser irradiation promotes Yes-associated protein (YAP) cytoplasmic translocation and amyloid-β-peptide (Aβ) inhibition. Aβ deposition is a known hallmark of Alzheimer's disease, while YAP translocation is involved in the regulation of Aβ-induced apoptosis. Zhang published a study demonstrating that 832.8 nm light irradiation is able to reduce Aβ-induced toxicity by inhibiting apoptosis through the activation of Akt/YAP/p73 signaling pathway [103].

Proliferation – Several cell types can have their proliferation levels increased by PBM. Keratinocytes, for example, showed an enhanced proliferation after 660 nm light irradiation, accompanied by an increased expression of Cyclin D1 and a faster maturation of keratinocytes in migration to the wound sites, via the expression of proteins involved in the epithelial proliferation process, namely p63, CK10 and CK14. This is useful for the improvement of epithelial healing [104]. Furthermore, fibroblasts irradiated with 632.8 nm light had their proliferation stimulated and their cell viability increased, demonstrating the stimulatory effect of PBM and the usefulness of this therapy in the wound healing process [105].

Vascular endothelial cells exposed to 635 nm irradiation proliferate faster than non-irradiated cells, showing a decreased VEGF concentration. This suggests that laser-induced cell proliferation is related to a decrease in VEGF concentration. 830 nm irradiation decreased TGF-β secretion by the endothelial cells [106].

Amid et al. published a review about the influence of PBM on the proliferation of osteoblasts. According to the studies reviewed by the authors, wavelengths between 600 nm and 1000 nm have been used, and resulted in positive effects on dentistry, on anti-inflammatory process and on osteoblastic proliferation [107].

Fibroblasts irradiated with 632.8 nm light had their proliferation stimulated and their cell viability increased, demonstrating the stimulatory effect of PBM and the usefulness of this therapy in the wound healing process.

Migration – Tendon healing requires migration of tenocytes to the injured area, with consequent proliferation and synthesis of extracellular matrix. Tsai and co-workers evaluated the effect of 660 nm light on rat Achilles tendon-derived tenocytes, and found that dynamin-2 expression was enhanced and the migration was stimulated in vitro. Inhibiting dynamin-2 with dynasore suppressed this stimulatory effect of PBM, leading to the conclusion that tenocyte migration stimulated by low-level light was mediated by the up-regulation of dynamin-2 [108].

Other cell types are also influenced by light irradiation. Melanocytes, for instance, showed an enhanced viability and proliferation after blue and red light irradiation. Melanocytes migration was enhanced by UV, blue and red light in lower doses, but a non-stimulatory effect was observed for higher light doses. Blue light seemed to be more effective compared to UV and red lasers [109]. Human epidermal stem cell migration and proliferation were increased alongside an increased phosphorylation of autocrine extracellular signal-regulated kinase (ERK), which contributed to accelerated wound re-epithelialization [110]. Finally, 780 nm irradiation seemed to be able to accelerate fiber sprouting and neuronal cell migration, at least in embryonic rat brain cultures. Large-size neurons with a dense branched interconnected network of neuronal fibers were also observed after laser irradiation. These results can be seen in Rochkind's work, and may contribute for future treatment modalities for neuronal injuries or diseases [111].

Protein synthesis – As mentioned before, PBM was able to increase the expression of proteins related to

the proliferation and maturation of epithelial cells: p63, CK10 and CK14 [104]. In fact, low level light can increase the expression of several other proteins. A good example is the enhanced collagen I expression by fibroblasts 2 days after 810 nm light irradiation, as demonstrated by Frozanfar and co-workers in 2013 [112]. Moreover, osteoblasts irradiated with 830 nm light increased the expression of proteins and proteoglycans such as osteoglycin and mimecan. Osteoglycin is a leucine-rich proteoglycan, once called osteoinductive factor, easily found in bone matrix, cartilage cells and connective tissues. They play a regulatory role in cell proliferation, differentiation and adhesion of osteoblastic cells, therefore PBM applied on the early proliferation stage of osteoblasts are important for the stimulation of bone formation, in concert with some growth factors and matrix proteins [113].

Stem cells – It appears that stem cells are particularly sensitive to light. PBM induces stem cell activity shown by increased cell migration, differentiation, proliferation and viability, as well as by activating protein expression [114]. Mesenchymal stem cells, usually derived from bone marrow, dental pulp, periodontal ligament and from adipose tissue, proliferate more after light irradiation (usually with wavelengths ranging from 600 to 700 nm). Since stem cells in their undifferentiated form show a lower rate of proliferation, this may be a limiting factor for the clinical effectiveness of stem cell therapies, PBM offers a viable alternative to promote the translation of stem cell research into the clinical arena [115].

Min and co-workers reported that the cell viability of adipose-derived stem cells was found to be increased after irradiation with 830 nm light. Their in vivo results also revealed elevated numbers of stem cells compared to the control group [116]. Epidermal stem cells can also be influenced by light, as demonstrated by Liao et al. The authors reveal that 632.8 nm light has photobiological effects on cultured human epidermal stem cells, such as an increase in proliferation and migration in vitro [110]. Soares observed a similar effect on human periodontal ligament stem cells irradiated with a 660 nm diode laser [117].

Tissue mechanisms

Muscles – We already mentioned the positive results for PBM in muscle recovery, reported by Ferraresi et al. The authors demonstrated the usefulness of PBM in muscle recovery after injury. The authors concluded that it takes between 3 and 6 hours for the PBM to exert maximum effect on the muscle physiology, consisting of increased matrix metalloproteinase activity and ATP synthesis. This effect could still be observed 24 hours after the laser irradiation [49].

Rochkind and co-workers have also worked with PBM applied to muscles, investigating the influence of low power laser irradiation on creatine kinase (CK) and the amount of acetylcholine receptors (AChRs) present in intact gastrocnemius muscle in vivo, as well as the synthesis of DNA and of CK in muscle cells in vitro. The authors found that PBM significantly increased CK activity and AChR level in one and two months, when compared to control animals. The biochemical changes on muscle cells might be due to a trophic signal for increased activity of CK, which leads to a preservation of a reservoir of high-energy phosphate that is available for rapid ATP synthesis [118].

Brain – Regarding the neurological field, PBM can lead to cognitive benefits and memory enhancement in case of brain damage caused by controlled cortical impact (CCI). Khuman and co-workers found that a 500 mW cm^{-2} laser irradiation (60 J cm^{-2}) for two minutes improved spatial learning and memory of mice with CCI, and this was not observed in sham-injured mice. The authors observed a brief increase in the temperature of brain, but it returned to baseline before 5 minutes of irradiation. They also observed reduction of microgliosis at 48 h. Low level light can be useful in traumatic brain injury (TBI) treatment, since suboptimal light doses demonstrated to affect spatial memory, as assessed by visible platform trials, even in the absence of non-spatial procedural learning, which is hippocampus-independent [82].

Near-infrared (NIR) light exerts a protective effect on neurons, but the mechanisms are not fully understood. However, two mechanisms may be involved, and the first that will be discussed is the direct action of NIR light on the cells, improving mitochondrial function, reducing inflammation, and helping the brain to repair itself. Xuan et al reported that transcranial NIR light could stimulate the process of neurogenesis in the hippocampus and subventricular zone (SVZ) in mice with CCI TBI [119]. These newly formed neuroprogenitor cells could travel to the injured region of the cortex to help in the repair of the damaged region. In another study the same group showed that BDNF was increased

in the hippocampus and SVZ at one week post TBI, and that at 4 weeks post TBI there as an increase in synaptogenesis in the cortex showing that new connections between existing brain cells could be stimulated by light [120].

The second mechanism is based on the hypothesis that NIR can trigger a systemic response, this time not so directly, suggesting the involvement of one or more circulating molecules or cell types. This assumption is based on studies reporting remote effects on tissues after irradiation of NIR light on specific sites, such as skin wounds. Another study reported brain protection in mice after remote irradiation with NIR light to the dorsum of the animals, without any direct irradiation on the head. One possibility to explain these remote effects is the stimulation of mast cells and macrophages, which could help to protect cells in the brain, as well as the modulation of inflammatory mediators, like the down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines. Another possibility is the involvement of bone-marrow derived stem cells, since NIR light can increase the proliferation of c-kit-positive cells located in the bone marrow of the skull, which are then recruited to damaged tissues, especially the myocardial infarct site. These progenitor cells can, alongside with immune cells, secrete trophic and pro-survival factors such as nerve growth factor (NGF) and VEGF. Finally, mitochondria itself could be secreting an unidentified extracellular signal, called by Durieux et al. a "mitokine", which is then transmitted to remotely located cells[82].

Nerves (repair and pain) – Some clinical studies have demonstrated the efficacy of laser-induced analgesia [121], [122], usually with a low power red or near-infrared laser, and it seems that the pain reduction is due to a conduction block of central and peripheral nerve fibers and to the release of endorphins. In this field, for instance, Chan and co-workers used a Nd:YAG pulsed laser (1064 nm) with average power 1.2 W and power density 0.3–0.45 J cm^{-2} in a randomized, double-blind clinical trial, and demonstrated the efficacy of this treatment on pulpal analgesia of premolar teeth[123].

Analgesia mediated by low level light therapy is due to various effects, such as light absorption by mitochondrial chromophores (mainly Cox) biomodulation, vasodilation, stimulation of cell division, release of NO, increase in cortisol levels and protein synthesis, increase in intracellular calcium concentration and increased activity of the antioxidant enzyme superoxide dismutase. Serra and Ashmawi investigated recently if serotonin played a role in PBM-induced analgesia, but their results indicated that this effect is mediated by peripheral opioid receptors, but not by peripheral serotoninergic receptors [124].

Low-level light therapy can be used for inhibition of pain and for pathological conditions associated with the nervous system. In 2011, Yan et al. postulated that PBM could suppress afferent fiber signaling as well as modulate synaptic transmission to dorsal horn neurons, including inhibition of substance P, and this can lead to long-term pain depression [125]. PBM exerts potent anti-inflammatory effects in the peripheral nervous system, can reduce myocardial infarction, promotes functional recovery and regeneration of peripheral nerves after injury, and can improve neurological deficits after stroke and TBI [82].

Light with irradiance higher than 300 mW cm^{-2}, when absorbed by nociceptors, can inhibit Aδ and C pain fibers, slowing of conduction velocity, reducing of the compound action potential amplitude, and suppression of neurogenicin inflammation. In case of PBM, the light can block anterograde transport of ATP-rich mitochondria in dorsal root ganglion neurons. This inhibition is completely reversible within 48 hours, and leads to the formation of varicosities, which are usually associated with the disruption of microtubules (interruption of fast axonal flow can reduce ATP availability, which is necessary for the polymerization of microtubules and for the maintenance of the resting potential) [83].

Healing (bones, tendons, wounds) – Regarding bones, low power laser irradiation is not believed to affect osteosynthesis, but it is likely that it creates environmental conditions that accelerate bone healing. PBM stimulates proliferation and differentiation of osteoblasts in vivo and in vitro, leading to an increased bone formation, accompanied by an increase in the activity of alkaline phosphatase (ALP) and in osteocalcin expression. This indicates that laser irradiation can directly stimulate bone formation and, according to Fujimoto et al., this effect can be attributed to an increased expression of insulin-like growth factor (IGF), although other differentiation factors might be involved as well,

such as BMPs. BMPs-2, −4, −6 and −7 are members of the TGF-β superfamily, and potent promoters of osteoblastic differentiation and of bone formation (promoting the change of mesenchymal cells into chondroblasts and osteoblasts) [126].

According to Fujimoto, BMP-2 might be most involved in the effects of PBM on bone. PBM stimulated mineralization in vitro via increased gene and protein expression of BMPs and Runx-2, as well as differentiation of osteoblasts into MC3T3-E1cells. Since BMPs are one of the most important and potent bone-inductive mediators and are expressed in skeletal tissues, it is possible that the bone nodules formed after PBM are mediated in part by BMP-2 expression [126].

The balance between oxidants and antioxidants is directly related to the time and quality of the wound healing process[127]. This process can be divided in four overlapping phases: hemostasis, inflammation, proliferation and remodeling or resolution. Hemostasis is initiated as soon as the blood vessels are damaged, and consists on the adherence of platelets to the extracellular matrix and further releasing of growth factors (mostly platelet-derived growth factor, PDGF and TGF-β), culminating in the production of thrombin which acts on fibrinogen to produce a fibrin clot. Thrombin also acts as a chemotactic agent and proliferating agent on monocytes, keratinocytes, fibroblasts and endothelial cells, therefore a defective thrombin activity can lead to a delay in the wound healing process. Hoffman reported that PBM could be beneficial in promoting healing when there is a defect in the hemostasis process [128].

Hair – Different mechanisms have been proposed to explain the reason for the first light-mediated effect observed by Mester in 1968 (hypertrichosis in mice [2]) but now widely used clinically to restore hair growth in adult humans [129]. Some researchers have hypothesized that this effect was due to polycystic ovarian syndrome present in 5 out of 49 female patients under laser treatment for facial hirsutism, others suggested that even if the heat generated by PBM was not able to ablate cells from the hair follicle, the small amount of heat supposedly produced could induce follicular stem cells to proliferate and differentiate, due to the increased level of heat shock proteins. Another possibility relies on the release of certain factors that could affect the cell cycle and induce angiogenesis [129]. The exact mechanism still needs clarifying, but the effects of PBM on hair growth are already well described.

Hair growth is divided basically in three phases: anagen, catagen and telogen. The anagen is the growth phase and can last from 2 to 6 years. Catagen phase lasts from 1 to 2 weeks and consists of club hair transitions upwards toward the skin pore, while the dermal papilla separates from the follicle. In the telogen phase, the dermal papillae fully separate from the hair follicle. It lasts from 5 to 6 weeks, until the papillae move upward to meet the hair follicles again and the hair matrix begins to form new hair, returning to the anagen phase. It has been observed that PBM is able to stimulate telogen hair follicles to enter the anagen phase, as well as to prolong the duration of the anagen phase itself. PBM is also capable of increasing the rate of proliferation of anagen hair follicles and to prevent premature catagen phase entry. This could be due to induced protein synthesis by the transcription factors activated by PBM, followed by cell migration and proliferation, alteration in cytokines levels, growth factors and inflammatory mediators. NO is also augmented in LLL treated tissues, usually dissociated from Cox, and since it is a well known vasodilator, it is likely that there is a vasodilation effect on hair follicles after PBM that could help hair growth. Some inflammatory mediators also have their expression inhibited by PBM (such as IFN-γ, IL-1a, IL-1b, TNF and Fas-antigen) and, considering that inflammation is highly disruptive for hair follicles, the anti-inflammatory effect of PBM could be useful in the treatment of hair conditions such as alopecia areata [129].

High Fluence Low Power Laser Irradiation (HF-LPLI)

Fluence, according to the International System of Units, is the energy density integrated over the unit surface of a sphere. Just like PBM using low fluences of light, high-fluence low-power laser irradiation (HF-LPLI) stimulates mitochondrial chromophores, but this time it overstimulates them, which in turn activates the mitochondrial apoptosis pathway, altering the cell cycle, inhibiting cell proliferation and even causing cell death. HF-LPLI (usually fluences above 80 J cm^{-2}) induces apoptosis by activating caspase-3, and mitochondrial permeability transition after HF-LPLI is the main mechanism of mitochondrial injury. In 2010, Sun et al. reported that signal transducer and activator of transcription 3

(Stat3) was involved in HF-LPLI-induced apoptosis in vitro, and this effect is time- and dose-dependent. Steroid receptor coactivator (Src) seems to be the main upstream kinase of Stat3 activation, and the increased ROS generation plays a key role in this process [130].

Recently, Wu et al. found that HF-LPLI, using light at 633 nm and 120 J cm^{-2}, could ablate tumors via activation of mitochondrial apoptotic pathway after ROS generation. The evidence is based on the inactivation of caspase-8, activation of caspase-9 and by the release of cytochrome C. When this high dose is used, light inactivates Cox (instead of activating Cox), inducing a superoxide burst in the electron transport chain and, finally, produces oxidative damage against cancer cells [29]. Chu and co-workers already observed that PBM could induce a mitochondrial permeability pore transition when higher levels of ROS are produced. As a consequence, the decrease of mitochondrial transmembrane potential causes the permeabilization of the mitochondrial outer membrane and, subsequently, the release of cytochrome c and caspase cascade reaction [131].

Cho also observed the interference that a protein, called survivin, could affect the outcomes of HF-LPLI. Light treatment can activate survivin by inducing an increase in its phosphorylation levels. The activated survivin is able to inhibit the permeabilization of the mitochondrial outer membrane, and therefore prevents the release of cytochrome c, the activation of Bax and caspase-9. Cho then concluded that survivin mediates self protection of tumor cells against HF-LPLI-induced apoptosis, through ROS/cdc25c/CDK1 signaling pathway [131].

CONCLUSIONS

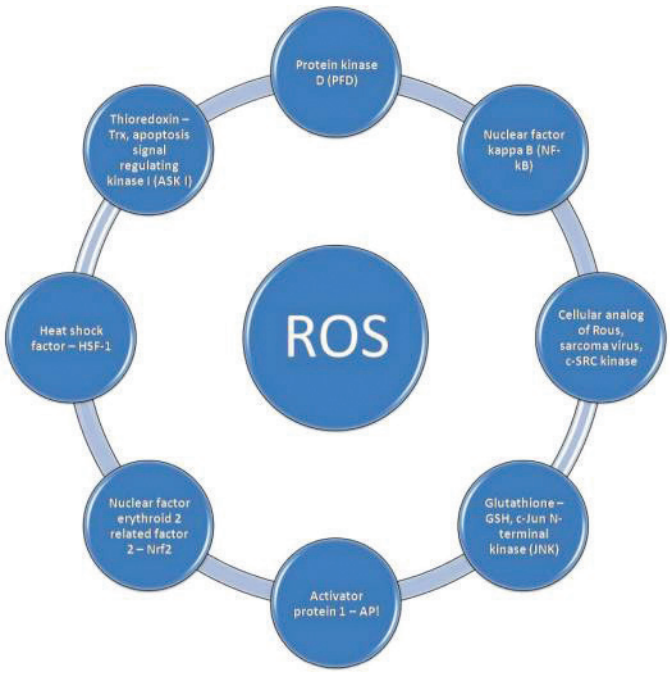
Low levels of red/NIR light can interact with cells, leading to changes at the molecular, cellular and tissue levels. Each tissue, however, can respond to this light-interaction differently, although it is well known that the photons, especially in the red or NIR, are predominantly absorbed in the mitochondria [132]. Therefore, it is likely that even the diverse results observed with PBM share the basic mechanism of action. What happens after the photon absorption is yet to be fully described, since many signaling pathways seem to be activated. It seems that the effects of PBM are due to an increase in the oxidative metabolism in the mitochondria [133]. Different outcomes can occur depending on the cell type, i.e. cancer cells that tend to proliferate when PBM is delivered [88]. In this review we have not discussed the response of cells and tissues to wavelengths longer than NIR, namely far IR radiation (FIR) (3 µm to 50 µm). At these wavelengths water molecules are the only credible chromophores, and the concept of structured water layers that build up on biological lipid bilayer membranes has been introduced to explain the selective absorption [134]. Nevertheless FIR therapy has significant medical benefits that are somewhat similar to those of PBM [135], and it is possible that activation of light/heat sensitive ion channels could be the missing connection between the two approaches.

As we have shown, PBM can regulate many biological processes, such as cell viability, cell proliferation and apoptosis, and these processes are dependent on molecules like protein kinase c (PKC), protein kinase B (Akt/PKB), Src tyrosine kinases and interleukin-8/1a (IL-8/1a). The effects of light on cell proliferation can be stimulatory at low fluences (which is useful in wound healing, for instance), but could be inhibitory at higher light doses (which could be useful in certain types of scar formation such as hypertrophic scars and keloids) [131].

The applications of PBM are broad. Four clinical targets, however, are the most common: shining light on injured sites to promote healing, remodeling and/or to reduce inflammation; on nerves to induce analgesia; on lymph nodes in order to reduce edema and inflammation; and on trigger points (a single one of as many as 15 points) to promote muscle relaxation and to reduce tenderness. Since it is non invasive, PBM is very useful for patients who are needle phobic or for those who cannot tolerate therapies with non-steroidal anti-inflammatory drugs [83].

The positive outcomes depend on the parameters used on the treatment. The anti-inflammatory effect of light in low intensity was reported on patients with arthritis, acrodermatitis continua, sensitive and erythematous skin, for instance [136]. With the same basic mechanism of action, which is the light absorption by mitochondrial chromophores, mainly Cox, the consequences of PBM are various, depending on the parameters used, on the signaling pathways that are activated and on the treated tissue. In order to apply PBM in clinical procedures, the clinicians should be aware of the correct parameters

and the consequences for each tissue to be treated. More studies have to be performed in order to fill the gaps that still linger in the basic mechanisms underlying LLLT and PBM.



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RED LED PHOTOBIOMODULATION REDUCES PAIN HYPERSENSITIVITY AND IMPROVES SENSORIMOTOR FUNCTION FOLLOWING MILD T10 HEMICONTUSION SPINAL CORD INJURY

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CASE REPORT

ABSTRACT

Background

The development of hypersensitivity following spinal cord injury can result in incurable persistent neuropathic pain. Our objective was to examine the effect of red light therapy on the development of hypersensitivity and sensorimotor function, as well as on microglia/macrophage subpopulations following spinal cord injury.

Methods

Wistar rats were treated (or sham treated) daily for 30 min with an LED red (670 nm) light source (35 mW/cm^2), transcutaneously applied to the dorsal surface, following a mild T10 hemicontusion injury (or sham injury). The development of hypersensitivity was assessed and sensorimotor function established using locomotor recovery and electrophysiology of dorsal column pathways. Immunohistochemistry and TUNEL were performed to examine cellular changes in the spinal cord.

Results

We demonstrate that red light penetrates through the entire rat spinal cord and significantly reduces signs of hypersensitivity following a mild T10 hemicontusion spinal cord injury. This is accompanied with improved dorsal column pathway functional integrity and locomotor recovery. The functional improvements were preceded by a significant reduction of dying (TUNEL+) cells and activated microglia/macrophages (ED1+) in the spinal cord. The remaining activated microglia/macrophages were predominantly of the anti-inflammatory/wound-healing subpopulation (Arginase1+ED1+) which were expressed early, and up to sevenfold greater than that found in sham-treated animals.

Conclusions

These findings demonstrate that a simple yet inexpensive treatment regime of red light reduces the development of hypersensitivity along with sensorimotor improvements following spinal cord injury and may therefore offer new hope for a currently treatment-resistant pain condition.

Background

The experience of pain serves as an essential survival mechanism that motivates us to protect ourselves from harm; however, following spinal cord injury, the development of treatment-resistant neuropathic pain often ensues, bringing no advantage to the sufferer but severely reducing the quality of life. Chronic pain affects a vast sector of the population for which the socioeconomic cost exceeds that of heart disease, cancer and diabetes [1]; thus, successfully treating neuropathic pain would bring significant benefits.

The non-invasive application of light, at wavelengths that penetrate transcutaneously [2], has begun to emerge as a potential therapy for improving functional outcomes from a variety of neural injuries [3]. Photobiomodulation with wavelengths ranging from 630 to 1100 nm has demonstrated positive effects in animal models of neurodegenerative

diseases such as Alzheimer's [4] and Parkinson's [5], genetic models of dementia [6], as well as acute nervous injuries to the retina [7–9], optic nerve [9, 10], sciatic nerve [11–15] and spinal cord [16]. In humans, photobiomodulation has been reported to be effective against a variety of pain conditions including mucositis [17], carpel tunnel syndrome [18–20], orthodontic pain [21], temporomandibular joint pain [22], neck pain [23] and neuropathic pain resulting from amputation [24].

Inflammatory mediators have long been implicated in the development and maintenance of pain [25–28]. These chemical mediators are controlled by a variety of immune cells including the balance of pro- and anti-inflammatory microglia/macrophage subpopulations [29–35]. As in non-neural tissues, macrophages can be activated by T helper cell type 1 (Th1) or type 2 (Th2) to generate opposing immune responses following spinal cord injury [30, 31]. Th1-activated microglia/macrophages (M1) have been considered potentially damaging to healthy tissues, as they induce a pro-inflammatory response and have been shown to inhibit axonal regeneration [30]. Conversely, Th2-activated microglia/macrophages (M2) have been considered protective, as they have a role in suppressing the pro-inflammatory response by producing anti-inflammatory mediators [30, 31]. Following spinal cord injury, there is evidence suggesting that the M1 response prevails over a more transient M2 response, and this observation has been proposed to contribute to the poor regenerative capacity of the spinal cord following injury [30, 31]. Consistent among various in vitro and in vivo studies, including spinal cord and peripheral nerve injury models, are reports of reduced levels of pro-inflammatory cell mediators, including as IL-6, iNOS, MCP-1, IL-1β and TNFα in response to treatment with various wavelengths including 633 nm [36], 660 nm, 780 nm [37], 810 nm [16] and 950 nm [14]. Coincidently, these pro-inflammatory cell mediators are secreted by M1 cells; thus, we were curious to examine the effect of light treatment on microglia/macrophage populations.

There are various wavelengths used throughout the literature which demonstrate biological effects. In an attempt to find the better wavelength option for treating nervous system injuries, one study compared the effects of two wavelengths in a variety of CNS injury models, to find that 670 nm treatment resulted in better outcomes for a number of parameters when compared to 830 nm [9]. Our aim therefore was to evaluate the effect of the 670 nm wavelength following spinal cord injury on a variety of functional parameters, namely the development of hypersensitivity to innocuous stimuli (allodynia), as well as on (tactile) sensory pathway conduction and locomotor recovery, and to see if there were alterations to the M1/M2 sub-populations. We found that red light treatment significantly reduced the severity of hypersensitivity while improving sensorimotor function and that these improvements were preceded by an anti-inflammatory microglia/macrophage cell population in the injury zone.

METHODS

Hemicontusion spinal cord injury

All animal work was approved by the ANU Animal Experimentation Ethics Committee. Hemicontusion spinal cord injuries were performed on 7-week-old Wistar rats under isoflurane (1.7–2.3 % v/v) anaesthesia. Following hair removal, a laminectomy of T10 vertebral body and removal of dura and arachnoid was performed, followed by a spinal cord hemicontusion using a customized impactor system [38] comprising of a cylindrical 10 g weight with a 1-mm diameter tip that was guided onto the right dorsal horn and dropped from 25 to 50 mm above the spinal cord.

Treatment and experimental groups

Injured animals were divided into 670-nm-treated (SCI+670) and sham-treated (SCI) groups. SCI+670 rats received 30 min of 670 nm irradiation commencing 2 h after surgery and then every 24 h after locomotor assessment for the remainder of the recovery period. A commercially available 670 nm LED array (WARP 75A, Quantum Devices, Barneveld, WI; 75 mm2 treatment area) was used for treatment. Spectral characteristics and power output (Fig. 1) of the LED were measured using a spectrometer (CCS175, Thorlabs) and custom made power meter that was calibrated against a commercially available power meter (PM100D, ThorLabs). Treatment was delivered through a transparent treatment box which was used to confine the animal within its home cage. This resulted in a 7-mm distance between the dorsal surface of the animal and the LED array and delivery of 35 mW/cm^2 (fluence = 63 J/cm^2) of 670 nm at the contact surface of the animal's dorsum. SCI rats (n = 29) were restrained in the identical way as the

SCI+670 group (n = 29), but without the LED device switched on to control for 30 min restraint in the transparent treatment box. Three additional control groups were included: an intact uninjured group (control; n = 7) was untreated and did not receive any sham operations or sham treatment; a sham-injured group (shamSCI; n = 8) underwent the spinal surgery, but without the contusion, and was subjected to sham treatment; a sham-injured 670-nm-treated group (shamSCI+670; n = 10) underwent spinal surgery without the contusion and received daily 30 min treatments.

Light penetration

Uninjured, unshaven animals (n = 6) were euthanised with sodium pentobarbital solution (325 mg/ml; Virbac; dosage, 100 mg/kg). The overlaying heart, great vessels and muscles were detached from the anchoring connective tissues and retracted to the side to expose the underlying vertebral column. The T10 vertebral body was eroded with a dental drill to expose the spinal cord from the ventral surface. The cadaver was placed on its back in an inverted transparent treatment box so that the dorsum of the cadaver could be positioned over the 670 nm LED array and the ventral surface of the rat was accessible to enable placement of a custom-built light measuring device. This device comprised of a photodiode chip (surface area, 0.62 mm2; maximal response (>95 %) to 630–685 nm; ODD-660W, Opto Diode Corp.) that was fixed to the bottom of an aluminium cylinder (height, 7.0 mm; external diameter, 8.7 mm). The top of the cylinder was sealed with a glass coverslip, and the entire probe was painted with black paint but leaving a small circular window (4.0 mm diameter) centred over the chip sensor. This left a ~2.4-mm lip between the external edge of the glass window and the external circumference of the cylinder. When pressed onto the ventral surface of the spinal cord, no light could penetrate from the side because the chip was located 7.0 mm behind the 4.0-mm aperture; thus, only light rays between 71° and 90° are able to

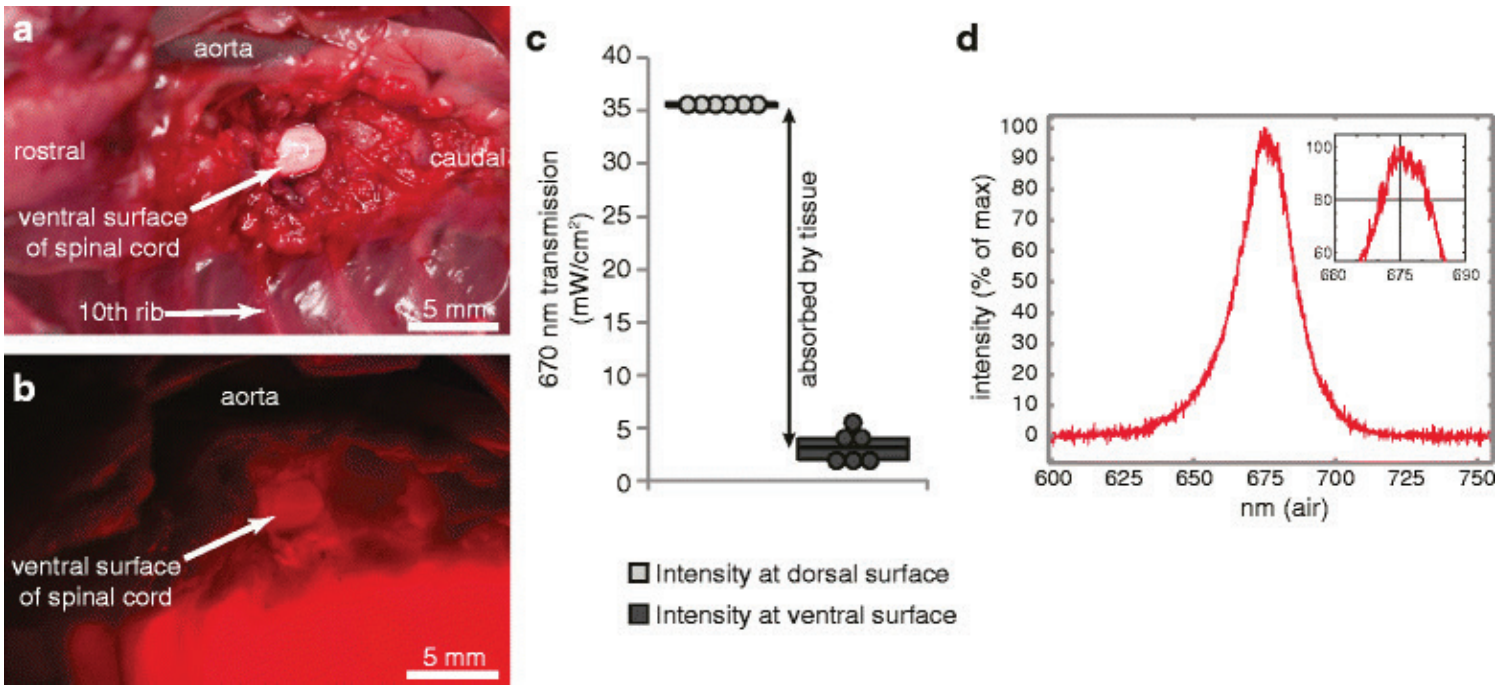

Fig. 1 Externally applied red light penetrates through the entire rat spinal cord. aPhotograph shows the ventral surface of the spinal cord following removal of the T10 vertebral body in a cadaver rat. Topography of the vertebral column is shown centred around the 10th vertebral body under normal light conditions. b The identical region as shown in a, with a 670 nm LED array light source (35 mW/cm2) placed directly on the dorsum of the animal and with ambient lights switched off. Note the visible red light illuminating from the ventral surface of the cord (exposed, arrow) indicating excess penetration through dorsal layers of the hair, skin, muscle, bone and spinal cord. c Intensities measured by a 670 nm power meter are shown for six freshly sacrificed cadaver rats (each dot represents the mean of triplicate readings). Readings shown are taken at the light source (through the Perspex restraining box, intensity at dorsal surface) and at the ventral surface of the spinal cord as shown by the white arrow in b (intensity at ventral surface). Black arrow indicates proportion of light absorbed and/or scattered by intervening tissues. d Spectral analysis of the light source indicating central frequency of 675 nm

reach the surface of the sensor; angles deviating from 90° do not hit the entire surface of the photosensitive diode and therefore contribute less to the total power reading. The signal from the probe was amplified by a custom amplifier built for purpose. The key component was the logarithmic converter amplifier (AD8304, Analog Devices). The readings were then calibrated against a commercially available light power meter tuned at 670 nm (PM100D ThorLabs) by producing a calibration table for different radiant power (controlled by distance from the light source) and subsequently converted into intensity (power/unit area). The probe was used to determine light intensity from the 670 nm array through (i) the treatment box, (ii) the spinal cord and dorsal overlying structures and (iii) the equivalent space through the air to provide a measure of attenuation over the distance of the light path. Prior to activating the LED, ambient lights were switched off; however, we also confirmed that no photons were detected by the light meter with the ambient lights on. Three repeat readings were acquired for each measurement.

Example images were obtained with a D1X Nikon (5.3 megapixels) camera and 120-mm lens (Medical NIKKOR) with a ×2 adaptor and built in ring flash. Images were captured with both the ambient lights and LED array on and then repeated in the same position with the ambient lights off.

Temperature measurement

A temperature probe (ML309/MLT422, ADInstruments) connected to a data acquisition system (PowerLab 26T, LabChart v7.3.7, ADInstruments) was attached to the dorsum of the animals prior to, and 2 min after sham or light treatment on consecutive days from four sham- and four light-treated rats.

Sensitivity assessment

Sensitivity assessment was carried out on day 7 post-injury prior to locomotor and electrophysiological assessments. To assess hypersensitivity, a nylon filament (OD: 1.22 mm) was used to deliver innocuous tactile stimuli over six defined regions over the animals' dorsum: Above-Level (dermatomes C6-T3), At-Level (dermatomes T9-T12) and Below-Level (dermatomes L2-L5) on ipsi- and contralateral sides relative to the injury. The boundary for each of the six regions was marked on the animals' back, and 10 consecutive innocuous "pokes" were delivered in each boundary at an inter-poke-interval of approximately 1–2 s, or until the animal recovered from movement evoked from the previous poke if longer than 2 s. Prior to testing, the operator practiced the stimulus procedure. This ensured that each poke was as brief as possible, that the filament landed normal (90°) to the skin surface and that the final position of the filament handle was approximately half the distance to that of the distance at initial contact of the filament. This protocol ensured pokes of consistent duration and maximum force which was confirmed using a weighing balance (maximum bending force: 2.86 ± 0.09 g; n = 10 pokes). During sensitivity testing, animals were "semi-restrained" in a V-shaped plastic box. This restricted the animal's ability to avoid the testing procedure and thereby facilitated the operator's accuracy of each poke but enabled sufficient movement for the animal to display behavioural responses of interest. Testing was recorded using a webcam (Logitec HD Pro C920). Videos were assessed blind to the observer in slow motion play back by evaluating the response to each innocuous poke that was graded into one of four categories as (I) no response; (II) mild response characterised by acknowledgment of the stimulus, head turns, brief shuddering of the contacted skin, but no obvious pain avoidance behaviours; (III) medium response, characterised by moderate signs of pain perception, including moderate avoidance attempts by moving away from the stimulus and (IV) severe response, characterised by severe signs of pain perception, including attacking the stimulus and "desperate" avoidance attempts and escape behaviours including jumping, running, writhing or audible vocalization. The four categories, I–IV, were chosen because these behaviours are easily distinguishable. The frequency of each response category was multiplied by a weight; categories I–IV were multiplied by 0, 1, $\sqrt{2}$ and 2, respectively, to provide greater separation between ordinal pain behaviours between non-painful and painful [39], as well as to help minimise heteroscedasticity of the data. The sum of the 10 weighted responses provided a regional sensitivity score (RSS) for each region. This paradigm enables high-resolution measures of sensitivity to 10 innocuous pokes with each possible RSS ranging between 0 and 20. Scores from ipsi- and contralateral regions were pooled to determine level sensitivity scores (LSS) above, at and below the level of injury. An cumulative sensitivity score (CSS) was derived for each animal by summing the RSS from all

six regions; the maximum CSS possible is therefore 120. The hypersensitivity threshold was defined by the mean + 2 standard deviations (confidence interval of 95.5 %) of CSSs calculated from uninjured intact rats (control group).

Somatosensory assessment

Animals were anaesthetised with urethane (12.5 % w/v; 1.4 g/kg; i.p.) and maintained at 37 °C on a heating mat. A tracheotomy was performed, and animals were placed in a stereotaxic frame. The gracile nuclei were exposed through the foramen magnum by head flection and removal of overlying muscles and meninges. Both left and right sciatic and sural nerves were exposed by the removal of the overlying skin followed by a splitting incision of the gluteus maximum and semimembranosus muscles, respectively. The exposed nerves were isolated from adjacent connective tissues and bathed in paraffin oil. Silver wire bipolar hook electrodes were used to stimulate sural nerves, and a single hook silver wire electrode was used to record from sciatic nerves to ensure complete recruitment of all sural nerve fibres upon electrical stimulation (square wave pulse, 0.5–1.1 mA, 0.05 ms). A platinum wire electrode was used to record from a single midline position on the brainstem at a location that was established to provide evoked potentials of equal magnitude and latency from left and right sural nerve stimulation. Thirty-three individual evoked potentials were recorded and averaged from the sciatic nerve and the brainstem in response to repeated sural nerve stimulations. Signals recorded from the brainstem were then processed offline (MATLAB, MathWorks). The averaged signal was band-pass filtered (500–3350 Hz) and response magnitudes calculated from the integral of rectified signals (integral limits: 5.00 ms before and 8.75 ms after the primary peak) after subtraction from baseline levels obtained prior to the stimulus. Latency was measured from the filtered signal where it first exceeded 3 standard deviations (confidence interval 99.7 %) of background levels.

Locomotor assessment

Prior to surgery, animals were trained to run along an 80-cm custom build transparent walking-track with mirrors that reflected left and right sides and underneath of the animal. This enabled exquisite locomotor detail from all sides of interest to be video captured simultaneously from a single viewpoint. 2 h following surgery, initial recordings of animals running three consecutive times down the walking-track were acquired with a digital camera (Sony, NEX-VG20EH) at 50 frames per second, which provided adequate data for detailed gait analysis. Recordings were repeated every 24 h post-surgery for 7 consecutive days. Each video file was coded and assessed blind by one assessor. The BBB locomotor scale [40] for the left and right hind-limbs was used to generate locomotor scores from video files assessed in slow motion.

Immunohistochemistry and TUNEL

Animals from both groups (SCI, n = 15; SCI+670, n = 15) were divided into three recovery time points and sacrificed at 1, 3 and 7 days post-injury. At the end of designated recovery periods, animals were transcardially perfused with saline and 4 % buffered paraformaldehyde (w/v). Harvested spinal cords were cryoprotected in 30 % sucrose (w/v), cryosectioned at 20 µm in the longitudinal plane using a Leica CM1850 cryostat, and dorsal sections labelled with primary antibodies (1:200) against rat CD68 (ED-1 clone, MAB1435, Millipore), and Arginase-1 (AB60176, Abcam) or CD80 (AB53003, Abcam) to quantify microglia/macrophages (ED1+) and polarized subtypes M1 (CD80+ED1+) and M2 (Arginase1+ED1+), respectively. Tissue was subsequently incubated with the appropriate secondary antibodies (1:1000, Invitrogen, Alexa 594 conjugated chicken anti-goat #A21468, Alexa 488 conjugated goat anti-mouse #A31619, Alexa 594 conjugated goat anti-mouse #A31623, Alexa 488 conjugated donkey anti-rabbit #A21206). Slides were then incubated in Hoechst solution (2 µg/ml Sigma-Aldrich). Standard immunohistochemical controls were included.

To detect cells undergoing apoptosis/necrosis, a TUNEL assay was performed. Slides were incubated with 1:10 Terminal Deoxynucleotidyl Transferase (TdT) buffer (125 mM Tris-HCl, 1 M sodium cacodylate, 1.25 mg/ml BSA, pH 6.6) for 10 min and then 1-h incubation at 37 °C with reaction mixture [0.5 enzyme unit/µl TdT (Roche Applied Science) and 2.52 µM Biotin-16-dUTP (Roche Applied Science) diluted in 1:10 TdT buffer]. This was followed by 15 min incubation in 1:10 saline sodium citrate (SSC) buffer (175.3 mg/ml sodium chloride, 88.2 mg/ml sodium citrate, pH 7.0) and blocked with 10 % normal goat serum in 0.1 M PBS for 10 min before

incubating with secondary antibody in 0.1 M PBS (1:1000 dilution, Invitrogen, Alexa 488 conjugated streptavidin S11223) at 37 °C for 30 min.

All image analysis was performed blind to the experimental group. 2D images were constructed from three colour channel (red, green and blue) images acquired from a LED fluorescent microscope (Carl Zeiss Colibri) with a ×20 objective and digital camera (AxioCam MRc 5) with all settings kept constant for each channel. Cells with co-labelling were quantified with ImageJ (v1.46r) using the Cell Counter plugin that enables the placement of different classes of markers onto an image. Cytoplasmic markers, a class for each channel, were used to tag positive label in a single focal plane for all green and red channels that were examined independently. To define ED1+ cells, the accompanying DAPI+ nucleus (blue channel) was tagged for cells where ED1 staining was clearly complementing the DAPI surface profile. Double-labelled cells (i.e., ED1+Arginase1+ or CD80+) were evaluated by scrutinising all tagged DAPI+ cells for co-labelling in red and green channels. These cells were tagged again with another marker class. All markers were automatically quantified for each class by the software. Cells out of focus were not included. Cell counts were obtained from dorsal horn regions with viable tissue and quantified as the mean of duplicate images, each covering a minimum area 0.05 mm2. The areas of interest were defined and quantified prior to cell quantification and included the dorsal horn grey matter region as well as the white matter in the surrounding dorsal columns and lateral funiculus. Cell quantification is expressed as the number of cells per unit area (mm2).

Statistics

All data expressed as boxplots with individual data points in figures or as mean ± SEM in the main text, unless otherwise stated. Boxplots indicate the median (thicker line), upper and lower quartiles with whiskers extending to maximum and minimum values excluding outliers (more than 1.5 times respective quartiles). Statistical analysis was carried out using R or MATLAB, and a criterion alpha level of 0.05 was adopted as statistically significant. Data sets were tested for normality and homoscedasticity, and ttests and linear mixed models (multi-factor ANOVA) were applied for normally distributed data (indicated by *) or Wilcoxon rank-sum (indicated by †) where data was not normally distributed.

RESULTS

Red light penetrates the spinal cord

We first set out to demonstrate that red light can pass through superficial and deep structures underlying the dorsal exterior surface and penetrate the entire spinal cord (Fig. 1). The penetrating light could be seen with the naked eye (example, Fig. 1a, b). The dorsal surface of uninjured rats (n = 6) was exposed to the LED array and 670 nm light intensity measured at the light source surface through the transparent treatment box which directly contacts the rat dorsum during treatment (Fig. 1c, intensity at dorsal surface; 35.4 ± 0.05 mW/cm^2) and the ventral surface of the spinal cord, where light had to pass through an additional ~10 mm of the animals' tissues from dorsal surface (Fig. 1c, intensity at ventral surface; 3.2 ± 0.6 mW/cm^2). These data show that 91.1 ± 1.8 % of the light from the LED array was absorbed/dispersed by the tissues between the dorsal surface of the animal and the ventral surface of the spinal cord (Fig. 1c, black arrow). To indicate the approximate attenuation over the distance of light travelling from the light source through to the ventral spinal cord surface, we measured the intensity at the approximate distance (10 mm) through the air (33.0 ± 0.5 mW/cm^2). This demonstrated that the expected attenuation (~7 %) of light is negligible over the distance required to travel to the ventral surface of the cord.

Surface temperature changes following light treatment

We measured the surface temperature of rats directly before and 2 min after treatment. Twenty-seven readings from sham-treated and 25 readings from light-treated animals were acquired from four animals in each group over consecutive days of treatment. While there was no significant difference in the surface temperature of sham-treated animals (before, 33.6 ± 0.23 °C; after, 33.6 ± 0.25 °C), there was a small but significant increase 2 min after light treatment (before, 32.8 ± 0.36 °C; after, 33.5 ± 0.22 °C; p = 0.038, paired ttest).

Red light reduces allodynia following spinal cord injury

To examine the effect of red light on the development of neuropathic pain, we assessed sensitivity on six regions over the rat dorsum using a T10 hemicontusion spinal cord injury model that results in clear development of hypersensitivity in most

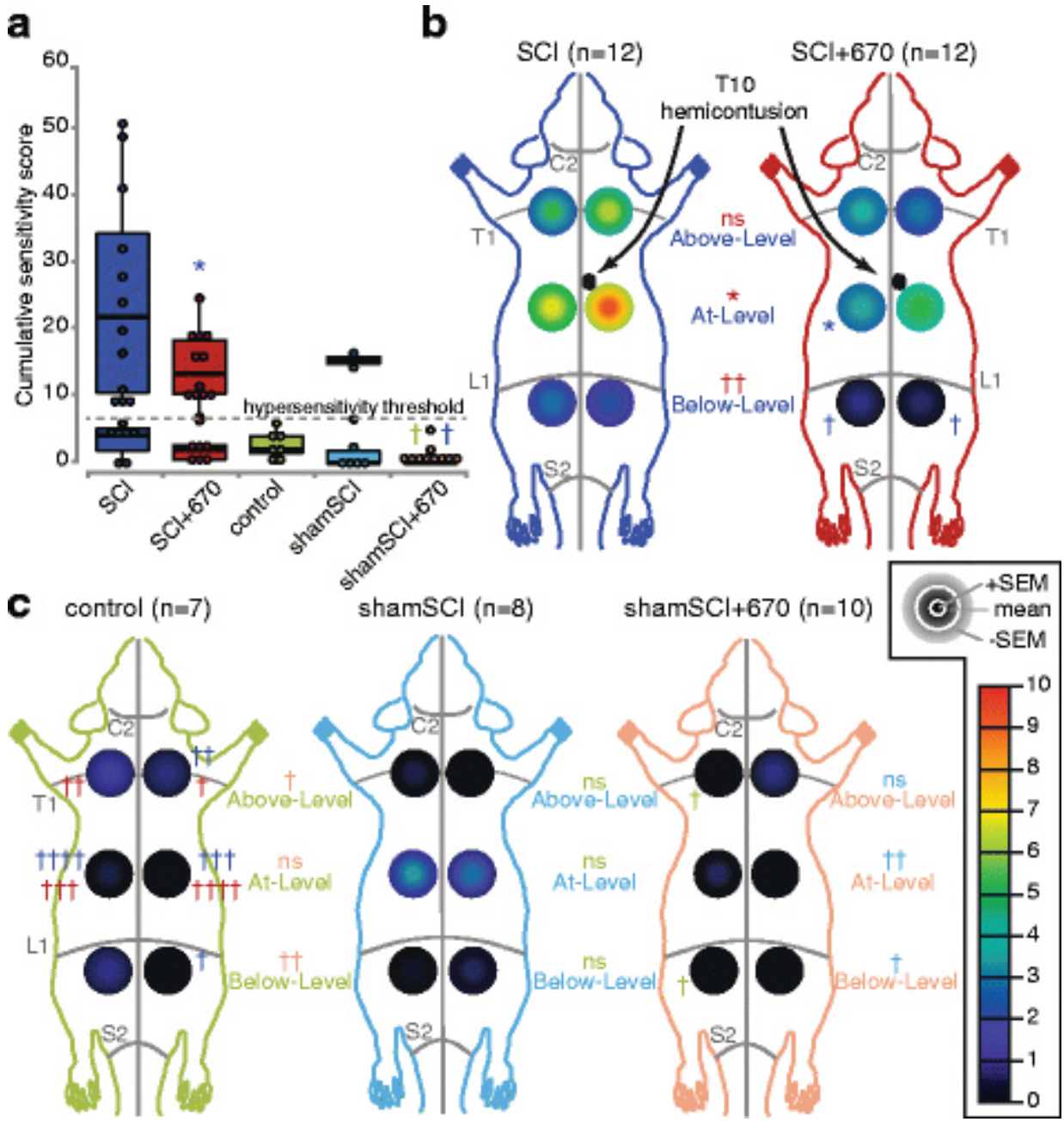

Fig 2 Hypersensitivity is reduced by red light treatment at 7 days post-T10 hemicontusion spinal cord injury. **a** CSSs (see the "Methods" section) for all groups are separated by the hypersensitivity threshold (6.9; indicated by *dotted green line*) into normosensitive (CSS < hypersensitivity threshold) and hypersensitive (CSSs > hypersensitivity threshold) subpopulations. **b** RSSs in hypersensitive sham-treated (SCI, *dark blue*) and 670-nm-treated (SCI+670, *dark red*) spinal cord injured animals (location of injury indicated). RSSs are represented as the mean ± SEM (colour-coded according to the insert: mean + SEM, mean, and mean − SEM concentrically represented) for the six tested regions (left and right sides; "Above-Level", "At-Level" and "Below-Level" relative to the injury). RSSs are overlayed on schematic representations of the rat dorsum, with C2, T1, L1 and S2 dermatomes, and the midline, indicated (*grey*). Individual RSSs and LSSs are compared between hypersensitive subpopulation of the two groups. **c** RSSs shown for normal uninjured rats (control, *green*), sham-injury + sham-treatment (shamSCI, *light blue*, data includes both normo- and hypersensitive subpopulations), and sham-injury + 670 nm treatment (shamSCI+670, *light red*). Pairwise statistical comparisons are indicated for RSSs and LSSs by respective group colours. Note: statistical comparisons of CSSs from shamSCI+670 group in (**a**) is to the normosensitive subpopulation of SCI (indicated in *dark blue*) and to control groups (indicated in *green*); Statistical comparisons of RSSs from control group in (**c**) is to SCI (indicated in dark blue) or to SCI+670 (indicated in *dark red*) in **b**. *$p < 0.05$ (Student's *t* test); † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, †††† $p < 0.0001$, (Wilcoxon rank-sum); ns, $p > 0.05$; *n* values indicated.

animals within 7 days. The T10 spinal hemicontusion resulted in 63 % of animals (n = 12) developing hypersensitivity in both sham-treated (SCI, n = 19) and light-treated (SCI+670, n = 19) groups at 7 days post-injury. The hypersensitive subpopulation of rats from the SCI group had a mean CSS (SCI, CSS: 25.3 ± 4.5) that was 3.7 × the hypersensitive threshold (Fig. 2a). The mean CSS was significantly reduced by 40 % (SCI+670, CSS: 14.5 ± 1.6; 2.1 × the hypersensitivity threshold) in the hypersensitive subpopulation of rats from the SCI+670 group. Light treatment significantly reduced At- (T9-T12 dermatomes) and Below- (L2-L5 dermatomes) LSSs, which arose from contralateral At-Level and both ipsi-and contralateral Below-Level regions (Fig. 2b). Compared to the uninjured control group (control, Fig. 2c), sham injury without light treatment (shamSCI, n = 8) had no significant effect on LSS or RSS despite two sham-injured animals developing At-Level hypersensitivity. Light treatment of sham-injured animals (shamSCI+670, n = 10) resulted in significant reductions of At- and Below-LSS compared to the shamSCI group (Fig. 2c). Thus, while the incidence of hypersensitivity was not altered by red light, the level of hypersensitivity was markedly reduced At- and Below-levels in T10 contused light-treated allodynic animals. Red light also caused a significant reduction in sensitivity in 670-treated sham-injured animals (shamSCI+670, CSS: 0.8 ± 0.5) compared to uninjured control animals (control, CSS: 2.8 ± 0.8) as well as normosensitive spinal cord injured animals (SCI, CSS: 3.5 ± 0.9), even though these animals were not hypersensitive.

Red light improves sensory conduction through dorsal column pathways

Could red light cause an anaesthetic-like effect on somatosensation that resulted in reduced sensitivity scores? To rule out the possibility that red light causes a reduced responsiveness to innocuous stimuli

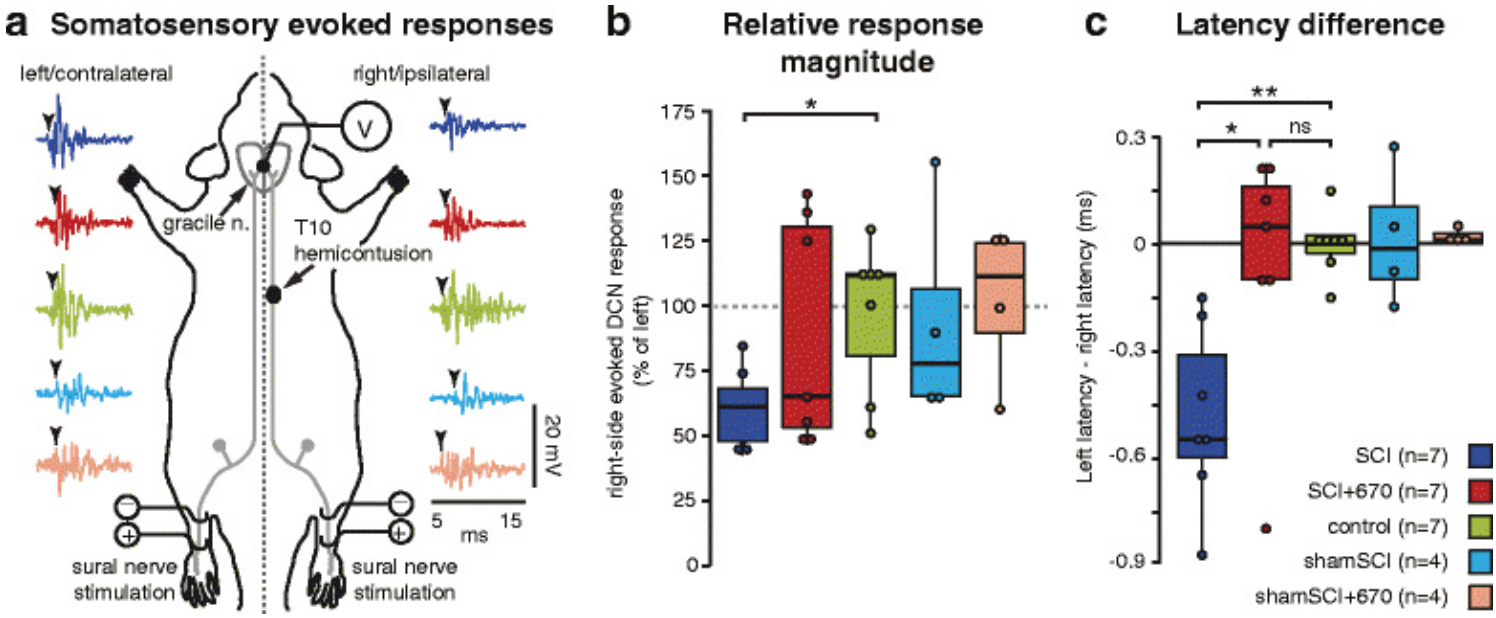

Fig 3 Dorsal column somatosensory functional deficits from T10 hemicontusion spinal cord injury is reversed by red light treatment. a Schematic of experimental paradigm for evaluating somatosensory (dorsal column pathway) functional integrity illustrating left and right dorsal column pathways (grey), T10 hemicontusion injury on right side, stimulation of sural nerves and location of recording electrode on midline of gracile nucleus. The same electrode position on the midline acquires somatosensory responses independently evoked from both left and right sural nerves, enabling direct comparable quantification of sensory pathways on both sides. Examples of responses (between 5 and 15 ms post-stimulus; 500–3350 Hz bandpass) evoked from left and right sides shown for respective groups (colour-coded as per legend in c and Fig. 2). Arrowheadsindicate latency of response onset. b Quantification (integral of rectified signals) of gracile nucleus response magnitudes (right expressed as a percent of left). c Difference in latencies of evoked responses between left and right sides. Note magnitudes and latencies from intact animals are equal on both sides (control group). *p < 0.05; **p < 0.01, Student's t test, Tukey's post hoc in c,

by bringing about a generalized inhibitory effect on somatic neural pathway conduction, we quantified the functional integrity of the sensory dorsal column pathway, at 7 days post-injury. The dorsal column pathways were activated by electrical stimulation of the left and right sural nerves, and a recording electrode was placed on the midline of the gracile nuclei (Fig. 3a). Stimulation of left and right nerves from control animals (n = 7) evoke responses of equal magnitude (Fig. 3b; right side: 101 ± 8 % of left side) and latency (Fig. 3c; left-right side latency difference: 0.09 ± 0.03 ms) on both sides when recorded from the same midline-positioned recording electrode, while sham-treated T10 hemicontusion spinal cord injury (n = 7) resulted in a 37 % reduction in magnitude (right side: 63 ± 16 % of left side) and a 0.48 ± 0.09 ms delay of the injured (right) pathway, when comparing the intact (left) side. Red light treatment (n = 7) rescued both the magnitude (Fig. 3b; right side: 93 ± 17 % of left side) and latency (Fig. 3c; left-right side latency difference: −0.05 ± 0.35 ms) deficits otherwise observed in the SCI group, indicating that red light treatment restored sensory pathway conduction, rather than impeding it. Furthermore, the rescued magnitude and latency deficits in the SCI+670 group indicates that their reduced sensitivity scores (Fig. 2) were unlikely to have resulted from a generalized reduction of somatic neural conduction.

We performed a variety of control experiments to validate our interpretations. There was no observable difference of conduction magnitudes or latencies in any of the sham-injured animals (shamSCI, n = 4; shamSCI+670, n = 4). There was no significant difference between gracile nuclei potentials evoked from the left sural nerve in any of the groups (SCI, 15.9 ± 1.8 μV · ms; SCI+670, 11.9 ± 2.4 μV · ms; control, 16.2 ± 3.6 μV · ms; shamSCI, 10.8 ± 2.6 μV · ms; shamSCI+670, 15.0 ± 2.8 μV · ms; p = 0.70, one-way ANOVA). Similarly, there was no significant difference of response latencies when evoked on the left side for all groups (SCI, 33.7 ± 0.3 μV · ms; SCI+670, 34.0 ± 0.4 μV · ms; control, 34.0 ± 0.4 μV · ms; shamSCI, 34.2 ± 0.5 μV · ms; shamSCI+670, 34.7 ± 0.3 μV · ms; p = 0.51, one-way ANOVA). These control experiments indicated

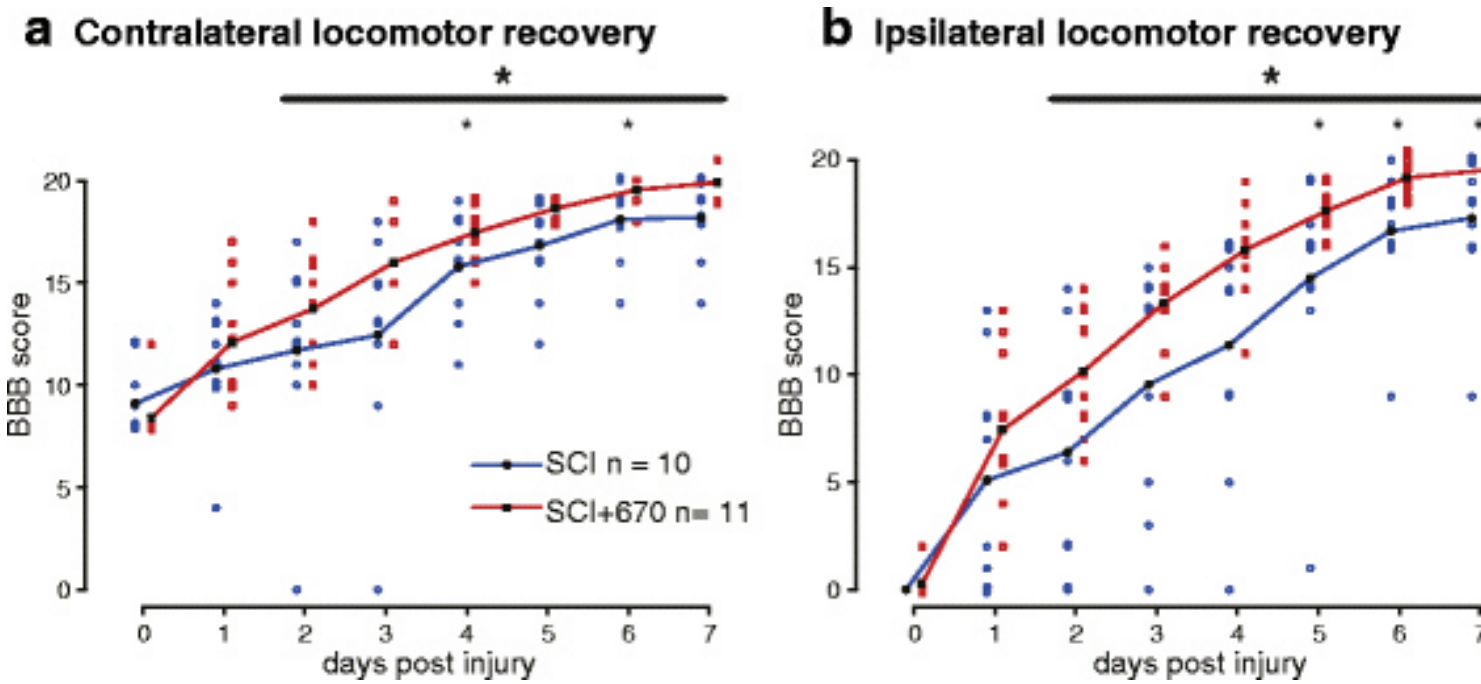

Fig 4 Locomotor recovery is improved by red light treatment following T10 hemicontusion spinal cord injury. Daily locomotor scores (BBB, see the "Methods" section) following a right-sided hemicontusion spinal cord injury are shown for the contralateral (a) and ipsilateral (b) sides. Red light treatment results in significant locomotor improvements on both sides over the period indicated by the black bar (large asterisk, linear mixed model with repeated measures). Point-wise comparisons between groups for individual time points are also shown (small asterisks, Student's t test). Individual data points are presented as open square or circular dots; lines indicate the group means. *p < 0.05; **p < 0.01.

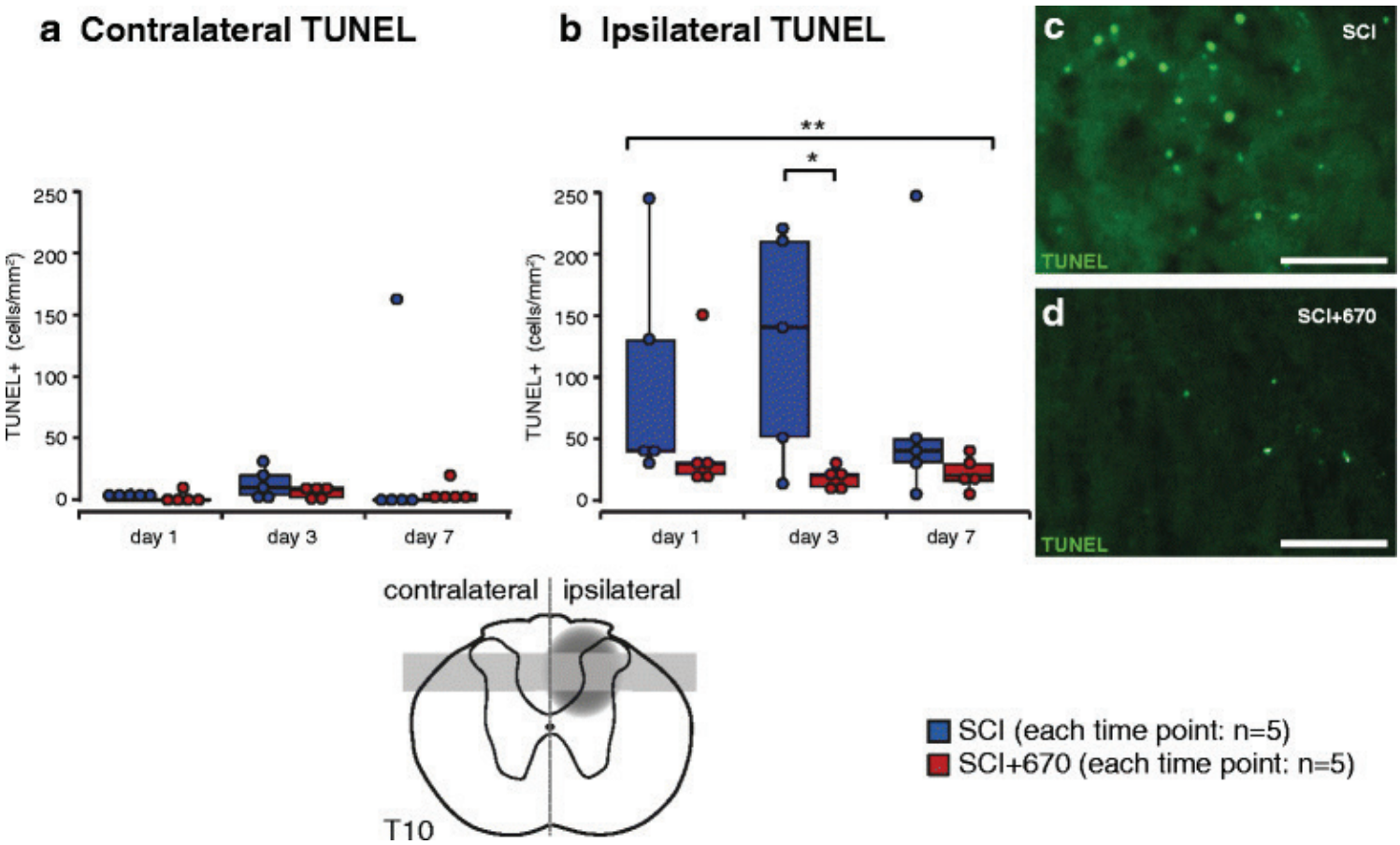

Fig 5 Cell death is reduced by red light following T10 hemicontusion spinal cord injury. Quantification of cells undergoing cell death (TUNEL⁺) contralateral (**a**) and ipsilateral (**b**) to the injury. Example images are from SCI (**c**) and SCI+670 (**d**) dorsal horn ipsilateral to the injury at 3 days post-injury. Schematic cross section of spinal cord (*bottom*) indicates location of injury (*dark grey* penumbra) and region of quantification (*light grey region*). *Scale bars*: 50 µm. *$p < 0.05$ (Student's *t* test); **$p < 0.01$ (linear mixed model).

that dorsal column pathway response magnitudes and latencies were similar between the different groups and largely unaffected contralateral to the injury.

Red light improves locomotor recovery

Could red light treatment cause motor deficits and thereby result in reduced sensitivity scores? To rule out the possibility that the red light impeded the animals' ability to perform escaping locomotor behaviours, daily locomotor recovery was examined blind to the experimental group (Fig. 4). We found that rather than impeding locomotion, the SCI+670 group (n = 11) demonstrated improved locomotor recovery as early as 2 days post-injury on the ipsilateral side and 3 days post-injury on the contralateral side compared to the sham-treated group (n = 10). Although a group effect of red light improvement was evident on the ipsilateral side (p = 0.026, linear mixed effects model with repeated measures), this failed to reach significance on the contralateral side (p = 0.055). There was a highly significant effect of time for both sides (p < 2e-16). Locomotor improvements observed in the SCI+670 group indicate that reduced sensitivity scores in light-treated animals (Fig. 2) could not have resulted from locomotor deficits.

Red light reduces cell death at the injury zone

To examine the effect of red light on cell death following injury, the number of TUNEL⁺cells was quantified at 1, 3 and 7 days post-injury in dorsal regions of the T10 spinal cord (Fig. 5, *n* = 5 for each time point). The SCI group resulted in an increased density of TUNEL⁺ cells in the dorsal spinal cord ipsilateral to the injury as early as day 1 (contralateral 1.5 ± 1.5 cells/mm²;

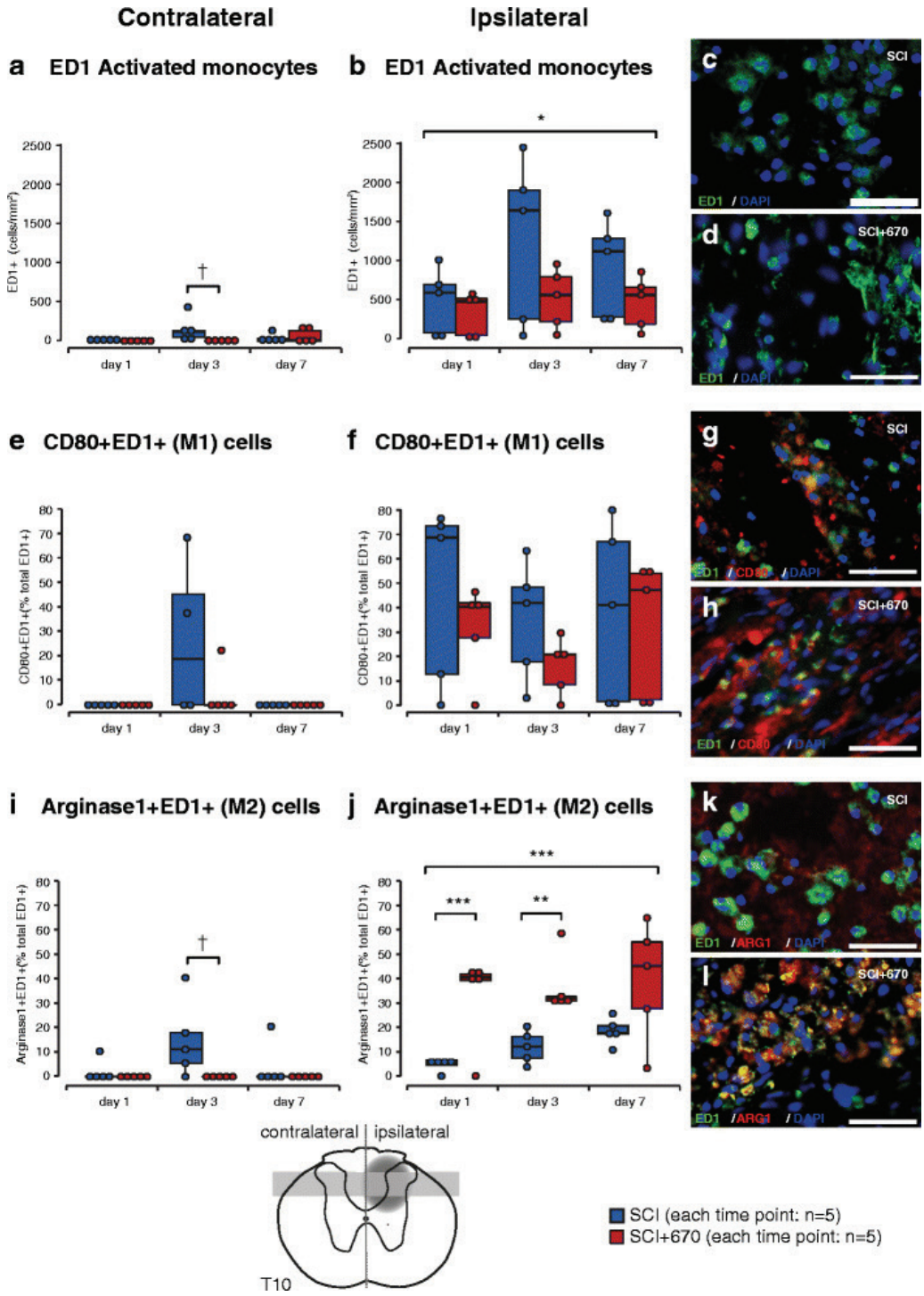

Fig. 6 Anti-inflammatory microglia/macrophages are promoted early by red light treatment following T10 hemicontusion spinal cord injury. a–d Total activated microglia/macrophages (ED1+) per mm2 contralateral (a) and ipsilateral (b) to the injury and example images from SCI (c) and SCI+670 (d) groups. e–h M1 (pro-inflammatory) microglia/macrophages (CD80+ED1+ double labelled) expressed as a proportion of total ED1+ cells contralateral (e) and ipsilateral (f) to the injury and example images from SCI (g) and SCI+670 (h) groups. i–l: M2 (anti-inflammatory) microglia/macrophages (Arginase1+ED1+ double labelled) expressed as a proportion of total ED1+ cells contralateral (i) and ipsilateral (j) to the injury and example images from SCI (k) and SCI+670 (l) groups. All example images are taken from the injury zone of the dorsal horn at 7 days post-injury. Schematic cross section of spinal cord (bottom) indicates location of injury (dark grey penumbra) and region of quantification (light grey region). Scale bars: 50 µm. *p < 0.05 (linear mixed model); **p < 0.01, ***p < 0.001 (Student's t test); † p < 0.05, †† p < 0.01, ††† p < 0.001 (Wilcoxon rank-sum).

ipsilateral 96.8 ± 41.1 cells/mm^2), reaching maximum levels by day 3 (contralateral 13.1 ± 5.6 cells/mm^2; ipsilateral 126.8 ± 41.5 cells/mm^2). The contralateral side had much fewer cells where maximum levels were reached by day 7 (Fig. 5; contralateral 32.5 ± 32.5 cells/mm^2; ipsilateral 74.2 ± 43.7 cells/mm^2). Red light treatment resulted in a significant group reduction of TUNEL$^+$ cells in the ipsilateral side, notably significant at the day 3 time point when TUNEL$^+$ cells were maximal in the sham-treated group (1 dpi: 49.6 ± 25.2 cells/mm^2; 3 dpi 18.2 ± 3.9 cells/mm^2; 7 dpi 22.0 ± 6.1 cells/mm^2). There was no significant difference in TUNEL labelling on the contralateral side between groups (1 dpi: 2 ± 2 cells/mm^2; 3 dpi 6.2 ± 2.1 cells/mm^2; 7 dpi 5.0 ± 3.9 cells/mm^2).

Red light reduces total activated microglia/macrophages but promotes the expression of the anti-inflammatory/wound-healing (M2) subtype

Inflammation has long being implicated in the development of neuropathic pain [27]. We therefore quantified activated microglia/macrophages (ED1$^+$ cells) at 1, 3 and 7 days post-injury in dorsal regions of T10 spinal cord (Fig. 6a–d, n = 5 for each time point). T10 spinal contusion resulted in an increase in ED1$^+$ cell density as early as day 1 post-injury, reaching maximum levels by day 3 in the ipsilateral side. Maximum levels were also reached at day 3 on the contralateral side, but there were negligible ED1$^+$ cells at days 1 and 7. Light treatment significantly reduced ED1 expression ipsilateral to the injury to approximately half that of the SCI group. Despite the low levels of ED1$^+$ cells in the contralateral side, red light treatment also resulted in a significant reduction of ED1$^+$ cells at the 3-day time point.

Microglia/macrophages can adopt pro- or anti-inflammatory states [30]. To determine the effect of red light treatment on the expression of pro-inflammatory (M1) cells, cells co-expressing CD80 and ED1 were quantified as a proportion of total ED1+ cells (Fig. 6e–h, n = 5 for each time point). The proportion of CD80+ED1+ cells ipsilateral to the injury was maximal at day 1 and remained greater than 40 % of the ED1 population at days 3 and 7 in more than half of animals. CD80+ED1+ cells were only found at day 3 on the contralateral side which coincided with the maximum number of ED1+ cells at that time point. Red light treatment did not have a significant impact on the proportion of M1 cells on either the ipsi- or contralateral sides. Note that no CD80+ED1+ cells were encountered at days 1 and 7 contralateral to the injury as ED1+ cells were also in small quantities at these time points (Fig. 6a).

To determine the effect of red light treatment on the expression of anti-inflammatory/wound-healing (M2) microglia/macrophages, ED1+ cells co-expressing Arginase-1 were quantified as a proportion of total ED1+ cells (Fig. 6i–l, n = 5 for each time point). In the SCI group, Arginase-1 expression increased with time ipsilateral to the injury ($p = 0.0048$, one-way ANOVA) but peaked at day 3 contralateral to the injury at the time when most ED1+ cells were present in that region. Ipsilateral to the injury, the SCI+670 group displayed significantly increased proportions of Arginase1+ED1+ cells from day 1, reaching approximately sevenfold that of the SCI group. This greater Arginase1+ED1+ proportion in light-treated animals was maintained at over one third of ED1+ cells for the entire duration investigated for the majority of animals. No Arginase1+ED1+ cells were detected contralateral to the injury in the SCI+670 group; however, there were very few ED1+ cells in this region (Fig. 6a). The group effect failed to reach significance contralateral to the injury ($p = 0.0628$) despite a significantly greater level of Arginase1+ED1+ cells in the SCI group.

DISCUSSION

We demonstrate that following spinal cord injury, 35 mW/cm^2 of red (670 nm) light transcutaneously applied for 30 min/day for 7 days to the dorsal surface of rats is sufficient to reach the entire spinal cord and reduce the expression of pain behaviours. These reduced signs of allodynia are not due to sensorimotor deficits, as red light treatment improves both sensory and motor function. Alleviated hypersensitivity, improved tactile/proprioception (dorsal column) pathway functional integrity and locomotor functional outcomes are preceded by reduced numbers of dying cells and reduced numbers of activated microglia/macrophages around the injury zone. Furthermore, the proportion of anti-inflammatory/wound-healing (M2) microglia/macrophages is greatly enhanced by 24 h following light treatment.

We are confident that the power output of the red light was sufficient to penetrate the entire rat spinal cord as red light could be seen with the naked eye illuminating through to the ventral surface of the cord in the cadaver models. While penetration through to the rat spinal cord was achievable with an intensity of 35 mW/cm^2, future studies would be required to determine the exposure parameters to achieve an equivalent level of irradiation in humans. Our finding of 91 % absorption (9 % excess penetration) is a conservative measure for two main reasons: (i) penetration measurements were obtained through the hair of unshaven animal cadavers (the injury site of all injured animals was shaven) and (ii) deoxygenated haemoglobin absorbs 670 nm significantly more than oxygenated haemoglobin [41, 42]. Measurements from freshly scarified animals are therefore likely to have increased levels of deoxygenated blood, and thus reduced penetration, compared to live animals. Another factor to consider is the small attenuation of light as a function of distance from its source. Our estimation indicates that over 93 % of the light would have reached the spinal cord ventral surface if no intervening tissues were present to absorb the light; thus, the effect of distance appears to be negligible. Nine percent excess penetration (i.e. 91 % absorption) from the surface of the skin (with hair intact) through all intervening tissue layers to the ventral surface of the spinal cord with a device delivering approximately 35 mW/cm^2 is consistent with a recent study that demonstrated an excess penetration of 6.6 % through the surface of the skin and the muscle overlying the spinal cord with a device producing approximately 16 mW/cm^2 in Fisher rat cadavers [9].

Temperature of sham-treated animals was not significantly different before and after treatment, while light-treated animals' experienced a significant 1.2 °C increase. This increase does not exceed the normal range for rat tail skin temperature variations which have been reported to oscillate by ± 2 °C within a 2-h time frame [43]. However, as there was a small but significant temperature increase 2 min after light treatment, it is likely that there was a larger temperature increase during the 30 min treatment period. We therefore cannot rule out the possibility that temperature increases did not impact on our findings. Nevertheless, red light treatment does result in significant functional and cellular improvements, regardless if temperature is a contributing factor. If temperature increases were to contribute toward improved outcomes, it would be in contrast to studies of hypothermic treatment which propose superior outcomes following spinal cord injury [44–46]. As the mechanisms of action for light-treatment improvements remain to be elucidated, future investigations isolating the effect of temperature and light are warranted.

To our knowledge, our study is the first to report a red light-induced locomotor improvement following a spinal cord injury, which contradicts the only other study by Giacci et al. [9] that examined 670 nm on locomotor recovery with a daily dose of 28.4 J/cm^2, an intensity of 15.8 mW/cm^2 for 30 min, i.e. less than half the intensity of the present study. The compounded effect of reduced intensity and a more severe contusion injury in their study may explain this difference, and furthermore, suggests that matching the appropriate light dosage to the injury severity is of paramount importance.

Our T10 hemicontusion injury model resulted in allodynia within 7 days in a subset of animals. We are confident that our injury model results in neuropathic pain because hypersensitivity developed above and below the level, as well as contralateral to the injury, i.e. at dermatomes that receive their innervation from outside the injury epicentre. This observation is also consistent with findings from an investigation using a C5 hemicontusion injury model and which also found a subset of animals developing allodynia from 7 days post-injury that lasted for least 42 days [47]. Our observation of allodynia on the animals' dorsum is also consistent with a T13 hemisection injury model that also results in clear development of hypersensitivity in most animals within 7 days and that remains persists for several weeks [48].

We found that red light treatment reduced the severity, but not the incidence of hypersensitivity at 7 days post-injury. As allodynia reached sensitivity levels of almost four times that of the hypersensitivity threshold, we would expect that a milder injury causing sensitivity scores closer to the hypersensitivity threshold boarder would result in a reduction of both the severity and incidence of hypersensitivity. The finding that the shamSCI+670 group demonstrated sensitivity significantly lower than that of the intact control and shamSCI groups was curious. Sham injury may have activated anti-nociceptive descending pathways such as periaqueductal grey/

raphe magnus-mediated inhibition of dorsal horn nociceptive inputs [49]. Thus, endogenous central anti-nociceptive mechanisms, compounded by a red light-induced anti-inflammatory microenvironment, could be responsible for the sham-injured-light-treated animals expressing less sensitivity than observed in uninjured animals. This speculation warrants further investigation as red light-augmented relief from pain would have significant clinical relevance for post-surgical pain treatment.

Quantification of pain behaviours relies on sensory and motor functional integrity. We are confident that the reduced expression of allodynia in red light-treated animals was not due to diminished general somatic sensation or impeded motor function because red light improved, rather than impeded these parameters. Locomotor recovery and sensitivity testing was scored blind to the experimental group, and therefore, any subjective bias was eliminated. Sural nerve evoked somatosensory potentials in the gracile nuclei provided an objective and precise measure of somatic sensory functional integrity of both left and right dorsal column pathways. Sural nerves were stimulated to recruit all nerve fibres, and therefore, input to the spinal cord was identical on both sides, while the recording conditions on the midline of the gracile nuclei were also identical during the acquisition of evoked potentials elicited from pathways of both sides. Therefore, the only difference in the responses between the left and right sides was due to alterations within their respective dorsal column pathways. We further confirmed this by demonstrating equal magnitudes and latencies of somatosensory potentials in the gracile nuclei when evoked from left and right sural nerves of intact and sham-injured animals. Thus, our data indicates that reduced expression of behavioural signs of pain following red light treatment is unlikely to have resulted from locomotor or sensory deficiencies, but rather, represents a true reduction of pain experienced by the light-treated rats.

While our study is the first to demonstrate red light-induced pain relief from spinal cord injury, it is consistent with peripheral nerve injury studies that report pain relief accompanied by light-induced alterations to the inflammatory response [13–15, 50, 51]. The functional improvements found in red light-treated animals were observed after a significant reduction in cell death was apparent at day 3 post-injury, a time coincident with maximal levels of activated microglia/macrophages in the injury zone of sham-treated animals. Our observations of reduced ED1+ cells in 670-nm-treated animals is consistent with that found in retinal damage [7], as well as another study that demonstrated similar proportions of ED1 cell suppression lasting up to 14 days post-corticospinal tract lesion in rats that received daily 810 nm diode laser treatments [16]. In the latter study by Byrnes et al., they also demonstrated functional improvements of some motor tasks, also consistent with improved locomotor function observed in our study. While we cannot speculate on the mechanisms for 810 vs. 670 nm wavelengths to suppress microglia/macrophage activation and improve motor function, it is noteworthy that both wavelengths evoke peak levels of cytochrome C oxidase activity and ATP production [52]. However, wavelength (i.e. 660 vs. 780 nm) has been shown to alter the expression of inflammatory mediators expressed by activated pro-inflammatory microglia/macrophages [37], and light dosage has been shown to alter the balance of M1/M2 cell expression [53]. These in vitro studies suggest that other mechanisms, unrelated to cytochrome C oxidase, may influence the inflammatory microenvironment following light treatment. Furthermore, they highlight the necessity for thorough investigations to establish the therapeutic limits of any wavelength under investigation.

While others have demonstrated the impact of wavelength and dose on inflammatory cells in vitro [37, 53], to our knowledge, we are the first to demonstrate the effect of 670 nm light on the polarization of activated microglia/macrophages following spinal cord injury in vivo. The pattern and sequence of pro-inflammatory M1 (CD80+ED1+) cell activation, cell death, followed by anti-inflammatory/wound-healing M2 (Arginase1+ED1+) recruitment observed in sham-treated animals in our study is consistent with what is expected under conditions of spinal cord injury and repair [30, 31]. However, our data indicates that red light-induced reduction of cell death is preceded by the upregulation of M2 cells as early as 1 day post-injury. This is intriguing because the M2 cell expression preceded that of the M1 cells, indicating that red light drastically altered the normal sequence of inflammatory events. We therefore speculate that the early presence of protective M2 cells may have caused the reduced subsequent population of dying (TUNEL+) cells. Insufficient expression of the M2 subtype in spinal cord injury, in

contrast to peripheral nerve injury, has been suggested to be a contributing factor to the poorer regenerative capacity and functional outcomes in spinal cord injury compared to peripheral nerve injury [30, 31]. The present study found that red light had a strong impact on promoting the M2 cell types as early as 24 h after treatment which was followed by reduced levels of cell death and subsequent improvement of sensory and motor functional outcomes thereafter. This is consistent with previous suggestions that enhancing the M2 population during recovery from spinal cord injury may indeed significantly contribute to improving functional outcomes following spinal cord injury [31].

CONCLUSIONS

Modulating the severity of neuropathic pain by simply applying red light is an exciting prospect with great significant clinical relevance, despite not yet fully understanding the mechanism behind photobiomodulation. Our data demonstrates that red light treatment, a non-invasive and cost effective treatment, is able to significantly reduce the severity of pain in rats acutely after spinal cord injury, and these behavioural changes are accompanied by alterations to the alternatively activated macrophage population. Early pain intervention is considered important to avoid the prospects of developing chronic pain [54]. As 670 nm light therapy is FDA approved, it could be quickly adopted as an adjunct to early treatment of spinal cord injury. Not only could this minimise the severity of pain to sufferers, it may also provide collateral benefits which include functional improvements to other sensory/motor systems. However, translation to human patients requires further studies to determine exposure parameters such as the light intensity necessary to penetrate the human spinal cord.

ABBREVIATIONS

ANOVA: Analysis of variance
CSS: Cumulative sensitivity score
BBB: Basso, Beattie and Bresnahan
CNS: Central nervous system
FDA: Food and Drug Administration
LED: Light-emitting diode
LSS: Level sensitivity score
M1: Th1-activated microglia/macrophages
M2: Th2-activated microglia/macrophages
PBS: Phosphate-buffered saline
RSS: Regional sensitivity score
shamSCI: Sham-treated sham-injured group
shamSCI+670: 670-nm-treated sham-injured group
SCI: Sham-treated spinal cord injured group
SCI+670: 670-nm-treated spinal cord injured group
SSC: Saline sodium citrate
TdT: Terminal deoxynucleotidyl transferase
Th1: T helper cell type 1
Th2: T helper cell type 2
TUNEL: Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling

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AVAILABILITY OF DATA AND MATERIALS

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AUTHORS' CONTRIBUTIONS

JRP and DH conceptualised and designed the experiments; DH and SZ conducted the experiments; DH, JRP and SZ conducted the analysis; DH and JRP wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

CONSENT FOR PUBLICATION

Not applicable.

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THE EFFECT OF RED-TO-NEAR-INFRARED (R/NIR) IRRADIATION ON INFLAMMATORY PROCESSES

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ABSTRACT

ABSTRACT

Introduction:

Near-infrared (NIR) and red-to-near-infrared (R/NIR) radiation are increasingly applied for therapeutic use. R/NIR-employing therapies aim to stimulate healing, prevent tissue necrosis, increase mitochondrial function, and improve blood flow and tissue oxygenation. The wide range of applications of this radiation raises questions concerning the effects of R/NIR on the immune system.

METHODS:

In this review, we discuss the potential effects of exposure to R/NIR light on immune cells in the context of physical parameters of light.

DISCUSSION:

The effects that R/NIR may induce in immune cells typically involve the production of reactive oxygen species (ROS), nitrogen oxide (NO), or interleukins. Production of ROS after exposure to R/NIR can either be inhibited or to some extent increased, which suggests that detailed conditions of experiments, such as the spectrum of radiation, irradiance, exposure time, determine the outcome of the treatment. However, a wide range of immune cell studies have demonstrated that exposure to R/NIR most often has an anti-inflammatory effect. Finally, photobiomodulation molecular mechanism with particular attention to the role of interfacial water structure changes for cell physiology and regulation of the inflammatory process was described.

CONCLUSIONS:

Optimization of light parameters allows R/NIR to act as an anti-inflammatory agent in a wide range of medical applications.

KEYWORDS:

Immune system, photobiomodulation, reactive oxygen species (ROS), R/NIR radiation, anti-inflammatory, low-level light therapy (LLLT)

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THE EFFECTS OF PHOTOBIOMODULATION ON MC3T3-E1 CELLS VIA 630 NM AND 810 NM LIGHT-EMITTING DIODE

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CASE REPORT

ABSTRACT

Background

Photobiomodulation (PBM) has been explored as a promising therapeutic strategy to regulate bone cell growth; however, the effects of PBM on osteoblast cell lines remains poorly understood. In addition, as a light source of PBM, the light uniformity of light-emitting diode (LED) devices has not been given enough attention. MATERIAL AND METHODS Here, we sought to investigate the effects of PBM on MC3T3-E1 cells via 630 nm and 810 nm light from a newly designed LED with high uniformity of light. Cell proliferation, flow cytometric analysis, alkaline phosphatase (ALP) staining, ALP activity, Alizarin Red S staining, and quantitative real-time polymerase chain reaction (qRT-PCR) were carried out to assess treatment response. MC3T3-E1 cells were irradiated with LED devices (630±5 nm and 810±10 nm, continuous wave) for 200 seconds at a power density of 5 mW/cm^2 once daily. RESULTS Increases in cell proliferation and decreases in cell apoptosis were evident following irradiation. ALP staining intensity and activity were also significantly increased following irradiation. Level of mineralization was obviously enhanced in irradiated groups compared with non-irradiated controls. qRT-PCR also showed significant increases in mRNA expression of osteocalcin (OCN) and osteoprotegerin (OPG) in the irradiated groups. CONCLUSIONS Our results showed that LED PBM could promote the proliferation, ALP staining intensity and activity, level of mineralization, gene expression of OCN and OPG of MC3T3-E1 cells, with no significant difference between the 630 nm- and 810 nm-irradiated groups.

Material/Methods

Here, we sought to investigate the effects of PBM on MC3T3-E1 cells via 630 nm and 810 nm light from a newly designed LED with high uniformity of light. Cell proliferation, flow cytometric analysis, alkaline phosphatase (ALP) staining, ALP activity, Alizarin Red S staining, and quantitative real-time polymerase chain reaction (qRT-PCR) were carried out to assess treatment response. MC3T3-E1 cells were irradiated with LED devices (630±5 nm and 810±10 nm, continuous wave) for 200 seconds at a power density of 5 mW/cm^2 once daily.

Results

Increases in cell proliferation and decreases in cell apoptosis were evident following irradiation. ALP staining intensity and activity were also significantly increased following irradiation. Level of mineralization was obviously enhanced in irradiated groups compared with non-irradiated controls. qRT-PCR also showed significant increases in mRNA expression of osteocalcin (OCN) and osteoprotegerin (OPG) in the irradiated groups.

Conclusions

Our results showed that LED PBM could promote the proliferation, ALP staining intensity and activity, level of mineralization, gene expression of OCN and OPG of MC3T3-E1 cells, with no significant difference between the 630 nm- and 810 nm-irradiated groups.

MESH KEYWORDS:

Laser Therapy, Low-Level; Osteoblasts; Osteoporosis

BACKGROUND

Osteoporosis, a common skeletal disease, can result in a greater propensity for fragility fracture [1–3]. Modulating the function of osteoblasts represents a promising therapeutic strategy for disease intervention as the combination of decreased osteoblastic activity and increased osteoclastic activity strongly contributes to the unbalanced bone metabolism underlying disease progression [3,4].

Photobiomodulation (PBM), also known as low-level laser therapy or low-level light therapy, is considered a promising new method for modulating osteoblasts, which play a key role in bone formation. PBM has demonstrated the capacity to promote tissue regeneration and reduce inflammation, swelling, and pain [5,6]. The wavelength is typically considered to be between 600 and 1000 nm [5,7]. Although the mechanisms of PBM are not well understood, it has been shown that cytochrome C oxidase (CCO), an enzyme in the mitochondrial oxidative respiratory chain, performs a vital role in light absorption. Stimulation of CCO can promote the photodissociation of inhibitory nitric oxide from CCO, increasing the synthesis of adenosine triphosphate (ATP), accompanied by the modulation of reactive oxygen species (ROS), ultimately leading to an upregulation of gene transcription. As a result, PBM has beneficial effects on cell respiration, especially for hypoxic cells. While the effects of PBM are not thermal in nature [5], temperature-gated calcium ion channels may be a target [8].

Red and infrared light was widely used in the study of PBM on osteoblasts, especially 630 nm and 810 nm light. Despite the significant promise of PBM, considerable disagreement persists regarding the ability of PBM to modulate the growth of osteoblasts [9]. Some studies had demonstrated that PBM could upregulate the proliferation and differentiation of several osteoblast cell lines [10–15]; however, these observations could not be replicated in all studies [16,17]. This divergence may be attribute to the different irradiation parameters used in different studies. The therapeutic effects of PBM depend on wavelength, power density, exposure time, energy density, and total energy. Use of continuous or pulsed wave treatments are also thought to affect treatment outcomes. Because these key parameters were not well described in some studies, some results cannot be properly evaluated.

Although laser, emitting coherent light, and light-emitting diode (LED), emitting non-coherent light, were both the light source of PBM [18], laser was more often used in the previous study [5,10–17]. There were more and more studies on PBM using LED [19–21]. Many studies supported that LED had similar reactions with laser [13,16–18,22,23]. LED is of many advantages compared with laser, it is more safety and cost-effective, easy to operation, can be used to irradiate large area at once and made into wearable device [18]. However, the light emission angle of LED is much larger than laser, which determines the nonuniform power density in irradiation. As the light uniformity of LED is important for evaluating the effects of PBM, the power density in the irradiation region should be uniform. We designed a new LED device, by arranging the distribution of LEDs in the 2 sides of the edge, with a uniformity of power density >95%. In our study, we assessed the effects of PBM on MC3T3-E1 subclone 14 cells via newly designed 630 nm and 810 nm LED devices. Obviously enhanced proliferation and reduced apoptosis were found following irradiation. Alkaline phosphatase (ALP) staining intensity and activity were also significantly increased following irradiation. Compared with non-irradiated groups, calcium deposition was obviously increased in the irradiated groups. Finally, PCR revealed significant increases in osteogenic gene expression in the irradiated groups.

MATERIAL AND METHODS

Cell culture

The mouse MC3T3-E1 subclone 14 cells was provided by the Cell Bank of the Chinese Science Academy. MC3T3-E1 cells were cultivated in ascorbic acid-free MEM-a supplemented with 10% fetal bovine serum (Gibco, 10091148, CA, USA), 100 U/mL penicillin, and 100 U/mL streptomycin

(Gibco, 10378016, CA, USA). Cells were sub-cultured using 0.25% trypsin (Gibco, 25200056, CA, USA) containing ethylenediaminetetraacetic acid after reaching 90% confluence.

To induce osteogenesis, osteogenic medium containing 50 ug/mL L-ascorbic acid (Sigma-Aldrich, 795437, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, G9422, St. Louis, MO, USA), and 10 nm dexamethasone (Sigma-Aldrich, D4902, St. Louis, MO, USA) was added. Medium and osteogenic factors were changed every 2 days.

LED irradiation

MC3T3-E1 cells were incubated in ascorbic acid-free culture medium for 24 hours, enabling cells to attach to the bottom of well, at which point the medium was changed to osteogenic medium, then cells were irradiated at 5 mW/cm^2 for 200 seconds every day with LED devices (630±5 nm and 810±10 nm, continuous wave), which were designed by the Institute of Semiconductors, Chinese Academy of Science. The irradiation parameters in detail was listed in Table 1. By arranging the distribution of LEDs in the 2 sides of the edge, the uniformities of power density of light within 6 cm of the center of the LED devices were >95% (Figure 1A). To test the effect of PBM on proliferation, cells were divided into a non-irradiated (control) group, a 630 nm LED-irradiated group, and an 810 nm LED-irradiated group. To determine the effect of PBM on MC3T3-E1 differentiation, the non-irradiated group was subdivided into osteogenic and non-osteogenic (control) groups. The distance between the bottom of the cell culture plate and the irradiation surface of LED device was 5 cm (Figure 1B). The power density could be adjusted through a direct current power supply. The power density was calibrated every time before irradiation using a spectral power density meter (SENSING, SPD-370, Zhejiang, China).

Table 1 Irradiation parameters.

Parameter	
Wavelength	630±5 nm, 810±10 nm
Wave type	Continuous wave
Power density	5 mW/cm^2
Application time	200 s
Energy density	1J/cm^2
Number of applications	1-time per day
Energy deposited per cm^2	1 J
Distance of irradiation to bottom of plate	5 cm

Cell proliferation assay

MC3T3-E1 cells were seeded in 96-well plates (2000 cells/per well). Cell viability was evaluated 1, 3, and 5 days after the first irradiation treatment using Cell Counting Kit-8 (Beyotime, C0039, Shanghai, China). The optical density was measured at 450 nm via an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio Tek EPOCH TAKE 3, VT, USA).

Flow cytometric analysis

MC3T3-E1 cells were seeded in 6-well plates (4×10^5 cells/well). Cell apoptosis was detected using an annexin V-FITC/propidium iodide (PI) kit (Beyotime, C1062, Shanghai, China). One day after irradiation, the cells were rinsed with phosphate-buffered saline (PBS) and harvested. After centrifugation at 1300×*g* for 3 minutes, 10^5 cells were resuspended in 195 μL of annexin V-FITC binding buffer, then 5 μL annexin V-FITC and 10 μL PI were added. After incubation at room temperature for 20 minutes in the dark, the fluorescence of 10 000 events per sample were analyzed by flow cytometry (FACSCelesta; BD Biosciences, NJ, USA). Live cells (annexin V−/PI−), early apoptotic cells (annexin V+/PI−), and late apoptotic cells (annexin V+/PI+) were distinguished.

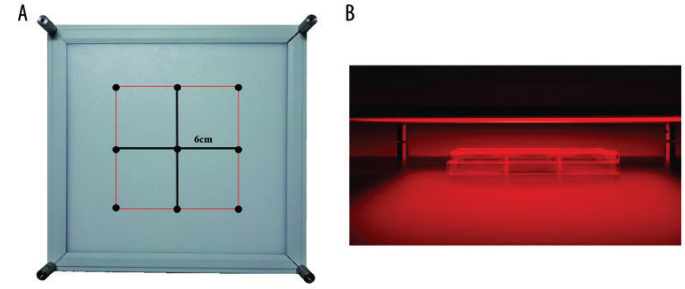

Figure 1 Overview of the light-emitting diode (LED) device. We arranged the distribution of many LED in the 2 sides of the edge and cover a glass in the irradiation surface to homogenize the light. (A) We measured the power density of 9 points in the interested field at a distance of 5 cm using a spectral power density meter.

$$\text{Uniformity} = \frac{\text{power density (minimum)}}{\text{power density (maximum)}} \times 100\%$$

The uniformity of light within 6 cm of the center of the LED device was >95%. (**B**) Overview of the irradiation process.

ALP staining

MC3T3-E1 cells were seeded in 24-well plates (8×10^4 cells/well), incubated for 7 or 14 days after the first irradiation, and washed with PBS. Cells were fixed with citrate-acetone-formaldehyde fixative solution for 30 seconds and rinsed in deionized water for 60 seconds. The alkaline-dye mixture (Sigma-Aldrich, 86C, St. Louis, MO, USA) was prepared and incubated for 15 minutes. After incubation, cells were rinsed with deionized water for 120 seconds.

ALP activity assay

MC3T3-E1 cells were seeded in 6-well plates (4×10^5 cells/well), incubated for 7 or 14 days after the first irradiation, and washed with PBS. Cells were harvested in 100 µL/well of lysis buffer (Beyotime, P0013J, Shanghai, China) containing 20 mM Tris (pH 7.5), 150 mM NaCl, and 1% Triton X-100. Cells were centrifuged at 12 000 *g* for 5 min at 4°C, after which the supernatants were used for ALP activity evaluation using the Alkaline Phosphatase Assay Kit (Beyotime, P0321, Shanghai, China). Samples were read using an ELISA plate reader at 405 nm. ALP activity was normalized to the total intracellular protein content, which was determined by the Enhanced BCA Protein Assay Kit (Beyotime, P0010S, Shanghai, China), with samples read using an ELISA plate reader at 562 nm. ALP activity is presented as mU/mg protein.

Extracellular matrix mineralization assay

To evaluate extracellular matrix mineralization, Alizarin Red S (pH 4.2) staining solution (Solarbio, G1452, Beijing, China) was used. MC3T3-E1 cells were seeded in 24-well plates (8×10^4 cells/well), and cultured for 21 days after the first irradiation, after which cells were washed with PBS without calcium magnesium. Cells were then fixed with citrate-acetone-formaldehyde fixative solution for 30 seconds. After fixation, cells were washed with deionized water and stained with the Alizarin Red S staining solution for 30 minutes. The unbound stain was removed with deionized water. Semi-quantitative analysis of Alizarin Red S staining was evaluated by eluting the bound stain with 200 µL of 10% cetyl-pyridinium chloride [8,24] in PBS for 2 hours at 37°C, as described previously [8,24]. To determine the amount of relative calcium deposition, the absorbance of 100 µL eluted solution was measured using the ELISA plate reader at 562 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR)

MC3T3-E1 cells were cultured for 21 days under the same conditions as that of the ALP activity assay. Twenty-one days after the first irradiation, cells were washed with PBS. RNA was collected and purified using the RiboPure Kit (Ambion, AM1924, USA) with the RNase/DNase-Free set. cDNA was synthesized from 1 µg RNA with ReverTra Ace qPCR RT Master Mix (Toyobo, FSQ-201, Osaka, Japan) and treated as follows: 5 minutes heat denaturation at 65°C, 15 minutes reverse transcription at 37°C, 5 minutes inactivation at 98°C, and hold at 4°C. SYBR Green Realtime PCR Master Mix-Plus (Toyobo, QPK-212, Osaka, Japan) was used for PCR amplification, which was performed as follows: 60 seconds at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 15 seconds at 60°C, and extension for 45 seconds at 72°C on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems,

Table 2
Primers used for quantitative RT-PCR.

Gene	Sequence (5′→3′)
GAPDH	F: CCAACTCTTTTGTGCCAGAGA
	R: GGCTACATTGGTGTTGAGCTTTT
OCN	F: CTGACCTCACAGATCCCAAGC
	R: TGGTCTGATAGCTCGTCACAAG
OPG	F: CCTTGCCCTGACCACTCTTAT
	R: CACACACTCGGTTGTGGGT

GAPDH – glyceraldehyde-3-phosphate dehydrogenase; OCN – osteocalcin; OPG – osteoprotegerin.

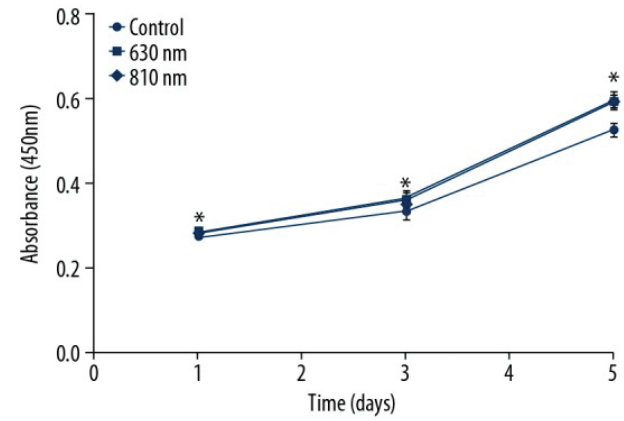

Figure 2
Effect of light-emitting diode (LED) photobiomodulation (PBM) on the proliferation of MC3T3-E1 cells. Cell viability assays were performed 1, 3, and 5 days after the first irradiation. * $P<0.05$.

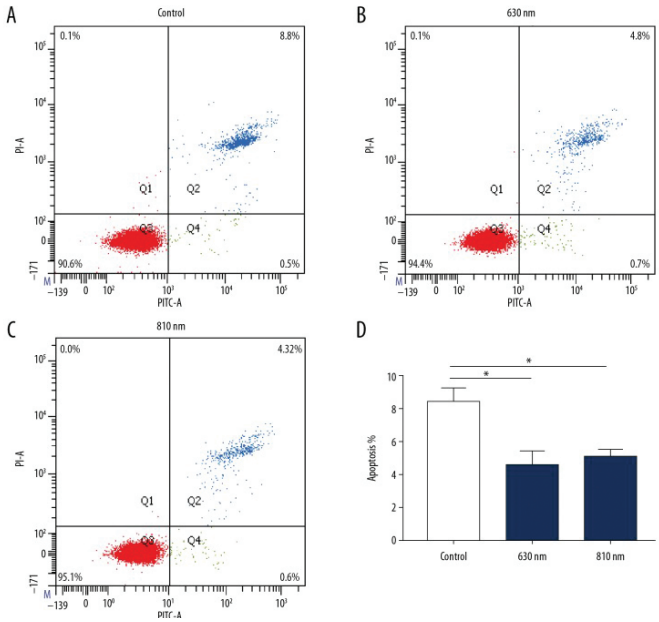

Figure 3
Effect of light-emitting diode (LED) photobiomodulation (PBM) on apoptosis of MC3T3-E1 cells. A representative histogram showing the distribution of annexin V and propidium iodide (PI)-labeled cells. Annexin V−/PI− (Q3) represented live cells, Annexin V+/PI− (Q4) represented early apoptotic cells, and annexin V+/PI+ (Q2) represented late apoptotic cells. (A) Control group. (B) 630 nm LED-irradiated group. (C) 810 nm LED-irradiated group. (D) A histogram depicting the percentage of apoptotic cells. Annexin V+/PI− (Q4)- and annexin V+/PI+ (Q2)-labeled cells were used for statistics. * P<0.05.

Thermo Fisher Scientific, MA, USA). The primers were found in the primer bank (*https://pga.mgh.harvard.edu/primerbank/*). Primer sequences are shown in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression [25].

Statistical analysis

All data were reported as means±standard deviations of each group and were evaluated via ANOVA followed by the Student-Newman-Keuls post-hoc test. Differences were considered statistically significant at $P<0.05$.

RESULTS

Effects of PBM on cell proliferation

The proliferation rate of MC3T3-E1 cells was significantly enhanced in the irradiated groups compared with non-irradiated controls from 1 to 5 days after the first irradiation, with no obvious difference between the 2 irradiated groups at any time point (Figure 2). Flow cytometric analysis showed that both 630 nm and 810 nm LED irradiation inhibited apoptosis of MC3T3-E1 cells (Figure 3). The results demonstrated that PBM had the capacity to promote proliferation and inhibit apoptosis in MC3T3-E1 cells.

Effects of LED PBM on ALP activity

ALP is an indicator of osteoblast differentiation. The addition of osteogenic medium significantly increased the intensity of ALP staining at 7 and 14 days. While the intensity of staining was higher in both the 630 nm- and 810 nm-irradiated groups, with similar effects between the 2 irradiated groups (Figure 4A). To better quantify these results, cells were examined using an ALP activity assay. The irradiated groups showed significantly higher ALP activity compared to non-irradiated groups, with similar effects seen between the 630 nm- and 810 nm-irradiated groups (Figure 4B). These results suggest that PBM may promote MC3T3-E1 cell differentiation.

Effects of LED PBM on extracellular matrix mineralization

Extracellular matrix mineralization results from calcium deposition and serves as a late marker of osteoblast differentiation. After 21 days, levels of Alizarin Red S staining indicated significant increases in calcification in both the osteogenic and irradiated groups; however, both the size and quantity of the calcifications in the irradiated groups were significantly greater than that of the osteogenic group (Figure 5A). To quantify the level of calcium deposition, we measured the absorbance of Alizarin Red S staining eluates at 562 nm. The absorbance was significantly higher in irradiated groups relative to non-irradiated controls, with similar levels of absorbance between the 2 irradiated groups (Figure 5B). These results indicated that PBM could facilitate extracellular matrix mineralization.

Effects of LED PBM on osteogenic gene expression

To confirm that PBM could promote differentiation of MC3T3-E1 cells, qRT-PCR was carried out to assess the effects of LED PBM on osteogenic gene expression. We examined the mRNA expression of osteocalcin (OCN) and osteoprotegerin (OPG), a key marker of late stage mineralization. OCN and OPG expressions were shown to be significantly increased in the irradiated groups compared to both the control and

osteogenic groups, consistent with the idea that PBM could promote MC3T3-E1 cell differentiation (Figure 6). Unlike previous assays, for OCN, this effect was significantly greater in the 810 nm-irradiated group, relative to the 630 nm-irradiated group.

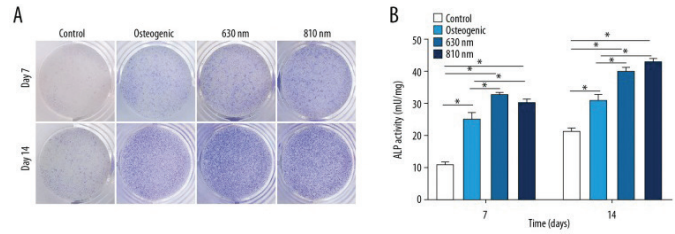

Figure 4
Effect of light-emitting diode (LED) photobiomodulation (PBM) on alkaline phosphatase (ALP) staining and activity in MC3T3-E1 cells. (A) ALP staining (blue), and (B) ALP activity after 7 and 14 days. ALP activity was normalized to total protein content and presented as mU/mg protein. * P<0.05.

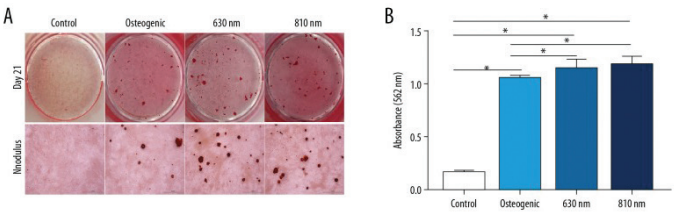

Figure 5
Effect of light-emitting diode (LED) photobiomodulation (PBM) on calcium deposition of MC3T3-E1 cells. (**A**) Calcium deposition was stained by Alizarin Red S and the calcium deposition nodules were stained red. Scale bar=1 mm. (**B**) Semi-quantitative analysis of calcium deposition. The absorption of eluate was measured at 562 nm. * *P*<0.05.

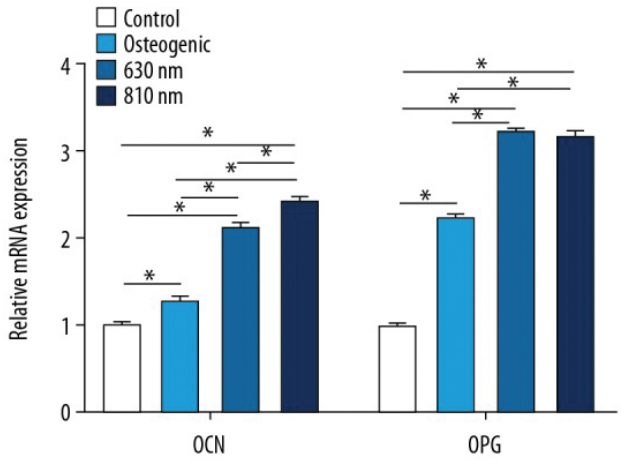

Figure 6
Effect of light-emitting diode (LED) photobiomodulation (PBM) on gene expression of MC3T3-E1 cells. Osteocalcin (OCN) and osteoprotegerin (OPG) expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR) after 21 days. The $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression. **P*<0.05.

DISCUSSION

Osteoporosis is a common aging disease that affects 200 million people worldwide [26], which is characterized by an increased risk of fragility. Alleviating bone resorption and increasing bone formation is the most common strategy used to both treat and prevent osteoporosis [1,27]. Several drugs have been developed for this purpose, with most classified as either anabolic or antiresorptive [1,26,28–32], targeting osteoblasts and osteoclasts, respectively. Despite good overall therapeutic profiles, the use of these drugs may be impeded by low adherence and adverse effects.

As a non-invasive therapeutic method, PBM had been used to promote tissue regeneration, alleviate pain, and reduce inflammation [33–36]. In recent years, PBM received considerable attention due to its effects on osteoblast lines [10–14,16,17]. Red and near infrared light have been widely used in PBM for regulating bone cells. Numerous studies showed that red and near infrared light (630–810 nm) were able to increase proliferation, ALP staining and activity, calcium deposition, and osteogenic gene expression in several cell types [10–14,16,17,37,38], including bone marrow-derived mesenchymal stem cells, MC3T3-E1 cells, hypoxic-cultured human fetal osteoblasts, and human adipose-derived stem cells. Increased ATP synthesis accompanied by upregulated ROS generation and transcription following PBM were shown to contribute to the proliferative effects [5]. Activation of Akt signaling may also mediate the osteogenic response of osteoblasts to red light [12]. Besides, activation of light-gated calcium ion channels may contribute to the promotive effects of 420 nm and 540 nm light in osteogenic differentiation [38].

Together, these studies provide strong evidence of a positive effect of PBM on osteogenic cell lines; however, contradictory findings have also been reported. Several studies have shown that PBM is not able to simultaneously promote proliferation and differentiation of osteoblast cells [12,16,17]. This inconsistent effect of PBM on osteoblasts has largely been attributed to differences in the parameters used in different studies. Of note, most studies showed that PBM could promote osteoblast proliferation and differentiation *in vitro*; similar effects have also been observed *in vivo*. Additional studies have also shown that PBM could increase the activity and density of osteoblasts [39,40].

Beyond its effects on osteoblasts, some studies have also demonstrated the potential of PBM to inhibit osteoclastogenesis [41,42]. Reduced ROS production has been proposed as a possible cause of reduced osteoclastogenesis following irradiation [41–43]. Moreover, many studies found that PBM could promote bone healing and improve bone quantity and strength in ovariectomized rats [39,40,44–46], in which near infrared light was more commonly used.

In recent years, LED had been widely used in other medical fields [19–21,47–51]. However, the light source of PBM in regulating osteoblasts was mainly focused on laser, and insufficient attention was paid to LED. The ultimate aim of studying the effect of LED PBM on osteoblasts was to treat osteoporosis. Although a previous study showed that 420 nm and 540 nm light was more effective than 660 nm and 810 nm light, due to the limitation of penetration depth [7], we did not choose on 420 nm and 540 nm light [38]. In our study, the effects of PBM on MC3T3-E1 cells were investigated using 630 nm and 810 nm LED lights.

The proliferation was obviously enhanced from 1 to 5 days following irradiation (Figure 2). In addition, the promotive effect was similar between the 2 irradiated groups. Meanwhile, PBM inhibited apoptosis of MC3T3-E1 cells, which might contribute to the enhanced cell viability (Figure 3). Increased proliferation and decreased apoptosis might facilitate osteogenic differentiation [52,53]. ALP, an indicative enzyme of osteoblast, was measured. The intensity of ALP staining was also significantly enhanced after irradiation relative to non-irradiated controls (Figure 4A). An ALP activity assay was further used to confirm these results, as these tests are more precise than ALP staining. Compared with non-irradiated groups, ALP activity was obviously enhanced in irradiated groups at all time points tested (Figure 4B), which gave more support to ALP staining. Calcium deposition, the indicative marker in the late stage of osteoblast differentiation, was evaluated by Alizarin Red S staining. We observed more red nodules in the irradiated groups compared to osteogenic groups (Figure 5A). Because the nodules were 3-dimensional with significant variations in size, we decided to elute the calcium deposits and measure the absorbance of the eluates to quantify the level of mineralization. The higher absorbance in the irradiated groups confirmed that irradiation could significantly promote calcium deposition (Figure 5B). The enhanced mineralization levels were further supported by increases in OCN and OPG gene expression in the irradiated groups, relative to controls (Figure 6). Interestingly, the OCN expression was significantly higher in 810 nm irradiation group than 630 nm group, which might show the superiority of 810 nm light in promoting osteoblast differentiation. These results showed that LED PBM had promotive effects on MC3T3-E1 cells. The similar promotive effects between the 630 nm- and 810 nm-irradiated groups might attribute to the similar amount of photons absorption by CCO. Considering the deeper penetration depth [54,55], 810 nm light was more appropriate in studying PBM on osteoporosis *in vivo*.

In our study, we presented one dose effect of PBM on MC3T3-E1 cells. However, biphasic dose response was very common in the study of PBM [8,16,56,57]. The definition of dose was still controversial [5,58]. Power density, energy density, and total energy should be paid equal attention in the study of PBM.

The data presented here suggest that LED PBM might be a promising option for the management of osteoporosis. As light penetration depth is strongly influenced by the wavelength used [54,55], and is closely associated with therapeutic effects, further studies will be needed to identify the optimal wavelength and dose for therapeutic use.

CONCLUSIONS

In our study, we designed new LED devices to test the effects of PBM on MC3T3-E1 cells. Our results showed that LED PBM could promote the proliferation, ALP staining intensity and activity, level of mineralization, gene expression of OCN and OPG of MC3T3-E1 cells, with no significant difference between the 630 nm- and 810 nm-irradiated groups. LED PBM may therefore represent a promising strategy to modulate bone metabolism.

ABBREVIATIONS

ALP	alkaline phosphatase
ATP	adenosine triphosphate
CCO	cytochrome c oxidase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LED	light-emitting diode
OCN	osteocalcin
OPG	osteoprotegerin
PBM	photobiomodulation
PBS	phosphate buffered saline
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction

FOOTNOTES

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CONFLICT OF INTEREST:

None

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TRANSCRANIAL AND SYSTEMIC PHOTOBIOMODULATION FOR MAJOR DEPRESSIVE DISORDER: A SYSTEMATIC REVIEW OF EFFICACY, TOLERABILITY AND BIOLOGICAL MECHANISMS

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ABSTRACT

HIGHLIGHTS

- Photobiomodulation (PBM) is a novel treatment under study for neuropsychiatric disorders.
- PBM modulates different cellular pathways, which are also relevant to MDD.
- Pre-clinical and clinical evidence suggest PBM is safe and potentially effective to treat MDD.
- Research on PBM for MDD is still preliminary and needs confirmation by large clinical trials.

ABSTRACT

Background

Photobiomodulation (PBM) with red and near-infrared light (NIR) –also known as Low-Level Light Therapy–is a low risk, inexpensive treatment–based on non-retinal exposure–under study for several neuropsychiatric conditions. The aim of this paper is to discuss the proposed mechanism of action and to perform a systematic review of pre-clinical and clinical studies on PBM for major depressive disorder (MDD).

Methods

A search on MEDLINE and EMBASE databases was performed in July 2017. No time or language restrictions were used. Studies with a primary focus on MDD and presenting original data were included (n = 17). References on the mechanisms of action of PBM also included review articles and studies not focused on MDD.

Results

Red and NIR light penetrate the skull and modulate brain cortex; an indirect effect of red and NIR light, when delivered non-transcranially, is also postulated. The main proposed mechanism for PBM is the enhancement of mitochondrial metabolism after absorption of NIR energy by the cytochrome C oxidase; however, actions on other pathways relevant to MDD are also reported. Studies on animal models indicate a benefit from PBM that is comparable to antidepressant medications. Clinical studies also indicate a significant antidepressant effect and good tolerability.

Limitations

Clinical studies are heterogeneous for population and treatment parameters, and most lack an appropriate control.

Conclusions

Preliminary evidence supports the potential of non-retinal PBM as a novel treatment for MDD. Future studies should clarify the ideal stimulation parameters as well as the overall efficacy, effectiveness and safety profile of this treatment.

KEYWORDS

Photobiomodulation, Low-level laser therapy, Low-level light therapy, Depression, Treatment, Mitochondria, Near-infrared

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