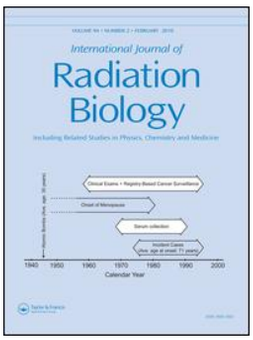

International Journal of Radiation Biology

ISSN: 0955-3002 (Print) 1362-3095 (Online) Journal homepage: http://www.tandfonline.com/loi/irab20

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To cite this article: Ruwaidah A. Mussttaf, David F. L. Jenkins & Awadhesh N. Jha (2019): Assessing the impact of low level laser therapy (LLLT) on biological systems: a review, International Journal of Radiation Biology, DOI: 10.1080/09553002.2019.1524944

To link to this article: https://doi.org/10.1080/09553002.2019.1524944

Published online: 07 Jan 2019.

INTERNATIONAL JOURNAL OF RADIATION BIOLOGY
https://doi.org/10.1080/09553002.2019.1524944

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REVIEW

Assessing the impact of low level laser therapy (LLLT) on biological systems: a review

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ABSTRACT

Purpose: Low level laser therapy (LLLT) in the visible to near infrared spectral band (390–1100 nm) is absorption of laser light at the electronic level, without generation of heat. It may be applied in a wide range of treatments including wound healing, inflammation and pain reduction. Despite its potential beneficial impacts, the use of lasers for therapeutic purposes still remains controversial in mainstream medicine. Whilst taking into account the physical characteristics of different qualities of lasers, this review aims to provide a comprehensive account of the current literature available in the field pertaining to their potential impact at cellular and molecular levels elucidating mechanistic interactions in different mammalian models. The review also aims to focus on the integral approach of the optimal characteristics of LLLT that suit a biological system target to produce the beneficial effect at the cellular and molecular levels.

Methods: Recent research articles were reviewed that explored the interaction of lasers (coherent sources) and LEDs (incoherent sources) at the molecular and cellular levels.

Results: It is envisaged that underlying mechanisms of beneficial impact of lasers to patients involves biological processes at the cellular and molecular levels. The biological impact or effects of LLLT at the cellular and molecular level could include cellular viability, proliferation rate, as well as DNA integrity and the repair of damaged DNA. This review summarizes the available information in the literature pertaining to cellular and molecular effects of lasers.

Conclusions: It is suggested that a change in approach is required to understand how to exploit the potential therapeutic modality of lasers whilst minimizing its possible detrimental effects.

ARTICLE HISTORY
Received 4 June 2018
Revised 22 August 2018
Accepted 24 August 2018

KEYWORDS
Low level laser therapy (LLLT); cell proliferation; DNA damage; DNA repair; light emitting diodes (LEDs)

1. Introduction

Laser therapy or low-level laser therapy (LLLT) has been widely used for over 50 years (Ginani et al. 2015). Evolutionary though, it emerged in its modern form after the invention of the laser in 1960 becoming a widespread treatment modality in a variety of clinical applications (Karu 1989; Kreisler et al. 2003; Posten et al. 2005). Investigators introduced a diverse set of terms to describe this potentially beneficial treatment tool (Lucas et al. 2002). Initially, expressions such as 'photobioactivation' and 'biostimulation' frequently relative to the stimulation effect of low level lasers were used (King 1989; Wu et al. 2012). Subsequently an inhibitory effect of this radiation were also noted, which led them to coin the term 'biomodulation' (Schindl et al. 2000).

Recently, a consensus decision was taken to use the terminology 'photobiomodulation' or 'PBM,' where some researchers gave LLLT a status of subjectivity and it is limited for actual laser specific interactions, this is not a requirement for in-coherent light emitting diodes (LEDs), which can work equally well (Hamblin 2017). On the contrary, other researchers reported that although LLLT is a well-established researchable and for much time used by clinician and researchers, it is not optimal. It is a broad term that could include photodynamic therapy (PDT) and optogenetics. These techniques use lasers and LEDs with low dose and require exogenous chromophores, unlike LLLT that utilize endogenous chromophores with low dose of light delivered at the target site. However, they also suggest using photobiomodulation (PBM), since it is more ideal, has specific definition for this application of light to be more accurate and can confirm its scientific principle (Anders et al. 2015). Specialists of medical field successfully used photobiomodulation in treating many health conditions when other methods had limited success, such as healing-resistant wound, chronic diabetic ulcers, injuries of spinal cord and nervous system and pain management (Tuner and Hode 2004). Nevertheless, photobiomodulation is not considered as a part of mainstream medicine as still not standard treatment (Karu 2013).

LLLT treatment has evolved over the years and is being developed as a sophisticated tool for therapeutic procedures and utilized clinically for several different ailments (Chung et al. 2012). The therapeutic treatments are based upon

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three principles: (a) to minimize inflammation, edema, and chronic disorders of joints by targeting brain, skin, joint, etc. (Bjordal et al. 2003), (b) to promote wound healing of superficial and deeper tissues, neurological damage, etc. (Gigo-Benato et al. 2005; Posten et al. 2005), and (c) to treat neurological disorders and pain (Chung et al., 2012). Recently, many studies on PBM therapy at infrared (IR) wavelengths, in particular from 700 nm up to the near infrared (NIR) have been carried out. (Barrett and Gonzalez-Lima 2013, Xuan et al. 2014, Salehpour and Rasta 2017). These studies have shown to produce more beneficial impacts than red light in many medical conditions. These include neural stimulation (by triggering direct activation of neural tissue) (Salehpour et al. 2017), photoaging (where IR radiation evidently has a biphasic effect), anti-tumour action (IR radiation capable of inhibiting the proliferation of cancer cells and enhances chemotherapy efficacy), brain neuroprotection (treatments for stroke, traumatic brain injury (TBI) in vivo models (Naeser et al. 2011, Salehpour et al. 2017) and neurodegenerative disorders for Alzheimer's and Parkinson's diseases. These studies have been summarized in Table 1. Therefore, a better understanding of the mechanisms using IR radiation, could support improved therapeutic effectiveness via new strategies of PBM therapy at IR wavelengths (Tsai and Hamblin 2017).

Laser is a device, which produces intense, monochromatic, coherent, and highly collimated beam of light (Fonseca et al. 2010). Laser light has quite pure frequency, which makes it useful for biomedical applications (Ratkay-Traub et al. 2001). Laser therapy involves visible red and near infrared (NIR) portions of the electromagnetic spectrum (390–1600 nm and 10^{13}–10^{15} HZ) because researchers have shown that these portions of the spectrum have been absorbed highly by the biological systems and bring about a beneficial therapeutic effects in living tissues (Hawkins et al. 2005). According to the portion of the spectrum (wavelength) that strikes the tissue and the intensity (power density or irradiance) of laser radiation, the photobiological impacts of laser therapy on tissue are different that lead to divide the laser therapy into two classes (Hawkins and Abrahamse 2006). Class I, which refers to radiation of wavelengths ranges ($<$390 nm and $>$10,600 nm) and high power and intensity levels, are used for ablation, cutting and sterilization, because of its thermal effect. Class II, which refers to radiation of wavelength ranges (390–10,600 nm), levels of power (10^{-3} to 10^{-1} W) and intensity (10^{-1} to 10° W/cm^2) and a dose of 10^{-2} to 10^{2} J/cm^2 (Posten et al. 2005).

However, there is some agreement on the best wavelengths of light and appropriate dosages to be used (irradiance and fluence), there is no agreement on the emission mode of laser light; whether continuous wave (CW) or pulsed light is more suitable for the various applications of PBM. However, pulsed lasers in PBM therapy are used widely in clinical research (Fonseca et al. 2010; da Silva Sergio et al. 2012); and for medical treatment (Vasheghani et al. 2009; Ahrari et al. 2014; de Meneses et al. 2015; Bayat et al. 2016). Two types of pulsed laser are used for PBM therapy: (a) super-pulsing gallium-arsenide (GaAs) diode laser, which has a wavelength in the region of 904 nm and pulse duration in the range of 100–200 ns, and (b) the semiconductor super-pulsing indium-gallium-arsenide (In-Ga-As) diode laser, which emits light at a similar wavelength (904–905 nm), producing very short pulses of light (200 ns) in the range of kilohertz (kHz) frequencies (Hashmi et al. 2010b). Therapeutically, the super-pulsed GaAs and In-Ga-As lasers are capable of deep penetration without the undesirable influences associated with continuous wave lasers (CW) (such as thermal damage), as well as allowing for shorter treatment periods. Pulsed lasers offer potential benefits, attributed to the pulse OFF times (pulse quench intervals) following the pulse ON times, so that pulsed lasers can deliver less tissue heating.

Low-intensity laser radiation is clinically a well accepted tool in medicine and dentistry (Amid et al. 2014, Table 2). It is known by its ability to incite a thermic, non-damaging photobiological action (McDaniel 2015). Unlike 'hard' high power lasers, LLLT provides low energy, only sufficient to induce stimulation response of body tissue. It has a wavelength-dependent manner able to change the cellular function, in the absence of significant heating (Surendranath and Arjun 2013). Hence, LLLT is also called 'soft' laser therapy or cold laser, as a low-energy laser has no thermal effects (Nelson 1993; Chung et al. 2012).

It was observed that the broad range of laser therapy included molecular, cellular and tissue level effects and the modes of action of LLLT may vary with different confounding factors and applications (Chung et al. 2012). To produce photo-biological action, photon absorption of laser radiation must occur (Hawkins et al. 2005). Endogenous or exogenous chromophores are the initial photoacceptor molecules (i.e. molecules that can absorb light at certain wavelengths) that are able to absorb the incident photon energy (Bjordal et al. 2001). A photochemical conversion of the photon energy absorbed by a photoacceptor has been demonstrated (Brondon et al. 2005). The absorbed energy of photon can be transferred to another molecule, which can then cause chemical reaction without alteration in temperature in the surrounding tissue (Mochizuki-Oda et al. 2002; Brondon et al. 2005). Some native component can be activated in the irradiated cell at certain wavelength, and consequently, biochemical reaction as well as cellular metabolism might be altered (Karu 1999).

Several studies suggested that mitochondria is the most sensitive component of cell to visible and near infrared light (Karu 1999; Karu et al. 2001) that result in increased production of adenosine triphosphate (ATP), increased deoxyribonucleic acid (DNA) synthesis, modulation of reactive oxygen species (ROS), nitric oxygen species (NOS) and the induction of transcription factors (Hamblin and Demidova 2006). Moreover, PBM at red and NIR wavelengths stimulate increasing intracellular calcium Ca^{2+} (Irvine and Schell 2001; Santana-Blank et al. 2005; Karu 2008; de Freitas and Hamblin 2016), however, recent studies emphasised that blue (420 nm) and green (540 nm) lights are more effective in increasing Ca^{2+} when applied at the same doses (Wang et al. 2016). Many researchers suggested that the response

Table 1. Review of published studies using LLLT to treat different diseases.

Study No.	Type of laser	Wavelength (nm)	Power (mW)	Energy density (J/cm^2)	Power density (mW/cm^2)	Emission model CW/Pulse	Types of diseases	Reference
1	Diode laser	810	10 W	3 and 30	5 and 50	CW	Zymosan-induced arthritis	(Castano et al. 2007)
2	He-Ne	632.8	10	3, 5, 10, 20, 25 and 50	64.6	CW	Neurodegenerative	(Song et al. 2012)
3	He-Ne	632.8	10	0.5, 1, 2 and 4		CW	Alzheimer's disease	(Meng et al. 2013)
4	Nd:YAG	1064	1.25 W			CW	Dental/Tooth extraction	(Vescovi et al. 2013)
5	GaAs	904	10	5.4	20	CW	Musculoskeletal diseases	(Bjordal et al. 2006)
6	Diode laser	830	30	1.1		Pulse	Painful stomatitis control	(Toida et al. 2003)
7	Diode laser	810	30	0.9	30	CW	Diabetic wounds	(Dancáková et al. 2014)
8	Diode laser	830	30			CW	Chronic diseases of inner ear	(Wilden and Dindinger 1996)
	He-Ne	632.8	20					
9	Diode laser	660	50	2		CW	Chronic lichenoid graft-vs.-host disease (cGVHD)	(Chor et al. 2004)
10	Diode laser	810		3	20	CW	Cortical neurons	(Huang et al. 2014)
11	He-Ne	632.8	400	1		CW	Alzheimer's Disease	(Farfara et al. 2015)
12	GaAlAs	860	30	3	3000	Pulse	Osteoarthritic (OA) pain	(Brosseau et al. 2005a,b)
			60			CW		
13	GaAs	808			10 and 20	CW	Traumatic brain injury (TBI)	(Oron et al. 2007)
14	GaAlAs	830	60	45	4000	CW	Lumbago	(Ohshiro and Shirono 1992)
15	Diode laser	660	30	7.5		CW	Lung neutrophils	(Aimbire et al. 2008)
16	Diode laser	660	40	20		CW	Burning mouth syndrome	(dos Santos Lde et al. 2011)
17	Diode laser	665, 730, 810 and 980		36	150	CW	Traumatic brain injury (TBI)	(Wu et al. 2012a)
18	Diode laser	660	24			CW	Periodontal disease	(de Almeida et al. 2008)
19	Diode laser	820	300	3		CW	myofascial pain (MP) dysfunction syndrome	(Öz et al. 2010)
20	GaAlAs	780	50	7.5		CW	Rheumatoid arthritis	(Ekim et al. 2007)
21	Diode laser	810		0.03, 0.3, 3, 10 and 30	25	CW	Cortical neurons	(Sharma et al. 2011)
22	GaAlAs	830	70	6		CW	Peripheral nerves regeniration	(Midamba and Haanaes 1993)
23	GaAlAs	810	1 W	4.8	80	CW	Orofacial granulomatosis	(Merigo et al. 2012)
				24				
24	Diode laser	830	100	3		CW	Chronic periodontitis	(Makhlouf et al. 2012)
25	Diode laser	780	30	6.3		CW	Temporomandibular joint pain	(Chang et al. 2014)
		830	500	100				
26	He-Ne	632.8	10	0.18–27		CW	Indolent ulcers	(Schindl et al. 1992)
27	Diode laser	808			110	CW	Hearing loss	(Tamura et al. 2015)
					165			
28	Diode laser	532	7.5			CW	Hearing loss	(Goodman et al. 2013)
		635				Pulse		
29	Diode laser	650	5			CW	Complaints of Tinnitus	(Salahaldin et al. 2012)
30	InGaAlP	660	10	2.5		CW	Acute zymosan-induced arthritis	(Carlos et al. 2014)
31	GaAs	904	20	2–20	11.2	Pulse	Chronic myofascial pain syndrome (MPS) in the neck	(Gur et al. 2004)
32	GaAs	904		29.5	246	Pulse	Salivary glands (xerostomia)	(Lončar et al. 2011)
33	Diode laser	630–670	10–100	2, 3 and 4		CW	Oral mucositis due to cancer therapy	(Bensadoun and Nair 2012)
		780–830						
34	Diode laser	660, 810 and 980		36		CW	Traumatic brain injury (TBI)	(Wu et al. 2010)
35	GaAlAs	670	5	2		CW	Chronic periodontitis Diabetes mellitus (DM)	(Obradović et al. 2013)
36	Ga-As-Al	780	22	7.7	100	CW	Rheumatoid arthritis (RA)	(Alves et al. 2013)
37	Diode laser	810		36	50	CW	Traumatic brain injury (TBI)	(Xuan et al. 2015)

(*continued*)

Table 1. Continued.

Study No.	Type of laser	Wavelength (nm)	Power (mW)	Energy density (J/cm^2)	Power density (mW/cm^2)	Emission model CW/Pulse	Types of diseases	Reference
38	Diode laser LED	685 640–685	200	2		CW	Reynaud's phenomenon	(Hirschl et al. 2004)
39	Diode laser	810			50	CW	Parkinson's disease (PD)	(Trimmer et al. 2009)
40	Diode laser	790	120	6		CW	Burning mouth syndrome	(Kato et al. 2010)
41	IR laser	830	35	3		CW	Lung inflammation	(Oliveira et al. 2013)
42	GaAs	904	150	6		Pulse	Carpal tunnel syndrome	(Dakowicz et al. 2011)
43	AlGaAs	780	30	22.5	750	CW Pulse Pulse	Renal interstitial fibrosis	(Oliveira et al. 2012)
44	GaAlAs	830	60	18	3000	CW	Knee osteoarthrosis	(Trelles et al. 1991)
45	AlGaAs	785	70	3		CW	Rheumatoid arthritis	(Meireles et al. 2010)
46	Diode laser	670	50	3		Pulse	Temporomandibular disorder (TMD)	(Núñez et al. 2006)
47	GaAs	904	45	5		CW	Muscle trauma	(Rizzi et al. 2006)
48	GaAlAs	980	300	4	1500	CW	Mucous membrane pemphigoid	(Cafaro et al. 2012)
49	Diode laser	660	5	4.5		CW	Acute lung inflammation	(de Lima et al. 2011)
50	GaAs	980	10 80 W	2–4		CW Pulse	Chronic low back pain (LBP)	(Hadi et al. 2009)
51	GaAlAs	980	300	4	1000	CW	Oral lichen planus	(Cafaro et al. 2014)
52	GaAlAs	660	30	57.14	428	CW	Periodontal disease (PD)	(Garcia et al. 2011)
53	InGaAlP	660	40	2	1000	CW	Ulcers in patients with leprosy sequelae	(Barreto and Salgado, 2010)
54	GaAlAs	815	250	12		CW	inflammation in retrodiscal tissues in patients with temporo mandibular joint	(Kucuk et al. 2010)
55	GaAlAs	808	500	5	1.8	CW	Bisphosphonate related osteonecrosis of jaws	(Altay et al. 2014)
56	AsGaInP	660	50 100	12.5 25	1.25 2.5	CW	Third-degree burns	(Brassolatti et al. 2016)

Table 2. Parameters involved in LLLT applications.

Irradiation parameters	Unit of measurement		Definitions
Wavelength	nm	390–10,600	An electromagnetic radiation travels in discrete packets that also have a wave-like property.
Power	W	10^{-3}–10^{-1}	It is the amount of energy consumed per unit time, and can be calculated as: Power (P) = Energy (J)/Time (sec)
Power density	W/cm^2	10^{-1}–10^{0}	Often called Irradiance, or Intensity, is the power transmitted per unit area, and calculated as: Power density = Power (W)/Area (cm^2)
Energy density	J/cm^2	10^{-2}–10^{2}	Energy density is the common expression of LLLT dose The dose is the most important parameter in laser Phototherapy, and is usually calculated as Power/Beam Area × Time = J/cm^2.
Total irradiation time	sec	10–3000	It is the allowed interval through which the energy has delivered to the target system.

of some cells to blue or green light interacting by light-gated ion channels, which enable light to control electrical excitability, intracellular acidity, calcium influx and other cellular processes (Kulbacka et al. 2017; Roska and Juettner 2017; Roska and Lagali 2018). The most likely ion channel is light-gated channel rhodopsin, because the action spectra of the channel rhodopsin family displays peaks in the blue-green spectral region (Schneider et al. 2015). The precise mechanism of laser-tissue interaction has not been completely explained, thus restricting the means to offer a clinical treatment protocol at present (Amid et al. 2013).

The review of the available literature suggests that the variety of studies have been mostly in vitro, using a range of cell lines for different types of LLLT and varying some of their parameters, as summarized in Table 3. It is possible to select wavelength, power density, laser beam intensity profile, polarisation and exposure time. The available information suggests both positive and negative outcomes with respect to different parameters (Table 2).

It could be concluded that conflicting results have been published, which may be attributed to a disparity in study design, including the use of different laser wavelengths and numerous illumination parameters, in addition to different confounding factors, which influence the determination of different biological parameters.

2. Optical sources and biological interaction

Low-level laser irradiation has been used in clinical practice causing biostimulation. A number of diseases and physical conditions are mentioned to respond to laser therapy (photobiostimulation) (Basso et al. 2013). At the cellular and molecular level, there is still significant argument regarding the effectiveness of lasers in producing the desired practical responses (Basso et al. 2013). To illustrate the therapeutic effects, through optical stimulation processes, we introduce here briefly the available light sources and their potential to interact at the cellular and molecular level. Currently, these are not well supported by the literature.

Laser light is generated on the principle of light amplification of stimulated emission of radiation (Koutná et al. 2003). The beam energy of laser light is powerful because it is highly coherent (waves are all in phase), polarized, focused and monochromatic (a single wavelength). It was first used in ophthalmological field in the early 1960s, although the basic principle of laser was proposed by Einstein as back as in 1917 (Koutná et al. 2003). Lasers are commonly designated and named by the type of lasing material employed. The laser medium can be a solid state semiconductor, a gas, a liquid or a solid, as in Nd:YAG lasers, which employ a Nd:YAG rod as the lasing medium (Thompson 1988).

Laser light is characterised by its single wavelength, although some lasers, such as the dye laser, can be tuned over a wide range of wavelengths (Singh et al. 2012). Lasers are also classified according to their intensity and if they are pulsed or continuous wave (CW), in order to identify the risk of harm to the patient (Karu et al. 2004). In the medical field, lasers are classified as either high-power surgical lasers or low-power therapeutic lasers (Mbene 2008). Non-invasive or 'soft' lasers were introduced into medicine in the 1980s and since then have been seen as useful light sources for medical application (Koutná et al. 2003). The wavelengths of laser radiation used have been investigated to show their therapeutic use (Smith 1991).

LLLT or photobiomodulation is a form of phototherapy, which is designed to apply low levels of red and near-infrared light with wavelengths in the region of 390–10,600 nm and output powers up to 500 mW (AlGhamdi et al. 2012). LLLT is effective in a number of clinical situations where the wavelength of red and near-infrared region are effective in such therapies. However, both of these two wavelength spectra are different in their photochemical and photophysical properties (Smith 1991).

LLLT refers to the use of photon energy at low levels to alter biological activity with no-thermal reactions because there is little increase in the temperature of the irradiated tissue (AlGhamdi et al. 2012). Lasers of low-level intensity are suggested to be non-toxic, non-allergic and because of their ease of application, these techniques have gained wide application in many fields of health care (Koutná et al. 2003, Table 1). Phototherapy has been found to have significant effects on a variety of pathological conditions including pain attenuation, inflammation and induction of wound healing in non-heating effects (AlGhamdi et al. 2012).

From observations, it appears that LLLT has beneficial effects at the molecular, cellular and tissue levels (Tafur and Mills 2008). It has been found that medical treatment with LLLT at various intensities has stimulatory effect on cellular processes (Avci et al. 2013). Recently, it has been reported by several investigators that at low levels of red or near-infrared

Table 3. Review of published studies evaluating the effect of LLLT on different cell lines.

No	Cell types used	How the cells are grown	Type of LLLT	Quality of laser used	Biological effects determination	References
1)	Human skin fibroblast cells	Cultures in minimum essential medium with Earl's balanced salt solution and incubated at 37 °C in 5 and 85% humidity	He-Ne Laser	λ: 632.8 nm Energy density (ED) 5 J/cm^2	1) Non irradiated Hydroxyuria (HU) treated cells had a reduced number of cells in the central scratch compared to non-irradiated non treated cells, suggesting that HU inhibited cellular proliferation. 2) Irradiated HU treated cells showed an increased number of cells in the central scratch compared to non-irradiated treated cells. This increase was due to the stimulatory effect of irradiation with 5 J/cm^2. The addition of HU had no significant effect on cell viability. 3) The Trypan blue exclusion test showed no significant difference in percent viability between treated and non-treated cells. 4) Irradiated non treated cells showed a significant increase in the formazan dye, which is as a result of cleavage of XTT by the mitochondrial succinate dehydrogenase in actively proliferating cells, compared to non-irradiated non treated cells. 5) Cell viability, proliferation and DNA integrity assays showed that irradiated and non-irradiated N cells were not significantly affected at both 1 and 24 h post irradiation. 6) There was a significant decrease in damage at 24 h compared to 1 h incubation due to the activation of DNA repair mechanisms.	(Mbene et al. 2006)
2)	*E. Coli* AB1157, BW527, BW9091 and BW375	Cultures in exponential and stationary growth phase. *E. coli* suspensions (1–2 $\times$ 10^8 cells/mL, in 0.9% NaCl solution)	Laser HTM compact model, AlGaInP	Power: 10 mW λ: 658 nm	1) There is no alteration of survival fractions of these *E. coli* cultures when exposed to laser. 2) I was indicate that laser exposure induces filamentation in exponential *E. coli* AB1157, BW527, BH20, BW375 and BW9091 cultures at all emission modes. 3) Laser – induced stimulation of cell replication in *E. coli* cultures depends on the culture conditions, determining the particular metabolic state necessary for the division.	(da Silva Sergio et al. 2013)
3)	Stem cells	Does not maintion the culture procedure	He-Ne Laser Gallium-Aluminum-Arsenide (Ga-Al-AS)	λ: 632.8 nm λ: 600 nm Energy density: 0.5–4.0 J/cm^2 Power 1–500 mW	1) LLLT can increase enhance the proliferation rate of various cell lines. 2) The stimulation of cellular proliferation is dependent on the doses of laser irradiation, as lower doses increase the cell proliferation rate	(AlGhamdi et al. 2012)

(*continued*)

Table 3. Continued.

No	Cell types used	How the cells are grown	Type of LLLT	Quality of laser used	Biological effects determination	References
					and other cellular functions, while higher doses of LLLT have negative effects.	
4)	Mesenchymal stem cells (MSCs) and Cardiac stem cells (CSCs)	Cell cultured at 1.3×10^6 cm^2 in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 m mol/L Glutamine, 100 U/ml pencillin, 100 U/ml stroptomycin CSC cultured in a class 2 flow hood.	Diod (Ga-As)	λ 804 nm Power density: 50 mW/cm^2 Energy density: 1 and 3 J/cm^2 Exposure time: 20 sec or 60 sec	1) CSCs of (1 J/cm^2) 1 and 2 weeks post LLLT irradiation significant increase of seven-fold and two-fold, respectively in the number of cells compared to control. 2) Significant increase in the number of cells at the energy density 3 J/cm^2 after 1 week. 3) The number of MSC_S increased post LLLT of 50 mW/cm^2 for 20 sec and 60 sec	(Tuby et al. 2007)
5)	Fibroblast of skin cells, buccal mucosa and gingival			λ: 540 nm 600–900 nm Energy density: J/cm^2	1) Increased proliferation, maturation and locomotion as well as transformation to myo-fibroblasts. 2) Reduced production of pro-inflammatory prostagland in E2 3) Increased production of basic fibroblasts growth factors. 4) Increased proliferation at low doses and suppressed at high doses.	(Walsh et al. 1997)
	Macrophages				1) Increased ability to act as phagocytes, and greater secretion of basic fibroblasts growth factors. 2) Macrophages resorb fibrin as part of the demolition phase of wound healing more quickly with LLLT, because of their enhanced phagocytic activity during the initial phases of the repair response.	
	Lymphocytes			λ: 660 nm 820 940 nm	Lymphocytes become activated and proliferate more quickly	
	Epithelial cells				These cells become more motile and are able to migrate across wound sites with accelerated closure of defects.	
	Endothelium cells				Endothelium forms granulation tissue more quickly. Relaxation of vascular smooth muscles	
6)	Human Gingival Fibroblasts (Hgf3-Pi 53 NCBI code C50)	The cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS). This medium was also supplemented with	(Ga–Al–As) diode laser	λ: 810 nm Power: 50 mW Energy density: 4 J/cm^2 Exposure time: 32 sec	1) The differences between the case and the control groups were statistically significant on 48 h and 72 h after irradiation. 2) The results of this in vitro study revealed that good levels of cell proliferation could be achieved if enough time has been given to the	(Frozanfar et al. 2013)

(*continued*)

Table 3. Continued.

No	Cell types used	How the cells are grown	Type of LLLT	Quality of laser used	Biological effects determination	References
		2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.			cells to show the effect of laser irradiation on cell proliferation rate.	
7)	HeLa cells	They were grown as monolayers in scintillation vials	He-Ne laser	λ: 632.8 nm Power density: 10 W/m^2 Exposure time: 10 sec Energy density: 100 J/m^2	1) When the cells exposed to laser radiation for 60 min before exposure to γ-radiation, substantial differences was seen between the survival curve and the curve representing the survival of γ-irradiated cells. 2) Increased the number of cells after stimulation with He–Ne in the exponential phase of growth than that for the control.	(Karu et al. 1994)
8)	Yeast, HeLa		He-Ne laser	λ: 632.8 nm Power density: I$\geq 2 \times 10^{11}$ W/cm^2	The activity of some enzymes was determined and shows that the growth stimulation is accompanied by the respiratory activity increase with no accumulation of toxic intermediates of oxygen metabolism and by synthetic processes in cell predominance over degenerative once. The data indicated that the irradiation causes a cell metabolism rearrangement, the light playing the role of a trigger controller of the cell metabolism.	(Karu 1988)
9)	Human B-lymphoblasts	Human B-lymphoblast cells (NC 37) were grown in suspension in RPMI 1640 medium (Sigma, Germany) with 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. The cells were sub-cultured twice weekly in fresh RPMI 1640 medium.	He-Ne laser	λ: 632.8 nm Power: 10 W Diameter of beam: 0.75 cm Doses ranging 0.5-2.7 kJ/m^2	1) The cell viability measurement shows no significant change of the cell survival. 2) He-Ne lasers alone do not result in any DNA damage.	(Dube et al. 2001)
10)	Human alveolar bone fragments	Cells were cultured in α-Minimum Essential Medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 50 μg/mL gentamicin (Gibco), 0.3 μg/mL fungizone (Gibco), 10-7 M dexamethasone (Sigma, St.Louis, MO, USA), 5 μg/mL ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma)	GaAlAs diode laser	λ: 780 nm Power: 70 m W Diameter of beam 0.2 cm Energy density: 3 J/cm^2 Exposure time: 9 min	1) Cell growth was affected by time only in LLLT group 2) From day 10 to 14, LLLT treated cultured showed an increase of cell growth	(Petri et al. 2010)

(*continued*)

Table 3. Continued.

No	Cell types used	How the cells are grown	Type of LLLT	Quality of laser used	Biological effects determination	References
11)	Human gingival fibroblasts	A cell line of human gingival fibroblasts named LMF was grown in DMEM with either 5% nutritional deficit or 10% (FBS)	Diode laser	λ: 670 nm, 780 nm, 692 nm 786 nm Energy density (fluence) 2 J/cm^2 Exposure time: 9 min	1) The irradiated cell number of cell cultured in 5%nutrition deficit more than that control cell cultured in idial conditions 2) In the same fluence, IR laser induced a higher cell proliferation than visible laser when the output powers are different. 3) Lasers of equal output power presented the similar effect on cell growth independently of their wavelength.	(Almeida–Lopes et al. 2001)
12)	Human Macrophages	The macrophage J774 cell line was grown in (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C and in a wet environment with 5% CO_2. Cell growth was assessed every 24 h using an inverted phase microscope	Diode laser	λ: 780 nm Power: 70 mW Energy density: 3 J/cm^2 λ: 660 nm Power: 15 mW Energy density: 7.5 J/cm^2	1) After 1 day of culture, activated and 780 nm irradiated macrophages showed lower mitochondrial activity (MA) than activated macrophages, but activated and 660 nm irradiated macrophages showed MA similar to activated cells. 2) After 3 days, activated and irradiated (660 nm and 780 nm) macrophages showed greater MA than activated macrophages, and after 5 days, the activated and irradiated (660 nm and 780 nm) macrophages showed similar MA to the activated macrophages.	(Souza et al. 2014)
13)	MG-63	Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 100 IU/ml penicillin, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 1% glutamine and 2% HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), supplemented with 10% fetal bovine serum. Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2	Diode laser	λ: 940 nm Energy outputs: 1–5 J Intensities: 0.5, 1, 1.5 and 2 W/cm^2	Pulsed low-level laser with low-energy density range appears to exert a biostimulatory effect on bone tissue.	(Huertas et al. 2013)
14)	Osteoblastic (MC3T3) cell line	Cells were grown in sterile Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM = F-12) (Invitrogen, Mount Waverley, Australia) supplemented with	Diode laser	λ: 830 nm Power: 30 mW Energy density: 10 J/cm^2	Reduction in cell proliferation compared to non-irradiated controls.	(Renno et al. 2010)

(*continued*)

Table 3. Continued.

No	Cell types used	How the cells are grown	Type of LLLT	Quality of laser used	Biological effects determination	References
		heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), and 200 ml penicillin +200 mg = ml streptomycin (Invitrogen)				
15)	Human osteoblast cell line	Cells were maintained in sterile medium (Dulbecco's Modified Eagle's Medium): Nutrient Mixture F-12 (DMEM = F-12) (Invitrogen, Mount Waverley, Australia) supplemented with heat-inactivated fetal bovine serum (FBS) (Cambrex, East Rutherford, NJ), and 200 ml penicillin + 200 mg = ml streptomycin (Invitrogen)	He-Ne laser 632 nm	λ: 632nm Power: 10 mW Energy density: 0.43 J/cm^2	LLLT promotes proliferation and maturation of human osteoblasts in vitro, and a significant 31–58% increase in cell survival	(Stein et al. 2005)
16)	Human monocytic THP-1 cell line	THP-1 cells were grown in 50 ml culture flask, the flask containing 20 ml of medium plus cell, at 37 °C with 5% CO_2 in a humidified incubator.	Diode laser 850 nm	λ: 850 nm Power: 9.5 mW Energy density: (0.6–27 J/cm^2) power density of 29.6 mW/cm^2	PBM promotes proliferation of human monocyte in vitro, and a significantly increased cell survival due to increasing membrane integrity and mitochondrial activity.	(Mussttaf et al. 2017)
17)	Stem cells from exfoliated deciduous teeth (SHED)	Cells were maintained in Eagle's minimum essential medium alpha modification supplemented with 10% FBS and 1% penicillin and streptomycin solution (penicillin–streptomycin, Gibco, Invitrogen) at 37 °C and 5% CO_2 in incubator.	InGaAlP red laser	λ: 660 nm Energy density: (1.2–6.2 J/cm^2)	Improved cell viability and proliferation of SHED after laser irradiation, except for 1.2 J cm^{-2}.	(de Souza et al. 2018)

light illumination, LLLT can prevent cell apoptosis (Huang et al. 2009; AlGhamdi et al. 2012), stimulation of mitochondrial activity, increased cell turnover, recruitment and proliferation, modulation of the cellular metabolites (Di Giacomo et al. 2013). It was suggested that LLLT might promote changes in the cellular redox state, playing an important role in sustaining cellular activities and induce photobiostimulative processes (Silveira et al. 2009). In addition to the above, pre-exposure of PBM had a protective effect against many external agents such as hydrogen peroxide (H_2O_2) and UV radiation (Canuto et al. 2015; Sergio et al. 2015). There is an evolutionary standpoint confirming that NIR pre-exposure can protect cells from the hazards of UV exposure and that re-exposure for NIR radiation could be important for protection maintenance (Continenza et al. 1993; Lettnin et al. 2016).

3. Optical properties of tissues

When the laser light strikes biological tissues, part of this light is absorbed, part is reflected or scattered and the rest transmitted. Reflection phenomenon is produced due to a change in refractive index of air and tissue. Snell's law can be used to explain this phenomenon:

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{n_2}{n_1} \tag{1}$$

Where θ_1 is the angle between the incident light and the surface normal in the air, θ_2 is the angle between the ray and the surface normal in the tissue, n_1, n_2 are the refractive index of air and tissue, respectively (Niemz 2007).

Most of the light is absorbed by the tissue because the energy state of molecules is quantized; therefore, photonic absorption occurs only when its energy equals the energy difference between such quantized states. Absorption is the key for the desired impact on tissue healing. The magnitude of optical absorption is described in terms of the absorption coefficient μ_a, in units of cm^{-1} (Jacques 2013). The depth of penetration (mean free path) into the absorbing medium is defined by the inverse, l_a (Chung et al. 2012).

The primary step for tissue interaction is scattering behaviour of light in the biological tissue, which is followed by absorption, it is also important because it determines the magnitude distribution of light intensity in the tissue. Scattering of a photon is synchronous with a change in the propagation direction without loss of energy. Analogous to absorption, scattering is expressed by the scattering coefficient μ_s (cm^{-1}) (Palan 2007; Niemz 2007). The length until next scattering occurs is $1/\mu_s$ (cm). Scattering is not isotropic, having a physical property that has the same value when measured in different directions. Forward scattering prevail in biological tissue. This physical characteristic is expressed by the anisotropy factor giving absolute values for isotropic scattering ($g = 0$) to forward scattering ($g = 1$). In biological tissue, g can differ from 0.8 to 0.99 and can have a considerable role in a reduced scattering coefficient, μ_s' (cm^{-1}), which can be defined as:

$$\mu_s' = \mu_s(1-g) \tag{2}$$

The sum of absorption coefficient (μ_a) and scattering coefficient (μ_s) is called the total attenuation coefficient, that the beam is 'attenuated' (weakened) as it passes through the medium. Attenuation coefficient of the volume of a material characterizes how easily it can be penetrated by a beam of light, in other words, the fraction of an incident beam of photons that is absorbed or scattered per unit thickness of the target absorber, μ_t (cm −1):

$$\mu t = \mu s + \mu a \tag{3}$$

3.1. Light distribution in laser-irradiated tissue

Most of the recent evolutions in describing the transfer of light energy in tissue are based on transport theory (Chandrasekhar 1960) and radiative transfer, the physical phenomenon of energy transfer in the form of electromagnetic radiation. The propagation of radiation through a medium is affected by absorption, scattering processes and emission, (Chandrasekhar 1960, Lenoble 1985). According to transport theory, the radiance $L(r,\ s)$ of light at position r traveling in the direction of unit vector s is reduced by absorption and scattering, but it is increased by light that is scattered from s′ direction into direction s. Radiance is a radiometric measure that refers to the amount of light that passes through or is emitted from a particular area and drops within a given solid angle in a particular direction. Then, the transport equation, which describes the light interaction is:

$$s.\nabla L(r,s) = -(\mu_a\ +\mu_s)L(r,s) + \mu_s\int_{4\pi} p(s,s')\,L(r,\ s')d\omega' \tag{4}$$

Where $d\omega'$ is the differential solid angle in the direction s', and $p(s,s')$ is the phase function (Cheong et al. 1990; Chung et al. 2012).

Determining the distribution of light in an irradiated tissue is based on the transport equation requiring μ_s, μ_a and p. An exact solution for transport equation is often difficult, therefore, several approximations have been made concerning the illustration of the radiance and phase function. The approximate calculations of distributed light in tissue are related to the type of light irradiation (diffuse or collimated) and the optical boundary conditions (matched or unmatched refractive indexes) (Cheong et al. 1990).

4. The mechanism of laser-sub-cellular and cellular interaction

It is being suggested that the key underlying mechanism of action for most of the physiological effects attributed to LLLT is the stimulation of mitochondrial activity (Hashmi et al. 2010a; Di Giacomo et al. 2013). The first law of photobiology states that photons of low-power light must be absorbed by electronic absorption bands belonging to chromophores to produce significant effects on living biological systems (Huang et al. 2009). A chromophore (or photoacceptor) is a molecule of a compound, which imparts some colour to the compound (Huang et al. 2011).

According to the theory of quantum mechanics by Max Planck (1900), light energy consists of photons or discrete packets of electromagnetic energy. The individual photon energy depends on the wavelength; therefore, the dose energy of light depends on the number of photons, their wavelength and surface area through spot-size of the laser (Hamblin and Demidova 2006).

When photons from a laser are incident on living tissue are scattered, scattered photons are reflected or transmitted (Hamblin and Demidova 2006). Absorbed photons interact with chromophore molecules located within the tissue. The absorption of light leads to excitation of electrons to higher energy levels. The delocalized electrons of the energized molecule, which are excited rise from the ground state to an excited state (Smith 2005). This excited molecule must lose its extra energy, which must be conserved according to the first law of thermodynamics. Three possible pathways occur when LLLT is delivered into tissue.

4.1. Pathway 1

This is the most common pathway that occurs and is called internal conversion, the excited singlet state of a chromophore is transported from a higher to a lower electronic state. This transition takes place without photons emitting, known as non-radiative decay (Hamblin and Demidova 2006). The energy of the electronically excited state is coupled to rotational and vibrational modes of the molecule. Thus, this interaction increases the kinetic energy of the molecule, such that the excitation energy is transformed into heat. This process would not be expected to cause chemical changes to the molecule (Smith 1991).

4.2. Pathway 2

The second pathway that can occur is fluorescence. Fluorescence is re-emission of light by a substance that has absorbed light. It is a form of luminescence. The excited molecule tends to return to its stable state by emitting photons with a longer wavelength (i.e. lower energy than the absorbed photon) (Smith 2005). The resultant heat (from molecular vibrations) arises from the energy difference between the absorbed and emitted photons.

4.3. Pathway 3

The third pathway that can occur after the absorption of low-level laser light by a tissue photo-acceptor representing a number of photochemical processes. Although covalent bonds cannot be broken by low-energy photons, the energy is, however, sufficient for electrons to go from the first excited singlet state to the triplet state of the photoacceptor through intersystem crossing. Increasing the reaction rate allows transforming such as ground state molecular oxygen (a triplet) to singlet oxygen state (reactive oxygen species). Alternatively, the long-lived triplet of the chromophore may undergo electron transfer to form a radical anion, which can transfer an electron to oxygen to form a superoxide (Hamblin and Demidova 2006).

The photochemical pathway is the separation of a non-covalent bound ligand from a binding site on a metal in an enzyme. Cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain in eukaryotic cells, is the candidate enzyme for a photoacceptor (chromophore). A molecule imparts colour to a compound, mediating the transfer of electrons from cytochrome c to molecular oxygen. After absorbing red or near-infrared light, cytochrome c oxidase undergoes photochemical processes through the dissociation of binding of nitric oxide from the iron-containing and copper-containing redox centres in the enzyme (Hamblin and Demidova 2006). There is a growing body of evidence, which suggests that cytochrome c oxidase could act as a photoacceptor of light in the near-infrared spectral range (Silveira et al. 2009). It is also considered as the photosignal transducer in the region of visible and IR-A region (Karu 2010). This reactivity is due to four redox active metal centres: the bi-nuclear CuA, CuB, heme a and heme a3, all of which have strong absorbency in the red to IR-A range (Smith 2007; Karu 2010; Piazena and Kelleher 2010).

Many studies on the biological influence of LLLT have compared the action spectrum, a plot of the relative effectiveness of different wavelengths of light in causing a particular biological response and under ideal conditions, it should follow the absorption spectrum of the specific molecule and whose photochemical alteration causes the biological effect attributed to the absorption spectra. These studies have suggested cytochrome c oxidase as the primary photoacceptor (chromophores) (Smith 2005; Desmet et al. 2006).

Cytochrome c oxidase is the fourth enzyme in the inner membrane of cellular mitochondria (Habash et al. 2006; Huang et al. 2011; Di Giacomo et al. 2013), that plays a pivotal role in adenosine tri phosphate (ATP) synthesis (Silveira et al. 2009). Excitation of cytochrome c oxidase components with infrared light energy accelerates the rate of electron transfer and in turn increases the ability of mitochondria to produce ATP, which accelerates cellular metabolic processes (Silveira et al. 2009). Moreover, signal transduction to other parts of the cell has occurred, including cell membranes (Woodruff et al. 2004). Photobiological responses are the result of photochemical and/or photophysical changes after the absorption of non-ionizing electromagnetic radiation (Smith 1991).

Production of nitric oxide (NO) in mitochondria, especially in injured or hypoxic cells can inhibit respiration by binding to cytochrome c oxidase and displace oxygen (Brown 1995). This binding is proposed to dissociate by the PBM or LLLT effect and reverse the mitochondrial inhibition of respiration due to excessive NO binding (Lane 2006). The photobiomodulation effect of LLLT is able to occur a shift in the overall cell redox potential in the direction of greater oxidation by generating reactive oxygen species (ROS) and inhibiting reactive nitrogen species (RNS) (Alexandratou et al. 2002; Lavi et al. 2003; Lubart et al. 2005; Zhang et al. 2008; Cotler et al. 2015). The excited mitochondrial cytochrome c oxidase after absorbing NIR radiation photon generates ROS that

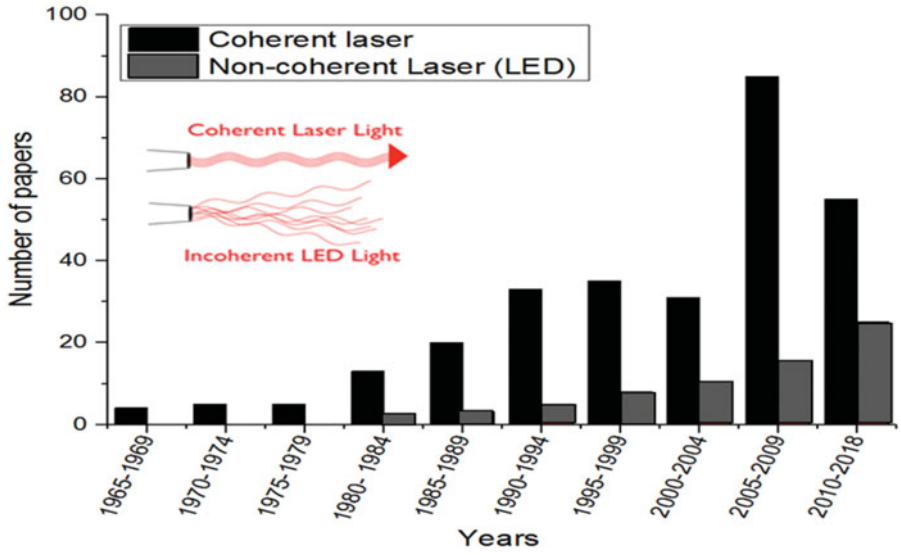

Figure 1. The number of papers published using lasers and LEDs sources in clinical and laboratory studies on the effect of LLLT from 1965–2018.

causes changing the oxidation state of the mitochondrial membrane (Gilmore 2006).

For the phototherapeutic effect to be observed, the appropriate wavelength of light and dose (fluency) of radiation are needed (Woodruff et al. 2004). However, phototherapy will not be effective on every system and in every situation. It has been emphasised that the magnitude of the phototherapy effect depends on the physiological state of the cell at the time of irradiation (Karu 1989).

5. Light emitting diodes (LEDs)

A light emitting diode (LED) is a semiconductor light source (Han et al. 2011). Henry J Round was the first who reported of light emission from carborundum (raw silicon carbide) in 1907. Oleg Losev, as a lot of people today believe, was the actual inventor of LED. He published his first paper in 1927 on emission of silicon carbide diodes. Losev set up the current threshold for the onset of light emission from the contact point between a silicon carbide crystal and a metal wire and recorded the spectrum of this light (Desmet et al. 2006; Suparman 2013). A LED is formed by p-n junctions (p-positive, n-negative), but not all semiconductors are suitable for use as LEDs (de Abreu Chaves et al. 2014) . The physical mechanism by which LED emits light is spontaneous emission (de Abreu Chaves et al. 2014) . They emit near-monochromatic, incoherent light (Ishida 2005), in a process called electroluminescence (Molinaroli 2001). LEDs are small, robust devices that emit a narrow band of electromagnetic radiation from the ultraviolet to the visible and infrared parts of the spectrum, from around 240 nm up to around 950 nm, according to their electronic structure (Ishida 2005), with a linewidth of around 10−30 nm. LEDs have been publicised as a comfortable, potentially highly selective light-based therapies for many indications (McDaniel et al. 2003). LEDs are also very controllable as light sources for non-thermal applications, acquiring a broad area of in medical applications (Avci et al. 2013).

5.1. Laser light vs. light emitting diode (LED)

Not all light is the same or has equal medical benefits (LED or LASER therapy). Recently, controversy has arisen around the comparison between low level laser therapy and light emitting diodes, which have completely different biological effects (Agnol et al. 2009). A number of studies have been published to determine the effectiveness of LLLT to LED light (Figure 1). The majority of the studies have found that although lasers have small focused spots, so only a small area of tissue ($<1\,cm^2$) is exposed to light; whereas LEDs usually have a large area ($100\,cm^2$), so much more area of tissue is exposed to light, lasers are far more effective (Agnol et al. 2009). Laser therapy can achieve much greater and deeper stimulative and therapeutically beneficial effects. Laser beams are easily manipulated using Gaussian beam optics, a simple analytical tool, to enable a laser beam to be fully controlled spatially, position, size, etc. As an LED is difficult to control in terms of position and spot size, it is limited for treatment of superficial tissue only. However, LED light has some beneficial effect where it is believed that it can have a photo-modulation effect on certain cellular and subcellular receptors. In addition, they have greater choice of wavelengths, are low cost and suitable for acute and chronic conditions (Darren Starwynn 2004). In Figure 1, it can be seen that there is a significant increase in the applications of laser sources with wavelengths of greater than 800 nm. Why should this be when there are no known new photoreceptors reported in recent years? This could be attributed to technological advances and differences in the way lasers and LEDs can be controlled. LED sources are limited by the fundamental properties of silicon, which means that their spectral bandwidth has an upper bound of around 950 nm. In addition, LEDs can be operated in continuous or modulated modes only. For a comparative analysis, laser sources will also be restricted to laser diodes. When light is incident on any material, in this case tissue, it will penetrate some distance into the tissue and this distance increases at longer wavelengths. The most significant factor in the application of

lasers compared to LEDs is their mode of operation. Laser sources can be driven in pulsed mode, which can deliver a stream of pulses where the power in each pulse is determined by the pulse width in ns. Thus, lasers are able to efficiently deliver energy to cells and tissues, whereas this is impossible with LEDs (Moskvin 2017; Sato et al. 2016). LEDs still have important applications for photobiomodulation, but coherent sources operated in pulsed mode are still able to deliver light in more controlled ways deeper into tissue compared to modulated incoherent sources (Hamblin 2016).

A number of studies have been published comparing these two modalities. Kubota and Ohshiro (2004) treated rat skin flaps with an 830 nm GaAlAs laser and an 840 nm infrared LED. They found an increasing flap survival area in a rat model after being irradiated with 830 nm laser. Flaps treated with the laser had better perfusion, a greater number of larger blood vessels and significantly enhanced flow rates, while flaps treated with an 840 nm IR LED showed no difference from the control group (Kubota and Ohshiro 2004). Berki et al. (1988) used a HeNe laser to stimulate cell activation in vitro. They observed increasing phagocytic activity along with immunoglobulin secretion, but this effect was not seen after irradiation of the cell cultures with LED light of the same wavelength and doses (Berki et al. 1988).

A comparative study has been performed by Haina et al. (1982) to show the effectiveness of a coherent HeNe laser compared with incoherent light of the same wavelength. Experimental wounds were 'punched out' in the muscle fascia of 249 Wister rats. They reported increasing granulation of tissue in the HeNe treated group, whereas there was less granulation in the incoherent light therapy group (Haina et al. 1982). Rockhind et al. (1989) conducted a study comparing five different wavelengths lasers. They gave a single transcutaneous irradiation dose to injured peripheral nerves. They observed reduced subsidence in functional activity following crush injury after HeNe laser irradiation. While the 830 nm IR laser was less effective, the 660 nm incoherent light was even less effective; 880 nm and 950 nm incoherent lights were completely ineffective (Rochkind et al. 1989). Laasko et al. treated patients with chronic pain using an 820 nm IR laser at 25 mW, a 670 nm laser at 10 mW and a 660 nm LED (Laakso et al. 1994). They found an elevated level of ACTH and beta endorphin in the laser therapy groups but not in the LED group (Laakso et al. 1994).

The effect of HeNe laser and incoherent LED light on leukocytes in migration inhibition assays has been studied by Lederer et al. (1982). They reported that irradiation with HeNe laser light affected leukocytes. However, incoherent light of the same wavelength and power density showed no influence (Lederer et al. 1982). Al (1989) investigated the role of coherent laser therapy in wound healing. They noticed that HeNe lasers with a dose of 1 J/cm^2 produced an acceleration of the healing process, but incoherent light of the same wavelength and dose was less favourable (Al 1989).

Other studies have indicated many reasons, which could lead to a preponderance of LED light than to laser light. NASA has stepped into developing LED light therapies for accelerating wound healing, photodynamic cancer treatment and much more. According to NASA: 'The near-infrared light emitted by these LEDs seems to be perfect for increasing energy inside cells. This means whether you are on Earth, in a hospital, working in a submarine under the sea or on your way to Mars inside a spaceship, the LEDs boost energy to the cells and accelerate healing' (Sommer 2001; Darren Starwynn 2004). Oliveira Sampaio and colleagues (2013) studied the effect of low-level light therapy on the healing of cutaneous wounds and their impact on fibroblastic activity during wound healing. They showed an increasing number of healthy animals after irradiation with laser light and a higher increase was seen when irradiated with LED. They concluded that using LED light caused a considerable biomodulation of fibroblastic proliferation on anemic animals. However, laser light was more effective in increasing proliferation on non-anemics (Oliveira Sampaio et al. 2013). A clinical study by Esper and Arisawa (2011) was carried out to show the effect of two phototherapy protocols on pain control in orthodontic procedure. They found that LED light therapy had a significant effect in the reduction of pain levels compared to laser light therapy. LED therapy showed a significant reduction in pain sensitivity (an average of 56%), when compared to the control group (Esper and Arisawa 2011).

Agnol et al. (2009) performed a comparative analysis of coherent laser light versus incoherent (light emitting diode) light for tissue repair in diabetic rats. They found that the coherent and incoherent lights produced similar effects during a period of 168 h after the lesions had been made. For the control group composed of diabetic animals, 72 h after creation of the lesion, it was observed that the therapy with LEDs had been more efficient compared with the laser for the reduction of the healing period (Agnol et al. 2009). Similar findings have been obtained by Klebanove et al. (2005) in a comparative study of the effect of laser and light emitting diode irradiation on healing and functional activity of wound exudate leukocytes (Klebanov et al. 2005). They deduced that coherent laser and incoherent light-emitting diode radiation have very similar effects on wound healing and activity of wound exudate leukocytes, and that the coherence of light is not required for this activity (Klebanov et al. 2005). Another study by Klebanov et al. (2006) has been carried out to explore the comparative effects of laser light and light emitting diodes on the production of superoxide dismutase (SOD) and nitric oxide (NO) in wound fluid of rats. The study indicated dose-dependent changes in superoxide dismutase activity and production of nitrites in wound fluid after irradiation with visible coherent laser and incoherent LED and the radiation coherence does not play any significant role in the changes of superoxide dismutase activity or nitrogen oxide formation (Klebanov et al. 2006).

The rapid evolution of light emitting diodes makes feasible the use of LEDs for medical treatment and light therapy (Yeh et al. 2010). The single frequency laser does not diffuse, whereas the LED light does. This diffusion allows the cell to be in control of the treatment (Ghuloom 2013). Moreover, LED light therapy has been considered non-significant risk by the FDA (Desmet et al. 2006). For this reason, it was

suggested that the use of light emitting diodes for treatment is much safer than laser therapy (Ghuloom 2013).

Given the above information, and from recently published studies (Lee et al. 2007; Oliveira et al. 2013), it has been shown that lasers have an important role in many medical conditions and with many positive research results (Avci et al. 2014; Cotler et al. 2015; Bell and Stout 2018), as well as LEDs, which are also important in many cases of the disease (Corazza et al. 2007; Xavier et al. 2010). Nevertheless, in most comparative studies that used laser and LED with the same qualities (wavelength, doses, intensity), it is confirmed that laser offer many advantages compared to LEDs (Leal Junior et al. 2009).

Given that LEDs are relatively cheaper and easier to use, it is not surprising that compared to lasers, the number of articles using LEDs for clinical applications are on the increase. There are, however, only a few studies where biological responses of these two types of sources have been compared concomitantly in the same biological model. This is, particularly so, at molecular and cellular levels with clinical implications or outcomes. In a comprehensive study, using various in vitro models derived from (a) 3T3 cells (standard fibroblast) stably expressing red fluorescence protein (DsRed), (b) EGFR expressing A431 cells (established from skin carcinoma) and, (c) stably luciferase-transfected 468-luc cells (originating from pleural effusion of mammary gland and breast tissue), the relative biological responses of these two sources have been compared (Sato et al. 2016). The study suggested no significant differences (in terms of cellular swelling, bleb formation) in non-EGFR-expressing 3T3 cells after irradiation with either sources. The cytotoxicity based on luciferase activity in 468-luc cells, however, showed a significant decrease of relative light units (RLU) related to near infrared photoimmunotherapy (NIR-PIT) induced reductions in ATP production and significant differences between the two sources, laser being more cytotoxic at the same energy level than LED (Sato et al. 2016). This enhanced cytotoxicity (determined by LDH cytotoxicity assay) by laser was further confirmed in spheroid (3D) cultures derived from A431 cells.

The in vitro studies by Sato et al. (2016) was further extended to in vivo mice models. Tumour volume was found to be reduced significantly in the laser NIR-PIT group compared with the LED NIR-PIT group of A431 xenograft mice. Furthermore, after NIR-PIT, the tumour irradiated by laser demonstrated lower IR700 – fluorescence intensity than the tumour irradiated by LED. Overall, the results suggested that lasers result in higher efficacy under in vitro and in vivo conditions and thus has superior therapeutic tumour effects compared to LEDs at the same energy level. This has obvious implications for the choice of NIR-light source for human clinical trials.

6. Effects of LLLT at cellular level

To assess the influence of low-level laser therapy at the cellular level, cell cultures are one of the best biological systems used to find out the effect of laser irradiation on cell proliferation rate. Various studies, which have used different types of laser therapy with a variety of cells, have been designed to improve understanding on the effect of LLLT at the cellular level (Figure 2). More recent studies have studied the biostimulatory effect of low level laser on cell proliferation processes.

Early work by Karu et al. (1994) have reported that the cytotoxic response of Hela cells to ionizing radiation can be influenced by irradiation with He-Ne laser (632.8 nm) with an energy density 100 J/m^2. They observed that there was a substantial difference between the survival curve of Hela cells treated with He-Ne laser for 60 min before exposure to γ– irradiation and the curve representing the survival of untreated γ -irradiated cells. Moreover, an increase in the number of cells was observed after stimulation with a He-Ne laser compared to the control group (Karu et al. 1994).

Pereira et al. (2002) examined a 632.8 nm He-Ne laser with an energy fluence of 0.053–1.89 J/cm^2 and a 904 nm (GaAs) laser with an energy fluence of 1.94×10^{-7} to 5.84×10^{-6} J/cm^2 on fibroblast cell cultures, which determined by using the Trypan blue dye exclusion assay. No difference in cellular proliferation for fibroblast cells exposed to a He-Ne laser versus untreated fibroblast cells could be found. On the other hand, with GaAs laser, a decrease in cellular proliferation of fibroblast cells compared to controls was observed. However, both He-Ne and GaAs lasers induced procollagen production (Pereira et al. 2002).

It was noted that with exposure to a 670 nm GaAlAs laser, an increase in myofibroblasts and collagen deposition was observed (Medrado et al. 2003). Furthermore, an increase in gingival fibroblasts after exposure to diode lasers (670, 692, 780, and 786 nm) was also found (Posten et al. 2005).

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Bouma et al. (1996) examined human monocytes and human umbilical vein endothelial cells (HUVECs) with a 904 nm GaAs laser at 40.18 mW/cm^2 power density. They found no difference in the cytokines level such as tumour necrosis factor TNFα, interlukin-6 and -8, E-selectin, intercellular adhesion molecule 1 and vascular cellular adhesion molecule 1 (Bouma et al. 1996). Schindl et al. (2003) reported that HUVECs irradiated with a 670 nm diode laser with a dose of 2–8 J/cm^2 resulted an increase in the proliferation of these cells that is determined by using a haemocytometer (Schindl et al. 2003). An in vitro study by Hass et al. (1990) showed an increase in human keratinocytes mortality after exposure to He-Ne laser and found no change in proliferation or differentiation (Haas et al. 1990). In contrast, Grossman et al. (1998) observed an increase in proliferation rate of keratinocyte cells after exposure to a 780 nm continuous-wave diode laser with a dose from 0 to 3.6 J/cm^2 (Grossman et al. 1998).

Researchers pointed out that using low-laser therapy with low doses can increase the proliferation rate of cultured cells when compared to high doses. Beyond a certain dose level,

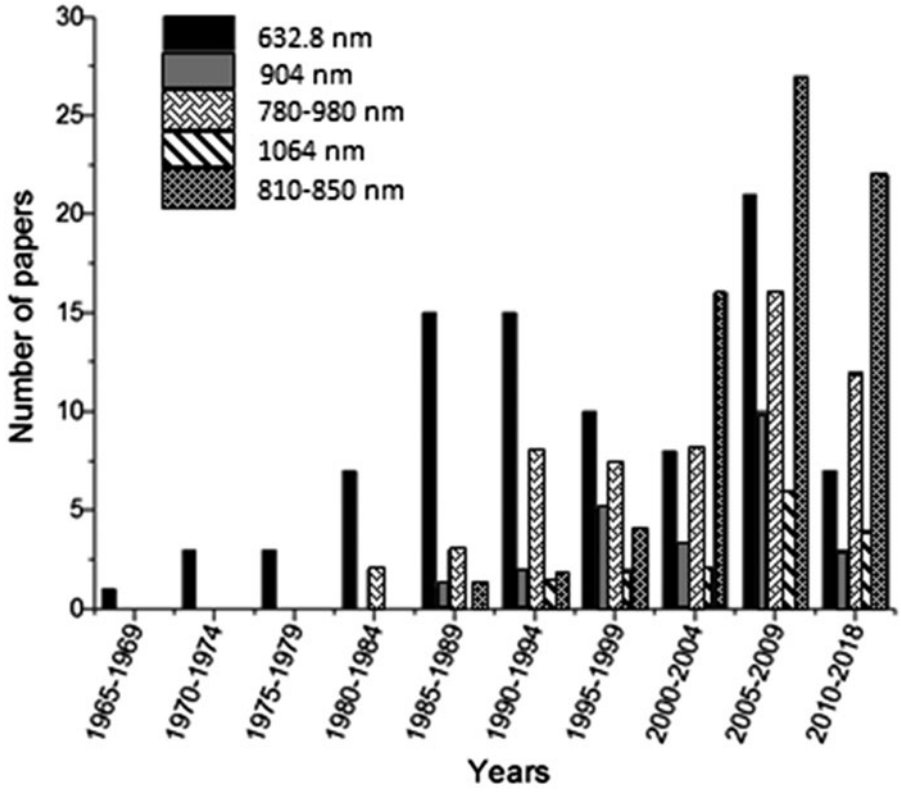

Figure 2. Papers published using different wavelengths light sources in clinical and laboratory studies on the effect of LLLT on cell functions from 1965–2018.

which is cell type dependent, high-dose levels have a detrimental effect on cell proliferation rates. AlGhamdi et al. (2012) have examined stem cells following exposure to a He-Ne laser at 632.8 nm and a GaAlAs at 600 nm, with a range of energy densities (doses) from 0.5 to 4.0 J/cm^2 and power densities from 1–500 mW and found that LLLT can increase the proliferation rate of various cell lines. They have confirmed that the stimulation of cellular proliferation is dependent on the dose level of laser irradiation. They concluded that lower doses increase the rate of cell proliferation and other cellular functions, In contrast, higher doses of low-level laser therapy have negative effects, significantly decreasing cell counts and the viability (AlGhamdi et al. 2012). Similar results have been obtained by Walsh and colleagues (1997), when they irradiated skin fibroblasts cells, buccal mucosa and gingival cells with semiconductor lasers at 540 nm and 600–900 nm with 0–56 J/cm^2 energy densities. Walsh noted increased cell proliferation at low doses, which was repressed at high doses. They also observed increased maturation and locomotion, transformation to myo-fibroblasts and increased production of basic fibroblasts growth factors.

Walsh (1997) used the same laser with the same energy densities to examine the effects on macrophage cells. A convergent result was observed, which included greater secretion of basic fibroblasts growth factors, increased ability to act as phagocytes and resorption of fibrin by macrophages. Walsh in another study used semiconductor lasers of 660, 820 and 940 nm to treat human lymphocyte cells showing activated cells with high proliferation rate. With the same wavelengths, Walsh noted the increased motility of epithelial cells and an ability to migrate across wound sites with quickened closure of defects.

Unlike AlGhamdi and Walsh, Petri et al. (2010) found that cell survival, as measured by MTT assay, was affected by time with LLLT after exposing human alveolar bone fragment cells to a GaAlAs diode laser of 780 nm with a power of 70 mW and energy density of 3 J/cm^2 (Petri et al. 2010). Recently, Forouzanfar (2014) has supported Petri's results when examining human gingival fibroblasts with a Ga-Al-As diode laser at 810 nm, output power of 50 mW and energy density of 4 J/cm^2. Forouzanfar noted that both good levels of cell proliferation and secretion of macromolecules can be regulated if enough exposure time of low level laser therapy has been given to the cells to determine whether LLLT could induce a bio-stimulatory effect on human cells. As well, they found a significant difference between the case and control groups on 48 and 72 h after irradiation (Forouzanfar 2014).

Tuby et al. (2007) obtained a positive result when they exposed mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) to a GaAs diode laser at 804 nm with an energy density between 1 and 3 J/cm^2 and an output power of 50 mW. The results showed a significant increase of seven-fold and two-fold in the number of CSCs after 1 and 2 weeks post irradiation of 1 J/cm^2 for 20 sec exposure and increased the number of MSCs and CSCs after 1 week post irradiation of 3 J/cm^2 compared to the control (Tuby et al. 2007).

Almeida-Lopes et al. (2001) used diode laser with 670, 692, 780, and 786 nm wavelengths and fluence (energy density) of 2 J/cm^2 to show the comparison of LLLT effects on the proliferation rate of cultured human gingival fibroblast cells. They found that in the same fluence and with different output powers, infrared lasers induced a higher proliferation rate of cells compared to visible laser. Whilst lasers of equal output power were shown to have similar effect on cell

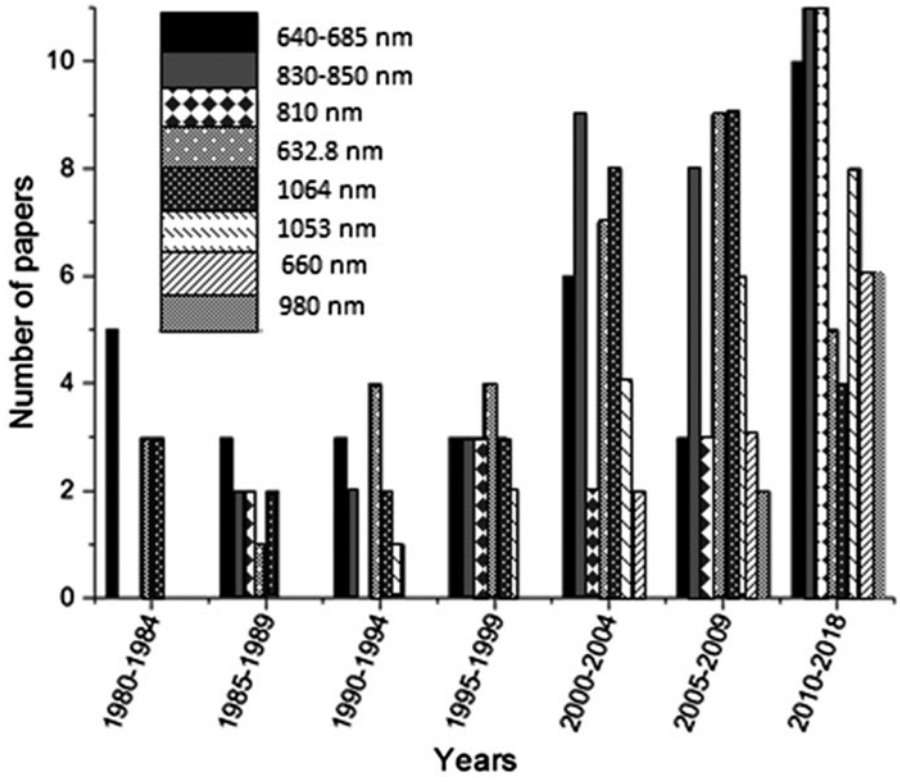

Figure 3. Papers published evaluating the effects of different sources for LLLT in clinical and laboratory studies on DNA from 1980–2018.

growth independent of their wavelengths (Almeida-Lopes et al. 2001).

7. Effect of LLLT at molecular level

LLLT has been in existence for more than four decades. It has been found beneficial in a wide variety of therapeutic applications (Mbene 2008). However, the possibility of induced DNA damage has now arisen; even though this damage could be repairable (Mbene 2008). Although phototherapy is used in the biomedical treatment of many diseases, the mechanisms of laser-molecule interaction remain unclear and the deleterious effects of laser irradiation are still controversial (Kujawa et al. 2004).

LLLT is usually performed with visible red or near infrared laser light and with typical accumulated doses. Since employing wavelengths within the red side of the optical spectrum, which is likely to be less damaging to DNA than sunlight, it is assumed that the doses per area of LLLT are safe when corresponding to the DNA damaging effects of a few minutes sunlight (Kujawa et al. 2004). If such irradiation induces DNA breaks, these breaks are likely to be repaired immediately; otherwise unrepaired damage could lead to mutations consequently leading to development of cancer in the long run (Albertini et al. 2008).

Different studies in eukaryotic and prokaryotic cells have reported adverse effects on cells and DNA damage after exposure to low-power laser therapy (Kong et al. 2009) (Figure 3). Experimental data about the effect of these light sources with different power, wavelengths and emission modes on DNA are, however, limited (Karu 2010). A study by Zhang et al. (2003) using microarray technologies indicated that low-intensity laser exposure (red light) at therapeutic doses promote expression of DNA repair genes following induction of DNA lesions by free radicals (Zhang et al. 2003).

It has been reported that the photo-reactivating enzyme (DNA photolyase) distinguishes one type of DNA damage as its substrate (i.e. the cyclobutane-type pyrimidine dimer), and combines with these dimers in the dark (Smith 1991). However, when exposing the enzyme-substrate complex to visible light, the enzyme uses the absorbed energy of light to split the dimer to produce repaired (original) DNA. Mbene (2008) treated wounded human skin fibroblast cells by He-Ne laser with 5 J/cm^2 and 16 J/cm^2 doses. Irradiation with 5 J/cm^2 and 16 J/cm^2 showed insignificant change in DNA damage, as determined by alkaline comet assay, at 1 h when compared to their respective controls. However, a significant decrease in DNA damage at 24 h incubation due to the mechanism of DNA repair was shown (Mbene 2008).

Fonseca et al. (2010) irradiated *E. coli* cells with low-intensity (AlGaInP) red laser with a power of 10 mW and with different fluencies (1, 4, and 8 J/cm^2). It was suggested that low-level red laser light induces DNA lesions as a result of the generation of free radicals. They suggested that biological effects induced by low-level laser fluence could occur due to the generation of free radicals. They suggested that considerable importance should be given to low-level lasers for their potential to induce DNA repair and changes in gene expression profile of the irradiated cells (Fonseca et al. 2010).

A study by da Silva Sergio et al. (2012) used an AlGaInP laser with a power output of 10 mW and with continuous or pulsed mode of irradiation. They found that low-intensity red

laser radiation could induce DNA lesions via oxidative mechanisms. Moreover, it was found that the survival mechanism against harmful radiation could be activated or induced after irradiation with monochromatic red light (da Silva Sergio et al. 2012). Kohli et al. (2001) examined *E. coli* cells with a He-Ne laser at 632.8 nm. They observed that irradiation with low-level He-Ne lasers induces photolyase gene (*phr*) and DNA repair genes investigated by *phr* gene expression assay. The magnitude of induction relies on fluence rate of the He-Ne laser and the time of incubation post irradiation. The study concluded that the stimulation of DNA repair may explain the higher survival cell against UV radiation (Kohli et al. 2001).

Dube et al. (2001) studied the effect of He-Ne laser 632.8 nm pre-irradiation on UVA-induced DNA damage in human B-lymphoblast cell line, as measured by comet assay. They found a decrease in UVA-induced DNA damage. However, the control cells showed higher DNA damage, the same rate of DNA damage in He-Ne laser pre-irradiated cells. The results suggested that He-Ne laser irradiation plays an important role in protecting the cells from UVA-induced DNA damage primarily through an influence on processes of preventing an initial damage of DNA (Dube et al. 2001).

Dillenburg et al. (2014) triggered epithelial cells with laser phototherapy (LPT) of energy density 4 J/cm^2 and 20 J/cm^2. They observed that laser phototherapy at a low-energy density of 4 J/cm^2 did not induce DNA damage or genomic instability as determined by comet assay. Interestingly, a low energy of LPT induced nuclear influx of the BRCA1 protein, which is involved in DNA repair process. Importantly, these findings suggest that LPT of low dose induces a safe level of reactive oxygen species (ROS), which accelerate healing (Dillenburg et al. 2014).

Ridha et al. (2012) used a He-Ne laser 632.8 nm to irradiate human lymphocytes. They concluded that the effect of low red laser light in maintaining cell survival may be attributed to the induction of endogenous radioprotectore and improvement of DNA repair due to induced enzymes involved in repair process (Ridha et al. 2012). More recently, Trajano et al. (Trajano et al. 2014) stated that at therapeutic fluences, exposure to red visible laser therapy alters the expression of genes related to the base excision and nucleotide excision pathways of DNA repair during wound healing (Trajano et al. 2014).

Although most of the aforementioned studies have shown the effect of LLLT on cell proliferation, conflicting results have been published. The studies also tried to explain the induction effect of LLLT on DNA repair mechanisms with varying results. All these contrasts may be related to a disparity in study design, including the use of different lasers, variations in parameters such as energy densities, wavelengths, exposure time, output power, etc.

8. Discussion

Interest in the field of LLLT has been rekindled in parallel with philosophical evolution towards minimally invasive laser therapies (Alam and Dover 2003). Although the action of lasers on biological tissue is mediated via photothermal effect, LLLT ideally causes low or imperceptible temperature changes, making LLLT known as'low intensity' or 'cold' lasers (Hamblin and Demidova 2006). Experiments of measuring the temperature following LLLT exposure have shown that the immediate increase in temperature of the irradiated tissue is negligible (±1 °C) (Hrnjak et al. 1994). Many researchers emphasises that the temperature remained unchanged in suspensions of different cells through LLLT irradiation (Boulton and Marshall 1986; Quickenden and Danniels 1993). Studies by Schneede et al. (1988) suggested that the temperature could raise by less than 0.065 °C, during irradiation with laser of 40 mW/cm^2, they used a microthermal probe in a monolayer of cells to measure the temperature (Schneede et al. 1988).

Lasers are distinctive and their unique properties of diffraction limited spot of sub-micro dimensions, yielding high-power density, ultrashort pulses, coherent radiation (i.e. the light waves are all in phase), and monochromaticity are all made use of (Smith 2005). However, many researchers have found no significant difference for photo stimulation regardless of whether the light used was generated by a laser source or from light of the same wavelength from a filtered incandescent lamp. This review shows an increasing number of articles in the literature on photo therapy in recent years using incoherent light sources, such as LEDs (Smith 2005).

These findings build on previous reviews of LLLT by including biological effects of LLLT at cellular and molecular levels. Although various studies included hypotheses explaining the mechanisms of laser action on biological systems, the understanding of the biological effects of laser therapy is still poor. This review has identified a growth in the number of studies. Many studies, often with conflicting results in this field, have been published (Smith 1991). These discrepancies may be attributed to a variance in study design, including the use of different lasers and inequalities in parameter selection. In addition, as indicated by Karu (1989), it may relate to the physiological state of the cell at the time of irradiation (Smith 1991). In general, for laser studies to be useful, all the characteristics of the light emitted from laser source or by LEDs must be specified (Smith 2005).

9. Conclusions

In conclusion, LLLT is a treatment method using laser light of low energy or intensity. It delivers a very low energy, enough to produce stimulation, but not destruction of the target system; therefore, it has been used extensively for diverse studies. Applications of this optical tool have also attracted criticism with respect to its reproducibility, despite several advantages. The present review has highlighted many subjects including the emergence of LLLT, the mechanism of LLLT interaction with the biological system, the optical properties of tissue, the cellular and molecular effect of LLLT as well as the types of lasers used for LLLT. However, it emerges that most studies concern dose and wavelength. There have only been a limited number of studies so far on the physical parameters of LLLT such as coherence and polarisation of light. The outcomes of this review revealed

that, in addition to low-intensity coherent lasers, incoherent light emitted from LED is used widely with a wide range of therapeutic applications. There were conflicting views as to whether coherent laser or incoherent LEDs has the most beneficial therapeutic impacts on biological systems. A relative comparison of biological responses with potential clinical implications using different sources (i.e. laser and LEDs) in the same model system has been very limited, which needs further elaboration. Furthermore, in spite of the large number of studies including different laser types, studies using the same parameters of LLLT to assess cell survival or effects on DNA are so far almost non-existent

More studies using LLLT with different properties are needed to investigate which laser with specific properties has a beneficial effect on biological system, in order to be included within the therapeutic tools and which has a deleterious impact to be excluded from uses (e.g. to treat malignant problems). Furthermore, local magnetic field as magneto-optical phenomenon can change the polarisation dependent absorption of laser light. These aspects need further studies in relation to therapeutic uses of LLLT.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

We acknowledge the financial support from the Ministry of Higher Education and Scientific Research, Republic of Iraq (PhD Studentship for RAM).

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Journal of Biomedical Optics

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Randa Zein, Wayne Selting, Michael R. Hamblin, "Review of light parameters and photobiomodulation efficacy: dive into complexity," *J. Biomed. Opt.* **23**(12), 120901 (2018), doi: 10.1117/1.JBO.23.12.120901.

Journal of Biomedical Optics 23(12), 120901 (December 2018)

REVIEW

Review of light parameters and photobiomodulation efficacy: dive into complexity

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Abstract. Photobiomodulation (PBM) therapy, previously known as low-level laser therapy, was discovered more than 50 years ago, yet there is still no agreement on the parameters and protocols for its clinical application. Some groups have recommended the use of a power density less than 100 mW/cm^2 and an energy density of 4 to 10 J/cm^2 at the level of the target tissue. Others recommend as much as 50 J/cm^2 at the tissue surface. The wide range of parameters that can be applied (wavelength, energy, fluence, power, irradiance, pulse mode, treatment duration, and repetition) in some cases has led to contradictory results. In our review, we attempt to evaluate the range of effective and ineffective parameters in PBM. Studies *in vitro* with cultured cells or *in vivo* with different tissues were divided into those with higher numbers of mitochondria (muscle, brain, heart, nerve) or lower numbers of mitochondria (skin, tendon, cartilage). Graphs were plotted of energy density against power density. Although the results showed a high degree of variability, cells/tissues with high numbers of mitochondria tended to respond to lower doses of light than those with lower number of mitochondria. Ineffective studies in cells with high mitochondrial activity appeared to be more often due to over-dosing than to under-dosing. [DOI: 10.1117/1.JBO.23.12.120901]

Keywords: photobiomodulation; low-level laser therapy; parameters; mitochondrial numbers; effective and ineffective studies.

Paper 180198VRRR received Apr. 4, 2018; accepted for publication Nov. 14, 2018; published online Dec. 11, 2018.

1 Introduction

Since Mester,[1,2] in 1968, accidently discovered the positive effect of a ruby laser beam on hair growth and wound healing in mice, researchers have attempted to uncover the scientific basis for this phenomenon as well to establish the range of optical exposure parameters that lead to successful clinical outcomes. The possibility of stimulating a wide range of cells to improve wound healing and cellular growth has created a science referred to as low-level laser therapy (LLLT) or photobiomodulation therapy (PBMT). As an understanding of basic concepts has emerged, the very wide range of factors contributing to positive outcomes in some cases and negative outcomes in others has stymied the development of definitive protocols.

The multitude of variables to be considered is formidable. More than 1000 research articles have reported that a range of factors can apparently affect the chances of success including wavelength, energy density, power density, total energy, total power, pulse structure, spot size, tissue absorption characteristics, and treatment repetition regimen. Further parameters of lesser importance requiring both control and study are use of combination wavelengths, delivery method (contact, punctual, broad beam), duration of treatment, inadvertent heating of tissue and even whether the source of photons is a laser, light-emitting diode (LED), or broad-spectrum light from a lamp.[3,4]

It has become apparent that, in order to achieve positive results with PBM, each of these dosimetric parameters must be controlled within a limited range of values. Of the many studies that have been conducted over the past 50 years, a number have attempted to determine the relative contribution of individual parameters to successful outcomes.

Consensus has (almost) been reached on one of the most important concepts in PBM. The so-called Arndt–Schultz law was originally proposed near the end of the 19th century. It states in original form that "For every substance, small doses stimulate, moderate doses inhibit, and large doses kill."[5] This concept[6] also forms the basis of the science of "hormesis," as reviewed by Calabrese and Mattson[7]

Pharmacological agents used at a therapeutic dose can be very beneficial while the same drug administered at a higher dose may be catastrophic. For many years, this Arndt–Schultz law has been used as a convenient concept to explain the cellular and tissue interactions with light.

Briefly, this law, when applied to PBM, states that, at very low levels of irradiation, photons are absorbed by subcellular chromophores present inside intracellular organelles, most notably, mitochondria. Absorption of energy by cytochrome C oxidase (CCO) in the mitochondrial respiratory chain is the primary initiating interaction triggering PBM effects.[8] Both adenosine triphosphate (ATP) production and oxygen consumption by the cells increase. This may lead to changes in nitric oxide (NO) levels, activation of secondary messenger pathways, activation of transcription factors, and growth factor production.[9] At this very low level, energy is absorbed by the cell but at such low amounts of energy that there are no observable gross changes (temperature or photochemical damage).

As the number of absorbed photons increases, stimulation of cellular metabolism, as noted above, begins to affect cellular activity, producing positive PBM effects. Both the number of photons and rate at which they are delivered has a significant influence on the response.[10,11]

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As the number of photons increases beyond a particular level, the cellular stimulation disappears, and if the number of photons is even further increased, inhibition and cellular damage occurs. Current theories suggest that the mitochondrial membrane potential having reached a maximum at the optimum dose declines back to baseline and can be lowered below baseline by excessive doses of light.[12] ATP reserves within the cell begin to be depleted by excessive doses of light compromising the positive cellular function. Production of excessive reactive oxygen species (ROS), which can be toxic, release of excessive free NO, which can damage cells, and activation of a cytotoxic mitochondrial-signaling pathway leading to apoptosis are also possible theories. At still higher levels of irradiation, depletion of cellular energy reserves or excess levels of the factors noted above become so significant that cellular metabolism falls below normal intrinsic levels and function is actually inhibited eventually leading to cell death.

This concept, represented by the Arndt–Schultz law of biphasic dose response, has become the foundational concept of PBM. However, the appropriate range of values of fluence and irradiance at which these significant transitions occur are not widely agreed upon. Numerous studies suggest that fluences ranging from 3 to 10 J/cm^2, at the cellular level, will produce the desired stimulation of metabolic activity.[13,14]

While this protocol has become widely accepted, some studies suggest that biostimulation will occur in the range of 0.5 to 1 J/cm^2 on an open wound and in the range of 2 to 4 J/cm^2 to a target through overlaying skin.[15] Another respected source suggests that doses used for superficial targets tend to be in the region of 4 J/cm^2 with a range of 1 to 10 J/cm^2.[16–18] Doses for deeper-seated targets should be in the 10 to 50/cm^2 range.[19–21]

While many studies have shown a positive effect of PBM,[17,18,21] a number have failed to show a benefit[22,23] and, in fact, some reports have shown negative outcomes at what are reported to be the same parameters of irradiation as other positive studies. Unfortunately, in many of the historical studies, important laser parameters were omitted or incorrectly presented.

Often, laser output total power is reported without consideration of the spot diameter at the surface of the target tissue. Therefore, power density, the most relevant parameter, is not reported and results are, predictably, inconsistent.

Sometimes the distribution of energy across the tissue surface is not noted in published studies introducing profound errors. As an example, most lasers are designed to emit in the TEM_{00} mode, which produces a Gaussian distribution of beam profile. By mathematical definition, cells in the exact center of the beam will be irradiated at precisely twice the indicated average output power while cells at the periphery of the irradiation spot will only receive about 13% of that power. If irradiation were to be delivered for 30 s, cells at the beam center would receive an energy dose of 6 J/cm^2 while those at the periphery would receive 0.39 J/cm^2. Obviously, the cellular response, taking into account the Arndt–Schultz law, will be different in each of these tissues. This could result in a conclusion of no-effect, positive effect, or negative effect, depending on which cells were observed in the analysis phase of the study.

Another basic concept that has been suggested to be relevant to the successful application of PBM is the Roscoe–Bunsen law of reciprocity.[24] This concept states that the most important parameter in PBM is the total quantity of photons absorbed by the target cells, and it is not important how quickly or how slowly these photons are delivered. This means that 100 mW/cm^2 applied for 60 s for a dose of 6 J/cm^2 will have the same effect as applying 1 W/cm^2 for 6 s (6 J/cm^2) or 6 W/cm^2 for 1 s (6 J/cm^2) using the same spot size.

Numerous studies have shown that, while this law is valuable for many parts of the parameter range, it does not hold true for the entire range.[18,25,26] The previously discussed theories of the biphasic dose response, supported by other studies, are the likely reason for this inaccuracy. Within a certain range of parameters, perhaps between 1 and 100 J/cm^2, and at power densities from 1 to 100 mW/cm^2, this linear reciprocity applies. However, beyond this range, reciprocity does not appear to apply. For instance, there exists a lower threshold (perhaps 0.5 mW/cm^2) below which the illumination time could be infinite and would be no different from daylight. Similarly, the upper threshold is fixed by the possible photothermal effect if the power density is too large. The irradiance values, that produce unacceptable heating of the tissue, are governed by the wavelength and are ~750 mW/cm^2 at 800 to 900 nm, about 300 mW/cm^2 at 600 to 700 nm, and as low as 100 mW/cm^2 at 400 to 500 nm. Furthermore, the illumination time is also important.[17] There exists a certain minimum length of time (few minutes) that the light needs to be on the tissue for the best effects to occur.[17]

The parameters of most importance in PBM are the power density (irradiance) measured in mW/cm^2 and the energy density (fluence) measured in J/cm^2. Many of the studies discussed here and, indeed, in most of the research literature, are based on the inaccurate statement of the laser output in Watts. Depending on the area irradiated by this beam of photons, the power density and the cellular effects produced will be very different.

As an example, 1 W delivered through a 400 μm diameter optical fiber will produce a power density of 796 W/cm^2 while the same 1 W delivered through an 8-mm diameter therapy hand-piece will produce a power density of only 2 W/cm^2.

Energy density is frequently reported in research literature but the spot area at the tissue is often omitted. This error makes it impossible to verify their findings or to see how they calculated the vital energy density information. Inconsistency in reporting these parameters is a major source of contradictory research findings and has done much to hinder the acceptance of PBM effects.

Another important factor that must be taken into account is the optical properties of the tissue itself.[27] Since the light is generally delivered as a surface spot shone onto the skin, the number of photons that actually penetrate into the tissue to arrive at the pathological lesion is highly variable.[28] The first issue to be addressed is light reflection from the surface of the skin,[29] which can be minimized if the optical probe is held in firm contact with the skin.[30] The second issue is scattering of light within tissue. Scattering is wavelength dependent with shorter wavelengths undergoing more intense Mie scattering than longer wavelengths.[31] The third issue is absorption of the light by chromophores that are not biologically active. These nonactive chromophores are chiefly hemoglobin (both oxyhemoglobin and deoxyhemoglobin), myoglobin, and melanin.[27] However, it should be noted that some authors have suggested that photodissociation of oxygen from hemoglobin[32] or NO from myoglobin[33] could be a relevant mechanism in PBM. There is a growing trend for researchers in PBM to undertake

modeling of tissue optical properties either by Monte-Carlo methods[34] or by use of tissue phantoms.[35]

1.1 *Mitochondria and Cells*

Mitochondria are highly important intracellular organelles whose main function is to act as "power plant" of the cell, generating ATP which is the main source energy for cellular activity and metabolism. Moreover, mitochondria play important roles in regulation of oxidative stress, calcium metabolism, apoptosis, and a host of signaling pathways.[36] It is believed that mitochondria originated when a primitive eukaryotic cell "captured" a primitive prokaryotic bacterium around the time the "great oxygenation event" occurred on the Earth.[37]

Mitochondria contain the electron transport chain responsible for transferring electrons from NADH through complexes I, II, III, and IV.[38]

When applying light to cells, mitochondria are the initial sites of light absorption and CCO (particularly, the CuA and CuB metal centers) are believed to be the photoacceptors.[39] Photon absorption results in setting in motion a cascade of reactions known as cellular signaling pathways leading to NO dissociation, ROS production, and increased ATP synthesis.[9]

The number of mitochondria in cells varies widely and it is strongly correlated with the metabolic requirements of the cell (how many chemical reactions the cell has to carry out) and may range from a few to thousands of individual organelles. Cells such as osteoblasts, keratinocytes, and fibroblasts have a lower number of mitochondria, whereas muscle cells, neural cells, cells composing internal organs (liver, kidneys, spleen, etc.), and myocardial cells contain a higher number of mitochondria. Broadly speaking, the proportion of mitochondria in a tissue type can be gauged by observing the color of the tissue (without containing any blood). For instance, dark colored tissues (liver, heart, kidney, gray brain matter) have a high concentration of mitochondria since CCO and other cytochromes are the most important cellular pigments, while light colored tissues (skin, bones, tendons) have few mitochondria. The following reports discuss how mitochondrial numbers and mitochondrial activity have been determined in different cells and tissues.[40–43]

Furthermore, mitochondria in stem cells and induced pluripotent stem cells are poorly developed and low in number; mitochondrial function and structure have even been suggested as indicators of stem cell competence.[44]

The hypothesis of the present review is that the effects of PBM on different tissues can be explained by taking into account two main factors. First, what is the content of mitochondria in the cells comprising the bulk of the tissue? Second, what is the depth? Cells *in vitro* are very superficial, skin and some connective tissues are moderately superficial, while other tissues are deeper, bones, joints, brain, organs, etc. Moreover, tissues with high mitochondrial numbers tend to be deeper than those with low mitochondrial numbers.

Therefore, studies were divided into two groups based on the number of mitochondria at the cellular level and the depth of the tissue level.

Cells of tissues with higher numbers of mitochondria were assembled in one group (brain cells, muscle cells, neural cells, macrophages, monocytes) and cells with fewer mitochondria were assembled in another group (keratinocytes, osteoblasts, chondrocytes, fibroblasts, stem cells). Tissues with abundant mitochondria exist in organs, such as muscle, heart, liver, kidney, cells.

The purpose of this review paper was to compare effective and ineffective studies on cells and tissues in each group. Every effort was made to find or calculate relevant parameters even if they were not explicitly stated in the paper.

2 Materials and Methods

This study was conducted following Preferred Reporting Items of Systematic reviews and Meta-analysis.

Research questions: Is it possible to propose a practical protocol of for PBM or LLLT? What are the best parameters that produce a positive result in different circumstances?

2.1 *Research Strategy for Article Identification*

Research was conducted using the following electronic databases: Springer, PubMed, Google Scholar, and Cochrane Database.

Keywords used: LLLT, PBM, LLLT and osseointegration, LLLT and bone graft, LLLT and cells, LLLT and bone regeneration.

After collecting the data, the titles, abstract, and conclusions were checked and unrelated, and obviously biased articles were excluded. Also, all case reports and literature reviews were excluded. Only studies dated from 2007 to 2016 were included.

Table 1 Eligibility criteria adapted from Cericato et al.[45] for selection of the studies.

Reason for exclusion	PubMed	Springer	Google Scholar	Cochrane	Total
Literature and/or systematic review	8	8	11	6	33
Article in language other than English	—	—	15	—	15
Letter from the editor, opinion articles	—	—	8	—	8
Fluence not mentioned	3	2	30	8	43
Use of very high fluence: density greater than 500 J/cm^2	4	2	8	8	22
Article did not mention power or fluence rate	5	2	16	6	29
Other (book chapter, appendix, bibliograghy, index	—	—	4	2	6
Total exclusion	20	14	92	30	156

Evaluations of articles were independently performed by two reviewers. The initial search yielded 250 articles. After exclusion of unrelated articles, only 190 remained. Using the exclusion criteria listed in Table 1 reduced this number to 34 articles.

2.2 *Assessment of the Studies*

After obtaining full texts of all 34 relevant articles, they were evaluated and scored following the checklist using eligibility criteria adapted from Cericato et al.[45] described in Table 1. Articles with scores from 0 to 8 points were considered low quality and were excluded. Article with scores from 13 to 15 points were considered high quality while scores from 9 to 12 were considered moderate quality. Table 2 presents the details of the 34 studies finally included in this review.

3 Effect of Varying a single parameter on PBM Efficacy

3.1 *I-Effect of varying wavelength on PBM Efficacy*

3.1.1 *In vitro studies*

It has been shown through many studies that CCO is the most important chromophore that absorbs light. Delpy and Cope[72] showed that over 50% of the light absorption between 800 and 850 nm was due to cytochrome c oxidase, with hemoglobin (oxy and deoxy) playing a minor role. CCO has two absorption bands, one in the red spectral region (~660 nm) and another in the NIR spectrum (~800 nm), which consequently are the wavelengths most often used in PBM[3].

In their study, Wang et al.[54] found that the mechanisms of action of 810 and 980 nm laser appeared to have significant differences. While the PBM effect occurred at both wavelengths, the chromophore was different between wavelengths. NIR wavelengths, such as 810 nm, stimulate mitochondrial activity and ATP production. At longer wavelengths, the mechanism of action of 980 nm relies on absorption by water leading to the activation of heat (or light)-gated ion channels and promotes cell proliferation via the TRPV1 calcium ion channel pathway.

The same study compared the effect on stem cell differentiation of these two different wavelengths, 810 and 980 nm. For each wavelength, different doses were used from 0.03 to 10 J/cm^2, spot size 4 cm^2, irradiance 16 mW/cm^2, power 64 mW, and time of irradiance (3 J/cm^2, 188 s) and (0.3 J/cm^2, 18.8 s). The irradiance was adjusted by varying the distance between the laser and the target cells.

Both wavelengths showed a biphasic dose response. At 980 nm, a peak dose response was seen at 0.03 and 0.3 J/cm^2 while 810 nm showed a peak response at 3 J/cm^2. Moreover, the dose of 0.3 J/cm^2 with the 980-nm laser had a better effect than any of the other groups.

A second study by Wang compared the effects of delivering four different wavelengths (420, 540, 660, and 810 nm) using the same parameters of 3 J/cm^2 at 16 mW/cm^2, on human adipose-derived stem cell differentiation into osteoblasts. They found that 420- and 540-nm wavelengths were more effective in stimulating osteoblast differentiation compared to 660 and 810 nm. Intracellular calcium was higher after 420 and 540 nm and could be inhibited by the TRP channel inhibitors, capsazepine and SKF96365. They concluded that using blue and green wavelengths activated the light-gated calcium channels rather than CCO.[61,73]

Table 2 Final list of studies that were included together with Cericato score.

Authors	Score (Cericato et al.)[17]
Fernandes et al.[46]	12
Mendez et al.[21]	12
Barbosa et al.[20]	11
Huang et al.[47]	11
Huang et al.[48]	12
Sharma et al.[49]	11
Oron et al.[50]	10
Chen et al.[26]	12
Souza et al.[51]	11
Ferraresi et al.[52]	12
Zhang et al.[53]	12
Wang et al.[54]	12
Amaroli[19]	10
Tschon et al.[55]	11
Pyo et al.[56]	12
Migliario et al.[57]	12
Khadra et al.[32]	11
Skopin et al.[58]	12
Salehpour et al.[59]	11
Wu et al.[58]	12
Lopes-Martins et al.[18]	11
Bozkurt et al.[60]	12
Wang et al.[61]	11
Alves et al.[25]	11
Oron et al.[62,63]	11
Castano et al.[17]	12
Salehpour et al.[64]	11
Leal junior et al.[65,66]	12
Ando et al.[13]	11
Zhang et al.[67]	12
Baroni et al.[68]	11
Leal Junior et al.[69]	11
Blanco et al.[70]	12
Disner et al.[71]	12

3.1.2 *In vivo studies*

Mendez et al.[21] compared, histologically, the effect of using two different wavelengths (GaAlAs 830 nm and InGaAl 685 nm) on repair of cutaneous wounds in rats. The control group received no treatment; group II was irradiated with 685 nm, using a fluence of 20 J/cm^2 with a spot diameter of 0.6 mm; group III was irradiated using 830 nm, 20 J/cm^2; group IV was irradiated with both 830 and 685 nm using a total of 20 J/cm^2; group V with 830 nm using 50 J/cm^2; group VI with 685 nm, 50 J/cm^2 and group VII using 830 and 685 nm, 50 J/cm^2. Laser therapy was repeated four times over 7 days at 48 h intervals. They concluded that better results were observed when combining both wavelengths of 830 and 685 nm and attributed this advantage to different absorption and penetration. When comparing the two wavelengths used separately, 830 nm showed better results. While combining the wavelengths provides valuable information, it was not appropriate to include it in the tables of effectiveness.

Barbosa et al.[20] compared the effect of light application on bone healing in rats using red and infrared wavelengths. Forty-five rats were divided into three groups after femoral osteotomy: Gr I was used as control; Gr II was submitted to laser treatment using a red wavelength (660 to 690 nm); and Gr III were treated using an infrared laser (790 to 830 nm). Laser therapy was applied immediately after osteotomy and repeated every 48 h, three times a week, for a total of nine sessions over 21 days. The output power was set at 100 mW, energy 4 J, spot size 0.028 cm^2, power density 3.5 W/cm^2 for 40 s producing a fluence of 140 J/cm^2. Animals were sacrificed, the femurs removed and subjected to optical densitometry analysis after 7, 14, and 21 days (five per group).[20] After 7 days, both laser-treated groups had significantly higher mean bone optical density compared with the control group but no significant difference between the two laser groups was seen. After 14 days, only Gr III treated with infrared energy showed significantly higher bone density than the control group. After 21 days, no significant difference of the mean bone density between the three groups was seen. They concluded that PBM accelerated bone repair in the initial phase and suggested that PBM in bone repair is both timing and wavelength dependent.

Al-Watban and Zhang[16] compared the efficacy of accelerating wound healing in diabetic rats using visible and NIR diode lasers at wavelengths of: 532, 633, 670, 810, and 980 nm. Each wavelength was delivered at doses of 5, 10, 20 and 30 J/cm^2, using the same power density for all the wavelength of 22 mW/cm^2 except for 633 nm (irradiance used: 15.5 mW/cm^2) and 532 nm (10 mW/cm^2). Results showed that there was a significant difference between the NIR and visible wavelengths with visible wavelengths being more effective than NIR. They also concluded that the optimum wavelength was 633 nm and the optimum dose was 10 J/cm^2.

These studies suggest that the relationship between wavelength and fluence is crucial. If the target is CCO, it is well accepted that red light (630 to 670 nm) or near-infrared light (780 to 940 nm) will have positive effects, using fluences in the stimulatory range of 3 to 10 J/cm^2.[16]

However, if the desired chromophore is ion channels within cells, the wavelengths that best affect the calcium channels are in the range of 420 to 540 nm.[54,61] Delivering just 3 J/cm^2 when using 16 mW/cm^2 will have the best effect. Using the higher wavelength of 980 nm may also have a beneficial effect for targeting water as a chromophore.[54]

Disner et al.[71] studied the effect of PBMT delivered to the head (over right prefrontal cortex) combined with attention bias modification (ABM) therapy on 51 human patients with elevated symptom of depression. PBMT was administered before and after blocks of ABM using 1064 nm, 3.4 W, irradiance of 250 mW/cm^2 (3,400 mW/13.6 cm^2 = 250 mW/cm^2) for 4 min and a cumulative fluence of 60 J/cm^2 (0.25 W/cm^2 × 240 s = 60 J/cm^2). They found that PBMT led to greater symptom improvement especially among participants, whose attention span was responsive to ABM, and they concluded that the beneficial effect of ABM could be improved by adjunctive interventions, such as right prefrontal PBMT.

3.2 *II-Effect of Varying Energy Density and Power Density on PBM Efficiency*

3.2.1 *In vitro studies with cells with high number of mitochondria*

Fernandez et al.[46] stimulated the M1 profile (macrophages can have two different phenotypes called M1 and M2 depending on the type of cytokines they produce) of macrophages by using two different sets of laser parameters: 660 nm, 15 mW, 0.375 W/cm^2, 20 s for 7.5 J/cm^2 and 780 nm, 70 mW, 1.75 W/cm^2, 1.5 s for 2.6 J/cm^2 (the spot area calculated by current authors from available information was 0.04 cm^2). Results showed that both lasers were able to decrease TNFα and iNOS expression but parameters used for 780 nm gave an additional decrease. Also, parameters used for 660 nm gave an up-regulation of IL-6 expression and production. They concluded that using 780 nm with high power and low energy density or 660 nm with low power and high energy density achieved similar results and the additional decrease by the parameters used with 780 nm suggest that this wavelength returned the cells to a nonstimulated state.

Lopes-Martins et al.[74] found a true biphasic response occurred in the neutrophils isolated from mice treated with different energy densities (1, 2.5, and 5 J/cm^2) with a maximum effect at 2.5 J/cm^2.

Huang et al.[47] irradiated cortical neuronal cells with a diode laser using 810 nm, 20 mW/cm^2, 3 J/cm^2, spot size of 5 cm, 150 s. They found that laser treatment reduced oxidative stress in primary cortical neurons *in vitro*.

Studies using PBM *in vitro* on cells with high numbers of mitochondria that reported positive results are summarized in Table 3. Ineffective parameters *in vitro* in cells with high numbers of mitochondria are reported in Table 7. In some cases, the same studies are included in both Tables 3 and 7 (effective and ineffective parameters) when the authors varied the parameters.

3.2.2 *In vitro studies with cells with lower numbers of mitochondria*

Tschon et al.[55] irradiated osteoblast–like cells using a 915-nm diode laser at the following parameters: 100 Hz pulsed mode, 50% duty cycle, and output power of 0.575 W. Laser energy was delivered in defocused mode using a concave lens to cover the growth area (1.91 cm^2) at distance of 19 mm (power density calculated by current authors from available information was 150 mW/cm^2). The laser was applied for 48, 96, and 144 s producing doses of 5, 10, and 15 J/cm^2

Table 3 Effective treatment of PBM: *in vitro* studies in cells with higher number of mitochondria.

Authors	Wavelength (nm)	Fluence	Irradiance	Cell type
Fernandes et al.[46]	780	2.6 J/cm^2	1.75 W/cm^2; 70 mW, 0.04 cm^2, 1.5 s	Macrophage
Huang et al.[47]	810	3 J/cm^2	20 mW/cm^2; 150 s, spot size 5 cm	Neural cells
Huang et al.[48]	810	3 J/cm^2	25 mW/cm^2, 2 min, spot size 5 cm	Neural cells
Sharma et al.[49]	810	0.03, 0.3, 3, 10, peak at 3 J/cm^2	25 mW/cm^2	Mouse cortical neuron
Oron et al.[50]	808	0.05 J/cm^2	50 mW/cm^2	Human neural cells
Chen et al.[26]	808	1 J/cm^2	44.7 mW/cm^2 170 mW, 3.8 cm^2, 22.4 s	Monocyte
Souza et al.[51]	780	3 J/cm^2	275 mW/cm^2 [Power = 70 mW, 1.5 s (2×) effective power 53.9 mW] Area = 0.196 cm^2 Beam spot area = 0.04 cm^2	Macrophage
Ferraresi et al.[52]	Cluster 40 LEDs (20 infrared 850 nm and 20 red 630 nm)	2.5 J/cm^2	28 mW/cm^2 50 mW (IR) and 25 mW (red) Cluster: 1000 mW (IR) and 500 mW (red) 45 cm^2, 90 s, distance: 156 mm	Myotube C2C12
Amaroli et al.[19]	808	3.0 J/cm^2	100 mW/cm^2 100 mW spot area: 1 cm^2	Paramecium
Amaroli[19]	808	64 J/cm^2	1000 mW/cm^2 100 mW, spot area = 1 cm^2	Paramecium
Chen et al.[26]	660	1 J/cm^2	0.8 mW/cm^2 6 mW, 7.5 cm^2 1250 s	Monocyte
Chen et al.[26]	660	2 J/cm^2	0.8 mW/cm^2 6 mW, 7.5 cm^2 2500 s	Monocyte
Souza et al.[51]	660	7.5 J/cm^2 effective fluence 1.15 J/cm^2	57.4 mW/cm^2 Power = 15 mW, 20 s Effective power 11.25 mW Irradiated area = 0.196 cm^2 Beam spot area = 0.04 cm^2	Macrophage
Fernandez et al.[46]	660	7.5 J/cm^2	0.375 W/cm^2 15 mW, 0.04 cm^2, 20 s	Macrophage

(energy density calculated by current authors from available information was 7.2, 14.4, and 21.56 mJ/cm^2), and specimens were examined after 4, 24, 48, and 72 h. *In vitro* scratch wounds treated with 5 and 10 J/cm^2 were the first to reach complete coverage after 72 h, followed by 15 J/cm^2, which reached complete healing after 96 h.

Pyo et al.[56] studied the effect of hypoxia and PBM on the expression of bone morphogenetic protein-2 (BMP-2); transforming growth factor-beta-1 (TGF-β1); type I collagen, osteocalcin; hypoxia inducible factor-1 (HIF-1) and AKT. Osteoblast cells were cultured under 1% oxygen tension and then exposed to hypoxia. These cells were then irradiated with an 808 nm diode laser; 1000 mW, continuous wave (CW) for 15 s for a stated energy density of 1.2 J/cm^2 at each session (power density calculated by current authors from available information was 80 mW/cm^2). Other cells were cultured 24 h more under hypoxia and irradiated a second and third time for a total energy density of 1.2, 2.4, and 3.6 J/cm^2. Finally, further hypoxia was applied to the cells after irradiation. Cells were not exposed to laser energy in the control groups and were incubated under hypoxia at 1, 24, and 48 h. Results showed that hypoxia did not affect osteoblast viability (in the control group) and BMP-2, but it resulted in a decrease in osteocalcin, TGF-β, and expression of type I collagen. However, PBM applied to hypoxic osteoblasts stimulated osteoblast differentiation and proliferation through an increased expression of BMP-2, osteocalcin, and TGF-β. In addition, PBM inhibited HIF-1 expression and inhibited production of Akt.

Migliario et al.[57] irradiated murine preosteoblasts (MC-3 T3 –E1) in order to evaluate the effect of PBM on ROS in cells labeled with an ROS marker. They used a diode laser at 930 nm, 1 W, irradiation time of 1, 5, 10, 25, and 50 s, for

a delivered fluence of 1.57, 7.87, 15.74, 39.37, and 78.75 J/cm^2 (spot area calculated by current authors from available information was 0.63 cm^2 and irradiance of 1.57 W/cm^2). The laser application was delivered three times at 0, 24, and 48 h. They found that ROS generation was dose dependent and doubled at higher fluences (25 to 50 J/cm^2). Also, laser irradiation was able to increase preosteoblast proliferation starting from a fluence of 5 J/cm^2. Increasing the fluence produced an increase in cell proliferation up to 25 J/cm^2 and then a decrease at 50 J/cm^2. The peak of cell proliferation occurred at 10 J/cm^2. These results are partially in disagreement with other studies that suggest that 1 to 5 J/cm^2 was optimal for cell proliferation. Contradictory results may be due to differences in irradiation parameters (wavelength, output power, energy density).

Zhang et al.[53] irradiated fibroblast cells with 628 nm. Power output was constant at 15 mW, irradiance 11.46 mW/cm^2, and distance of 0.75 cm. Samples were irradiated for various time periods to yield final energy doses of 0.44, 0.88, 2.00, 4.40, and 9 J/cm^2. They found a maximum increase in human fibroblast cell proliferation with a fluence of 0.88 J/cm^2 and a reduction in the proliferation at 9 J/cm^2.

Khadra et al.[75] investigated the effect of single and multiple doses on attachment and proliferation of human fibroblasts. Cells were cultured on titanium implants and divided into three groups: group I was used as a control, group II received GaAlAs 830 nm, output power 84 mW, 9 cm distance to the cells, a single dose of 3 J/cm^2, 360 s (spot area calculated by current authors from available information was 10 cm^2 and irradiance of 0.0084 W/cm^2), group III was divided into three subgroups and exposed to multiple doses (one dose on each of three consecutive days) of 0.75, 1.5, and 3 J/cm^2 corresponding to exposure times of 90, 180, and 360 s (spot area calculated by current authors from available information was 10 cm^2). Results indicated that samples exposed to multiple doses of 1.5 and 3 J/cm^2 showed a significantly proliferation. They concluded that the attachment of human fibroblasts to the titanium implant was enhanced by PBM. Both multiple and single doses significantly increased cellular attachment. Finally, 0.75 J/cm^2 did not promote proliferation and cell attachment.

Skopin and Molitor[58] studied the effect of using different doses and different irradiances on wound healing in fibroblast cultures using 980-nm diode laser. They applied an irradiance of: 26, 49, 73, 97, and 120 mW/cm^2 for a constant 2 min each, delivering 3.1, 5.9, 8.8, 11.6, and 14.4 J/cm^2. They found a significant increase in cell division when using 26, 73, and 97 mW/cm^2. This effect was negated at 120 mW/cm^2.

Al-Watban and Andres[76] studied the effect of He–Ne laser on the proliferation of hamster ovary and human fibroblasts. Irradiance was held constant at 1.25 mW/cm^2 using an accumulated dose over three consecutive days of 60 to 600 mJ/cm^2. They found a peak response at 180 mJ/cm^2. This study suggested that there is activation at a lower dose from 2 mJ/cm^2 with a peak at 180 mJ/cm^2. At higher doses, greater than 300 mJ/cm^2, there was bioinhibition.

Studies using PBM *in vitro* on cells with low numbers of mitochondria that reported positive results are summarized in Table 4. Ineffective parameters *in vitro* in cells with low numbers of mitochondria are reported in Table 8. In some cases, the same studies are included in both Tables 3 and 7 (effective and ineffective parameters) when the authors varied the parameters.

3.2.3 *In vivo studies in tissues with high number of mitochondria: heart, brain, muscle, inflammation*

Oron et al.[62] treated myocardial infarction with LLLT using an 810-nm laser. Fluence was held constant at 0.9 J/cm^2 while irradiance was varied to deliver 2.5, 5, and 25 mW/cm^2. A peak response was found at 5 mW/cm^2, while treatment was less effective when using 2.5 and 25 mW/cm^2.

Castano et al.[17] studied inflammatory arthritis in rats, comparing the effect of using high and low fluences (3 to 30 J/cm^2) delivered at high and low irradiance (5 to 50 mW/cm^2). Effective treatment was observed when using: 30 J/cm^2 at 50 mW/cm^2 for 10 min and 30 J/cm^2 at 5 mW/cm^2 for 100 min. Low fluence of 3 J/cm^2 at 5 mW/cm^2 for 10 min was also effective. Only the dose of 3 J/cm^2 at 50 mW/cm^2 for 1 min was ineffective. They concluded that at higher fluence (30 J/cm^2), the PBM effect on arthritis did not depend on irradiance as both high and low irradiance were effective, while at a lower fluence of 3 J/cm^2, only the lower irradiance was effective. Therefore, they concluded that the duration of the light exposure was of great importance. While some studies found (3 J/cm^2, 50 mW/cm^2) beneficial, this study did not. They suggest that because the duration was only 1 min, the light did not have sufficient time to produce a sufficient activation of cellular metabolism.[17]

Salehpour et al.[77] compared the therapeutic effect of a 10-Hz pulsed wave of NIR (810 nm) and red (630 nm) lasers with citalopram in rats that had been subjected to a model of chronic mild stress that causes depression. After inducing stress in rats, they were divided into: group I receiving PBM using NIR 810 nm and group II receiving 630-nm coherent light using identical parameters of: 10-Hz gated wave (50% duty cycle), fluence of 1.2 J/cm^2 per session, output power 35 and 240 mW, respectively, 2 ms duration for both type of lasers, beam diameter of 3 mm, contact mode, and spot size of 0.07 cm^2. Laser power was set at 6.2 W in the red wavelength and 39.3 W in the infrared wavelength for an irradiance of 89 and 562 mW/cm^2, respectively. The average fluence for each session was 1.2 J/cm^2 and totaling 14.4 J/cm^2 for the entire 12 session treatment. Finally, group III was treated with the antidepressant drug citalopram that works by decreasing cortisol levels. Results showed that PBM using 10-Hz pulsed NIR laser had a better effect than red laser and the same effect as citalopram.

Salehpour et al.[59] studied brain mitochondrial function in mice after inducing mitochondrial dysfunction by administration of D-galactose. This model is considered to be a model of age-related cognitive dysfunction. Animals were treated with wavelengths of 660 and 810 nm at two different fluences: 4 and 8 J/cm^2, 10 Hz, 4.75 W/cm^2, 88% duty cycle, 200 mW, in contact, three times a week, 48 h between sessions, and 7-mm diameter power meter sensor. They found poor results with both wavelengths at 4 J/cm^2 and an amelioration of the aging-induced mitochondrial dysfunction with 8 J/cm^2

Wu et al.[78] induced traumatic brain injury (TBI) in mice and treated the animals using 660, 730, 810, or 980 nm, single dose treatment of 36 J/cm^2 using an irradiance of 15 mW/cm^2, 4-min duration, 4 h after injury. They found a significant improvement for mice having moderate to severe injury only when using 660 nm and 810 nm. The most desirable effect was seen at 810 nm, and both 730 and 980 nm did not produce any benefit.

Table 4 Effective treatment of PBM: *in vitro* studies in cells with lower number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Cell type
Wang et al.[54]	420	3	16 mW/cm^2 4 cm^2, 188 s	Adipose stem cells
Wang et al.[54]	540	3	16 mW/cm^2 4 cm^2, 188 s	Adipose stem cells
Zhang et al.[53]	628	0.88	11.46 mW/cm^2 Output power 15 mW, 0.76 cm distance to the surface, area = 9.6 cm^2	Fibroblast
Zhang et al.[53]	628	2.0	11.46 mW/cm^2 Output power 15 mW, 0.76 cm distance to the surface, area = 9.6 cm^2	Fibroblast
Zhang et al.[53]	628	4.4	11.46 mW/cm^2 Output power 15 mW, 0.76 cm distance to the surface, area = 9.6 cm^2	Fibroblast
Khadra et al.[32]	830	1.5	8.4 mW/cm^2 84 mW, 10 cm^2, 9 cm distance to cells	Fibroblast
Khadra et al.[32]	830	3.0	8.4 mW/cm^2 84 mW, 10 cm^2, 360 s, 9 cm distance to cells	Fibroblast
Tschon et al.[55]	915	7.2	150 mW/cm^2, 100 Hz, 50% duty cycle, power 0.575 W, 48 s	Osteoblast
Tschon et al.[55]	915	14.4	150 mW/cm^2 50% duty cycle, power 0.575 W, 96 s	Osteoblast
Migliario et al.[57]	930	7.8	1580 mW/cm^2 1 W, 5 s, 0.63 cm^2	Preosteoblast
Migliario et al.[57]	930	15	1580 mW/cm^2 1 W, 10 s, 0.63 cm^2	Preosteoblast
Migliario et al.[57]	930	39	1580 mW/cm^2 1 W, 25 s, 0.63 cm^2	Preosteoblast
Pyo et al.[56]	808	1.2	80 mW/cm^2 15 s, 1 W	Osteoblast
Skopin et al.[58]	980	3.1	26.7 mW/cm^2	Fibroblast
Skopin et al.[58]	980	8.8	73 mW/cm^2	Fibroblast
Skopin et al.[58]	980	11.6	97 mW/cm^2	Fibroblast
Bozkurt et al.[60]	940	18	0.3 W/cm^2 0.3 W, 60 s, distance: 0.5 to 1 mm	Cementoblast
Wang et al.[73]	810	3	16 mW/cm^2 4 cm^2, 188 s	Adipose stem cells
Wang et al.[61]	980	0.3	16 mW/cm^2 4 cm^2, 18.8 s	Adipose stem cells

Lopes-Martins et al.[18] investigated the effect of PBM on muscular fatigue in rats during tetanic contractions. Four groups of 32 rats received different doses of PBMT (0.5, 1.0, and 2.5 J/cm^2), using parameters of 655 nm, spot area 0.08 cm^2, 25 mW, 2.5 mW; 31.25 mW/cm^2. Groups: 0.5 J/cm^2 (32 s), 1 J/cm^2 (80 s), 2.5 J/cm^2 (160 s). Only the groups of 0.5 and 1 J/cm^2 prevented the development of muscular fatigue in rats during repeated tetanic contractions.

Lopes-Martins et al.[74] in another study used 650-nm wavelength on acute inflammatory pleurisy in mice. Using the same power of 2.5 mW but different fluences of 3, 7.5, and 15 J/cm^2. They found that under these conditions, 7.5 J/cm^2 were more effective than either 3 or 15 J/cm^2.

De Almeida et al.[79] studied muscle performance after inducing muscle contraction in 30 rats. Using 904 nm, 15-mW average power and different energies (0.1, 0.3, 1.0, and 3.0 J) they

Table 5 Effective PBM treatment: *in vivo* on tissues with higher number of mitochondria.

Authors	Wavelength (nm)	Fluence	Irradiance	Tissue type
Alves et al.[25]	808	142.4 J/cm^2	1.78 W/cm^2 4 J, 50 mW, 0.028 cm^2, 80 s per point	Arthritis
Oron et al.[62,63]	810	0.3 J/cm^2	5 mW/cm^2 5 mW, area 1.1 cm^2, 60 s	Heart
Oron et al.[62,63]	810	0.9 J/cm^2	5 mW/cm^2	Myocardium tissue
Castano et al.[17]	810	30 J/cm^2	50 mW/cm^2	Arthritis
Castano et al.[17]	810	30 J/cm^2	5 mW/cm^2	Arthritis
Castano et al.[17]	810	3 J/cm^2	5 mW/cm^2	Arthritis
Salehpour et al.[59]	810	1.2 J/cm^2	560 mW/cm^2 39.3 W, spot size 0.07 cm^2	Brain
Salehpour et al.[64]	810	8 J/cm^2	89 mW/cm^2 6.2 W, spot size 0.07 cm^2	Brain
Wu et al.[78]	810	36 J/cm^2	15 mW/cm^2	Brain
Blanco et al.[70]	1064	250 mW/cm^2	60 J/cm^2	Brain (human)
Disner et al.[71]	1064	250 mW/cm^2	60 J/cm^2	Brain (human)
Ando et al.[13]	810	36 J/cm^2	50 mW/cm^2	TBI
Zhang et al.[67]	810	Fluence reaching the cortex 1.8 to 2.5 J/cm^2 Average irradiance 36 J/cm^2	150 mW/cm^2 Pulse freq 10 Hz, pulse duration 50 ms, 4 min	TBI
Salehpour et al.[68]	810	1.2 J/cm^2	89 and 562 mW/cm^2 35 and 240 mW 10 Hz, 50% duty cycle; 0.07 cm^2	Brain
Baroni et al.[68]	Cluster with 69 LEDs 660/850 nm	206.89 J/cm^2	6.89 W/cm^2 200 mW; 6 J per diode (30 s); 0.02 cm^2 30 J per application point $(5 \times 6$ J)6 application points: total energy 180 J	Femoral quadriceps
Zhang et al.[80]	635	0.96 J/cm^2	6.37 mW/cm^2 5 mW, laser beam width 10 mm, 150 s	Preconditioning myocardium
Salehpour et al.[59]	660	8 J/cm^2	4.75 W/cm^2 88% duty cycle, 200 mW, in contact three times a week, 7 mm diameter	Brain
Wu et al.[78]	660	36 J/cm^2	15 mW/cm^2	Brain
Lopes-Martins et al.[18]	655	0.5 J/cm^2	31.25 mW/cm^2 2.5 mW, spot area 0.08 cm^2, 25 mW, 32 s	Muscle
Lopes-Martins et al.[18]	655	1 J/cm^2	31.25 mW/cm^2 2.5 mW, spot area 0.08 cm^2, 25 mW, 80 s, 2.5 mW	Muscle

found that the 1.0 and 3.0 J groups showed significant enhancement ($P < 0.01$) in total work. They conclude that 1.0 J decreased postexercise muscle damage and enhanced muscle performance.

Studies using PBM *in vivo* in tissues with high numbers of mitochondria that reported positive results are summarized in Table 5. Ineffective parameters PBM *in vivo* in tissues with high numbers of mitochondria are reported in Table 9. In some cases, the same studies are included in both Tables 5 and 9 (effective and ineffective parameters) when the authors varied the parameters.

3.2.4 *In vivo studies in tissues with a lower number of mitochondria: skin, bone, cartilage*

Lanzafame et al.[15] treated pressure ulcers in mice with a 670-nm diode laser. Maintaining a constant fluence of 5 J/cm^2 and

Table 6 Effective PBM treatment: *in vivo* on tissues with lower number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Tissue type
Mendez et al.[21]	830	50	125 mW/cm^2 35 mW 0.6 cm diameter	Wound healing
Lanzarfane et al.[15]	670	5	8 mw/cm^2	Ulcer
Prabhu et al.[21]	632	2	4.0 mW/cm^2 7 mw, 1.75 cm^2	Wound healing
Gal et al.[21]	670	5	15 mw/cm^2	Wound tensile strength
Al-Watban et al.[21]	670	1 and 5	130 mW/cm^2 200 mW, 1.534 cm^2	Wound healing
Mendez et al.[21]	830	20	125 mW/cm^2 35 mW, 0.6 cm diameter	Wound healing
Barbosa et al.[20]	790	140	3500 mW/cm^2 100 mW, 4 J, spot size 0.028 cm^2	Bone
Barbosa et al.[20]	830	140	3500 mW/cm^2 100 mW, 4 J, spot size 0.028 cm^2	Bone

using different irradiances (0.7, 2, 8, 40 mW/cm^2), they found a significant improvement at 8 mW/cm^2.

Prabhu et al.[81] found a biphasic dose response on excisional wound healing in mice when using a He–Ne laser (632 nm, 7 mW, 4 mW/cm^2 at different fluences (1, 2, 3, 4, 6, 8, and 10 J/cm^2). A clear biphasic dose response occurred with a peak benefit at a fluence of 2 J/cm^2 and an inhibitory effect at the higher dose of 10 J/cm^2.

Gal et al.[82] compared the wound tensile strength in rats at different power densities using 670 nm. A positive effect was seen when using 4 mW/cm^2 delivered for 20 min, 50 s, (5 J/cm^2), but this effect was not seen when using 15 mW/cm^2 delivered for 5 min, 33 s, (5 J/cm^2) at the same wavelength. This suggests that delivering the same fluence at a lower irradiance over more time was more effective.

Al-Watban and Delgado[83] studied, *in vivo*, the effect of laser irradiation on burn wound healing in rats. They created a superficial burn with an area of 1.534 cm^2 and irradiated the wound with a diode laser at 670 nm, 200 mW, three times per week for 12 weeks at different doses of 1, 5, 9, and 19 J/cm^2. Only the groups receiving the lower doses of 1 and 5 J/cm^2 showed significantly better wound healing compared to the control, with the greatest effect obtained at 1 J/cm^2.

Studies using PBM *in vivo* in tissues with low numbers of mitochondria that reported positive results are summarized in Table 6. Ineffective parameters PBM *in vivo* in tissues with low numbers of mitochondria are reported in Table 10. In some cases, the same studies are included in both Tables 6 and 10 (effective and ineffective parameters) when the authors varied the parameters.

3.3 *III-Effect of Varying the Mode of Delivery on PBM Efficiency: CW or Pulsed*

In a comprehensive literature review,[84] Hamblin included 33 studies, nine of them directly comparing pulsed wave and CW. Six of these studies found that pulsed wave offered better results than CW; one study found that both modes were equally effective and only two studies reported better result using CW. Hamblin et al. concluded from this review that pulsed light may be superior to CW light, particularly for wound healing and poststroke management, whereas CW may be more beneficial in patients requiring nerve regeneration. In addition, they concluded that it is impossible to draw any correlation between pulse frequency and pathological condition. They found that no particular frequency appears to be more or less effective than others. Finally, this review reported that the following frequencies were beneficial: 2, 10, 25, 50 Hz when using (670 nm, 20 mW, energy density, 2 J/cm^2), 100 Hz when using (808 nm, 37.5 mW/cm^2, 0.9 J/cm^2) 292 Hz when using (800 mW/cm^2; 21.6 J/cm^2), 600 Hz when using (670 nm, 10 mW, 5 J/cm^2), 1000 Hz when using (808 nm, 7.5 mW/cm^2, 0.9–1.2 J, duty cycle, 30%), 1500 Hz when using (5 mW/cm^2); 3000 Hz when using (10 mW/cm^2) and 8000 Hz (N/A).

Gigo-Benato et al.[14] compared the effect of combined CW and pulsed laser (CW+PW) using 808 nm (CW) and 905 nm (PW) to either the CW (808 nm) or PW (905 nm) laser used separately. CW was applied at 29 J/cm^2 while the pulsed wave laser was applied at 40 J/cm^2. Results suggested that the combined laser was more effective in nerve regeneration than the CW alone or the PW alone.

Al-Watban and Zhang[16] evaluated the effects of using both pulsed and CW PBM in rats wound healing. After creation of elliptical wounds, animals were treated with a 635-nm diode laser, average power of 3.4 mW, spot size of 3.8 cm^2, wound size of 1.04 cm^2, irradiance of 0.89 mW/cm^2, treatment duration 18.7 min and fluence of 1 J/cm^2, three times per week. The dose was delivered using either CW or pulsed mode at: 100, 200, 300, 400, or 500 Hz. They found that the effect of using CW was more efficient than using pulsed laser and, when comparing different frequencies, 100 Hz had better effect on wound healing than the other frequencies.

This article contradicts Hamblin, who concluded that pulsed mode was more effective than CW in wound healing. Perhaps, Al-Watban found that CW was more efficient because he did not

Table 7 Ineffective treatment of PBM: *in vitro* studies in cells with higher number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Cell type
Sharma et al.[49]	810	30	25 mW/cm^2	Mouse cortical neurons
Chen et al.[26]	660	3	0.8 mW/cm^2 6 mW, 7.5 cm^2 3750 s	Monocyte
Chen et al.[26]	660	2	0.8 mW/cm^2 6 mW, 7.5 cm^2 2500 s	Monocyte
Amaroli et al.[19]	808	3.0	1000 mW/cm^2, 1 W, 1 cm^2 spot area	Paramecium
Amaroli et al.[19]	808	64	100 mW/cm^2, 1 W, 1 cm^2 spot area	Paramecium

use the same fluence in CW that he used in pulsed mode. Moreover, he used gated CW rather than true pulsed wave.[16]

Ando et al.[13] treated TBI in mice comparing pulsed and CW 810-nm laser irradiation. The parameters used were: 810-nm diode laser, irradiance of 50 mW/cm^2, spot diameter of 1 cm onto the injured head with a 12-min exposure giving a fluence of 36 J/cm^2. They found that 10 Hz produced better results than 100 Hz or continuous mode.

el Sayed and Dyson[85] compared the effect of four different frequencies (2.5, 20, 292 and 20,000 Hz) and found that only 20 and 292 Hz were beneficial.

Sushko et al.[86] investigated pain induced in mice by hypodermic injection of 20 ml of 5% formalin solution into the footpad. They irradiated the mice using 640 and 880 nm LED in continuous or pulsed mode for 10 min. They found that pulsed mode was more effective than CW and frequencies of 10 and 8000 Hz were most effective, whereas pulse repetition rates of 200 and 600 Hz were less effective.

Ueda and Shimizu[87] studied the effect of three different pulse repetition rates on osteoblast-like cells from rats using these parameters (830 nm, 500 mW, 0.48 to 3.84 J/cm^2) in CW mode and (1, 2, and 8 Hz) in pulsed mode. They found that 1 and 2 Hz markedly stimulated cellular proliferation, ALP activity, ALP gene expression, and bone nodule formation, and that 2 Hz was the best pulse repetition rate to stimulate bone nodule formation.

4 Review of Which Parameters Lead to Effective and Ineffective PBMT

It is difficult to compare studies done with different parameters, protocols, treatment objectives, and biological target tissues. Often, parameters are not completely presented or are of questionable accuracy. In this part of the review analysis, an attempt is made to draw at least some general inferences from the data presented in Tables 3–10.

Table 8 Ineffective treatment of PBM *in vitro* studies in cells with lower number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Cell type
Tschon et al.[55]	915	20.56	150 mW/cm^2 100 Hz, 50% duty cycle, power 0.575 W 144 s	Osteoblast
Migliario et al.[57]	930	1.57	1580 mW/cm^2 1 W,1 s, 0.63 cm^2	Preosteoblast
Migliario et al.[57]	930	78.7	1580 mW/cm^2 1 W, 50 s, 0.63 cm^2	Preosteoblast
Skopin et al.[58]	980	5.9	49 mW/cm^2	Fibroblast
Skopin et al.[58]	980	14.4	120 mW/cm^2	Fibroblast
Zhang et al.[53]	628	9.0	11.4 mW/cm^2 15 mW, distance of 0.75 cm	Fibroblast
Khadra et al.[75]	830	0.75	8.4 mW/cm^2 84 mW, 10 cm^2, 360 s, 9 cm distance to cells	Fibroblast
Wang et al.[73]	980	20	16 mW/cm^2 4 cm^2, 1 W	Adipose stem cells

Table 9 Ineffective PBM treatment *in vivo* on tissues with higher number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Tissue type
Oron et al.[62,63]	810	0.3	2.5 mW/cm^2 5 mW, area of irradiation of 1.1 cm^2	Heart
Oron et al.[62,63]	810	0.3	25 mW/cm^2 5 mW, area of irradiation of 1.1 cm^2	Heart
Salehpour et al.[59]	660	4	4.75 W/cm^2 10 Hz, 4.75 W/cm^2, 88% duty cycle, 200 mW	Brain
Salehpour et al.[59]	810	4	4.75 W/cm^2 10 Hz, 4.75 W/cm^2, 88% duty cycle, 200 mW	Brain
Wu et al.[78]	980	36	15 mW/cm^2	Brain
Alves et al.[25]	808	142.4	3.57 W/cm^2 4 J, 50 mW, 0.028 cm^2, 80 s per point	Arthritis
Lopes-Martins et al.[18]	655	2.5	31.25 mW/cm^2 2.5 mW, spot area 0.08 cm^2, 25 mW, 160 s, 2.5 mW	Muscle

Table 10 Ineffective PBM treatment *in vivo* on tissues with lower number of mitochondria.

Authors	Wavelength (nm)	Fluence (J/cm^2)	Irradiance	Tissue type
Lanzafame et al.[15]	670	5.0	0.7 mW/cm^2	Ulcers (wound healing)
Lanzafame et al.[15]	670	5.0	2.0 mW/cm^2	Ulcers (wound healing)
Gal et al.[82]	670	5.0	15 mW/cm^2	Wound healing
Lanzafame et al.[15]	670	5.0	40 mW/cm^2	Wound healing
Prabhu et al.[81]	632	10	4.0 mW/cm^2 7 mw, 1.75 cm^2	Wound healing
Al-Watban et al.[83]	670	9.0	130 mW/cm^2 200 mW, 1.534 cm^2	Wound healing
Al-Watban et al.[83]	670	19	130 mW/cm^2 200 mW, 1.534 cm^2	Wound healing
Kilik et al.[88]	636	5	1 mW/cm^2 Probe to wound 10 cm	Wound healing

4.1 *Wavelength*

Wavelength affects tissue penetration. Shorter wavelengths (600 to 700 nm) are considered best to treat superficial tissue, whereas longer wavelengths (780 to 950 nm) are preferred to treat deeper tissues. Red wavelengths penetrate 0.5 to 1 mm and near-infrared energy penetrates 2 mm before losing 37% of its intensity.[89–91]

The infrared wavelengths show better effects on bone repair compared to red wavelengths because red light has less capacity to penetrate compared to the infrared laser.

According to Karu,[8] wavelengths between 700 and 770 nm do not have any significant activity. Wu et al.[78] used a 730-nm laser on TBI in mice and found it to be ineffective while 660 and 810 nm lasers were effective. Gupta et al.[92] carried out a similar comparison on wound healing in mice and again found that 660- and 810-nm lasers were effective, while a 730-nm laser was not effective.

Barbosa et al.[20] concluded that the PBM effects of NIR were effective for more than 14 days, whereas the effects of red wavelength are lost after 14 days.

The combination of two wavelengths gives an additional effect of PBM. When comparing 830 and 685 nm, Mendez et al.[21] found that 830 nm offered better results. Much work still remains to define the optimal wavelengths. Nevertheless, NIR wavelengths are preferable for deep tissues and targets within the body, which require substantial doses of light.

4.2 *Laser Versus Noncoherent Light*

Both coherent lasers and noncoherent LEDs are used in PBMT. Laser beams are collimated and the light is more likely to be forward scattered within the tissue than noncollimated LED light.[5] This means that the penetration depth is likely to be deeper with lasers provided all the other characteristics are

identical. Moreover, lasers emit coherent light, while LED light is noncoherent. The coherence length is higher for smaller bandwidths. For instance, gas lasers such as He–Ne laser have very long coherence lengths. Diode lasers have somewhat greater bandwidths and consequently shorter coherence lengths. When coherent laser light interacts with tissue, small imperfections in the tissue structure lead to different phases occurring in the individual wavefronts leading to mutual interference patterns. These interference patterns are called "laser speckles" and the size of the speckles is related to the light wavelength. In the visible range, the sizes are less than 1 μm. Subcellular organelles (such as mitochondria) have dimensions of this order and a theory proposes that the laser speckles are better to stimulate mitochondria than noncoherent LED light.[93–95] A recent review concluded that there were no substantial differences between lasers and LEDs for PBM applications provided all the other light parameters were equal.[96]

4.3 *Fluence and Irradiance*

The photon intensity i.e., irradiance (W/cm^2 or spectral irradiance), must be adequate. Using higher intensity, the photon energy will be transformed to excessive heat in the target tissue and, using lower intensity, photons absorption will be insufficient to achieve the goal.

The dose also must be adequate (J/cm^2). Using low irradiance and prolonging the irradiation time to achieve the ideal fluence or dose will not give an adequate final result. The Bunsen–Roscoe law of reciprocity, termed the second law of photobiology,[97] does not hold true for low incident power densities.

There is no fixed value of dose or fluence that always produces a positive PBM effect. Even within different studies on the same animal models, there can be contradictory findings. For instance, three papers looked at peri-implant bone regeneration after PBM. Menezes et al.[98] found that 20 J/cm^2 was the best dose to deliver, whereas Massotti et al.[22] and Mayer et al.[23] found that 20 J/cm^2 was the worst dose to deliver.

The optimal doses are directly related to different factors:

- Wavelength
- Type of treatment being delivered: pain relief, wound healing, or tissue regeneration
- Power density or irradiance
- Energy density or fluence
- Depth of the target tissue being treated
- Spot size of the beam reaching the tissue surface and the actual target tissue.

In an attempt to determine whether the delivered fluence (J/cm^2) was more or less important than the irradiance (mW/cm^2), we constructed scatter plots (Figs. 1–4) of both the effective and ineffective studies arranged according to our categorization of the studies in Tables 2–9.

4.3.1 *In vitro studies*

Figure 1(a) shows the plot of *in vitro* studies in cells with higher numbers of mitochondria, whereas Fig. 1(b) shows the corresponding plot for cells with lower numbers of mitochondria. The following observations can be made. In all the effective studies, the fluence was relatively low (<7.5 J/cm^2) and in several cases, less than 1 J/cm^2. However, in the ineffective

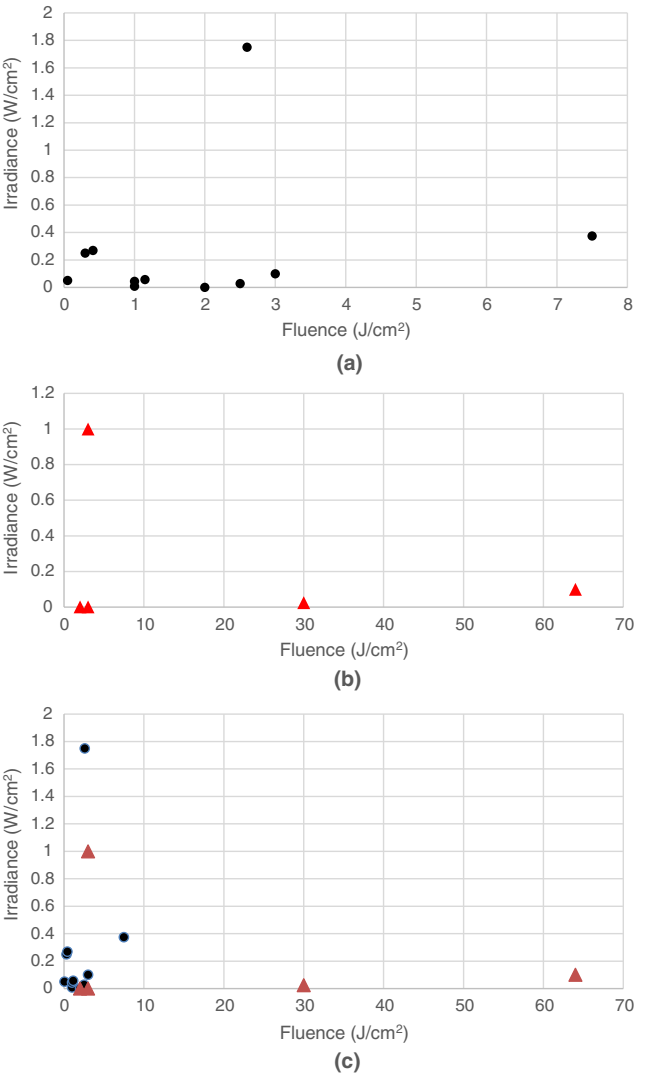

Fig. 1 Studies on PBM of cells *in vitro* with higher numbers of mitochondria. (a) Effective (positive studies), (b) ineffective (negative studies), and (c) combination of effective (positive studies), and ineffective (negative studies).

studies, the fluence values were larger (all >3 J/cm^2), and in two cases, very large values (30 and 65 J/cm^2). There were more studies in the effective group (11) than in the ineffective group (5). This suggests that high-mitochondrial cells respond well to PBM and that ineffective studies are more likely to be due to over-dosing than to under-dosing.

Figure 2(a) shows the effective *in vitro* studies in cells with lower mitochondrial numbers. Again, the positive studies outweigh the negative studies [Fig. 2(b)] (15 to 8). The fluence values in the positive studies in the lower mitochondrial number subgroup appeared to be overall higher than the fluences used in the positive studies in the higher mitochondrial number subgroup. The fluences used in the negative studies in the lower mitochondrial number subgroup were only a little higher than those in the positive studies, suggesting that over-dosing was not such a big problem as it was in the higher mitochondrial number subgroup [Fig. 1(b)]. There were three positive studies that used relatively high irradiances (>1.5 W/cm^2), as opposed to only one study in the positive high-mitochondrial subgroup.

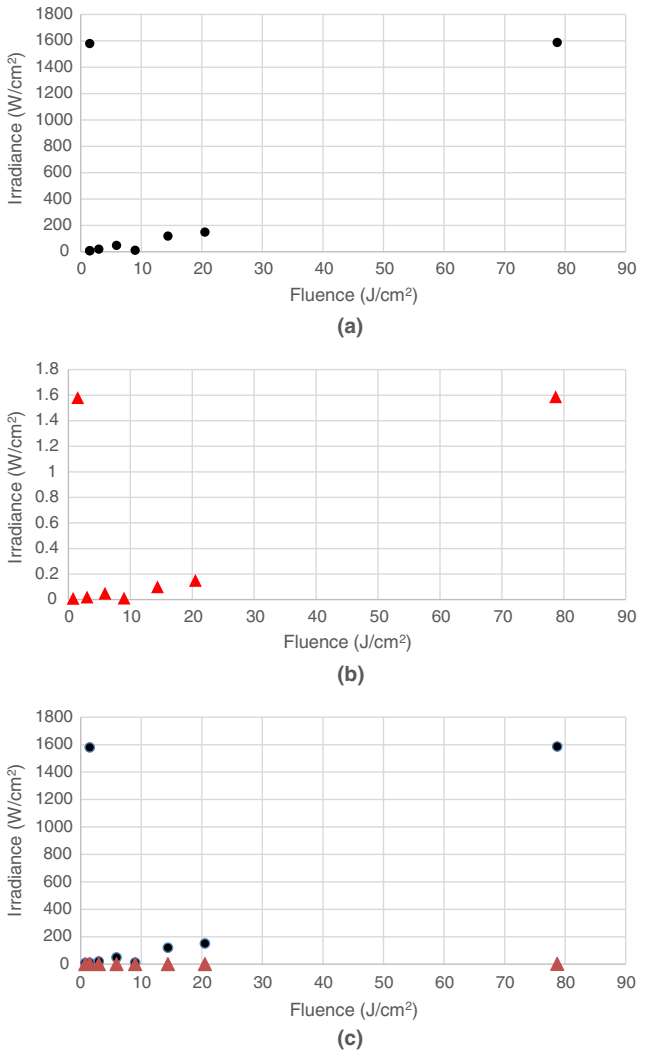

Fig. 2 Studies on PBM of cells *in vitro* with lower numbers of mitochondria. (a) Effective (positive studies), (b) ineffective (negative studies), and (c) combination of effective (positive studies) and ineffective (negative studies).

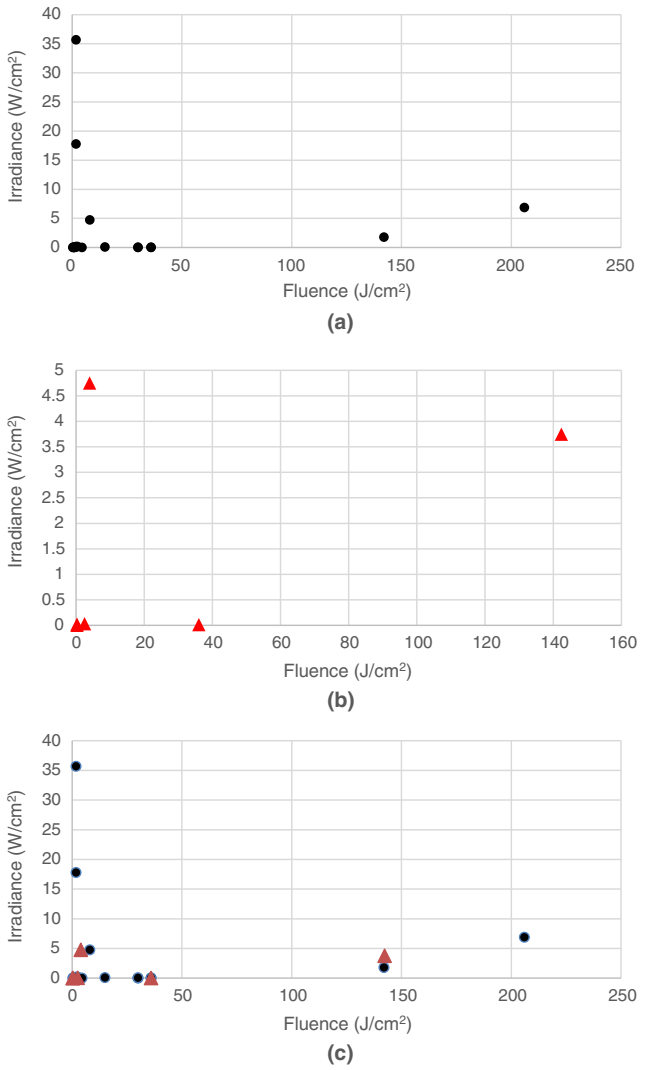

Fig. 3 Studies on PBM of tissues *in vivo* with higher numbers of mitochondria. (a) Effective (positive studies), (b) ineffective (negative studies), and (c) combination of effective (positive studies) and ineffective (negative studies).

4.3.2 *In vivo studies*

Figure 3(a) shows the plot of effective or positive studies *in vivo* on tissues composed of cells with higher numbers of mitochondria, whereas Fig. 3(b) shows the corresponding plot for ineffective or negative studies on tissues composed of higher mitochondrial number cells. Here, a difference is seen when comparing the two plots and with the analogous two plots from the *in vitro* studies. In the *in vivo* case, the fluence values in the effective studies subgroup [Fig. 3(a)] are higher than those in the ineffective studies subgroup [Fig. 3(b)]. This is the opposite of what was found in the *in vitro* case with cultured cells [compare Figs. 1(a) with 1(b)]. Hence, these observations tend to suggest that failure, *in vivo*, could be due to under-dosing while failure, *in vitro*, could equally well be due to over-dosing. *In vivo*, the depth of the tissue is important, while cells, *in vitro* culture, are generally a single monolayer. It is a fact that tissues with higher numbers of mitochondria (brain, heart, muscles, inflammatory cells) tend to be deeper within the body than tissues with lower numbers of mitochondria (skin, tendons, cartilage). There are, of course, some exceptions (bones and bone marrow), which have lower numbers of mitochondria but are still deep within the body.

Figure 4(a) shows the plot of effective treatment in tissue with a lower number of mitochondria, whereas Fig. 4(b) shows the plot of ineffective treatment on tissue with a lower number of mitochondria.

The following observation can be made:

The fluence values used in the positive studies are much higher than those in the negative studies, particularly when the tissue is deeper (such as bone). In addition, some studies used very low fluences of less than 1 J/cm^2 to treat superficial tissue (wound healing) and had positive results.

Fluences used in the negative studies are generally less than 10 J/cm^2, most of them used low irradiance. There are three studies that use lower fluence in combination with higher irradiance and produced positive results.

This would suggest that ineffective studies for tissue with lower mitochondria are more likely to be due to under-dosing rather than over-dosing. Fluence and irradiance are both important in determining the success of *in vivo* studies.

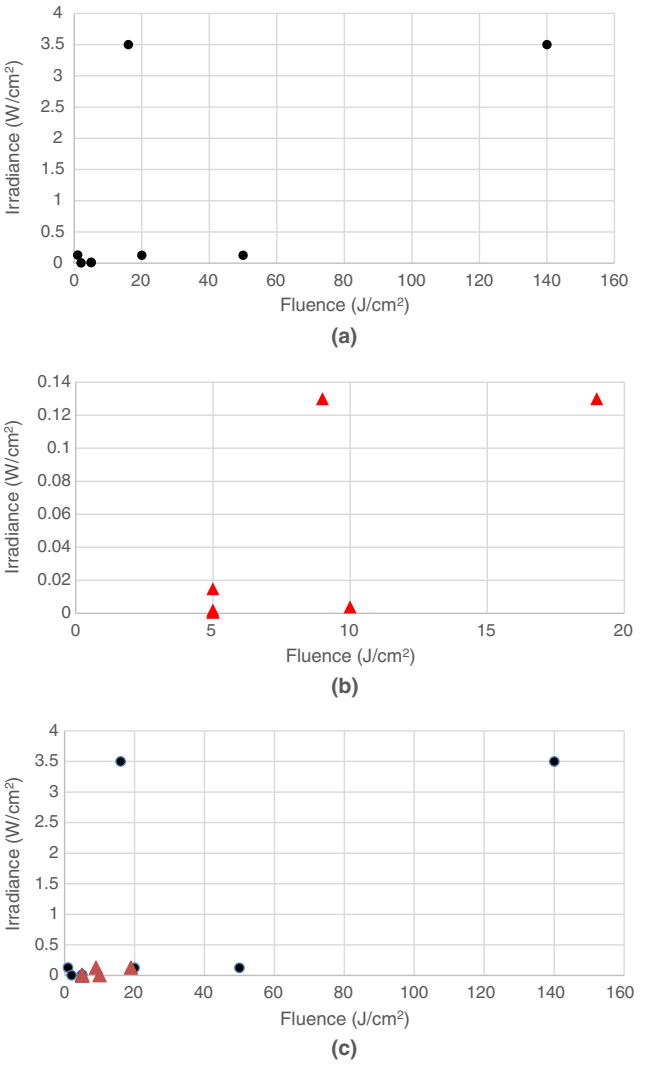

Fig. 4 Studies on PBM of tissues *in vivo* with lower numbers of mitochondria. (a) Effective (positive studies), (b) ineffective (negative studies), and (c) combination of effective (positive studies) and ineffective (negative studies).

5 Conclusions

The limitation of this analysis was the relatively small number of studies that passed our inclusion and exclusion criteria. Nevertheless, some tentative conclusions can be drawn from the analysis that we can at least propose for other researchers to confirm or refute, as more well-documented studies continue to be published in the coming years.

1. Cells with higher numbers of mitochondria respond better to PBM than cells with lower numbers of mitochondria.
2. Ineffective studies on cells with higher numbers of mitochondria are as likely to be due to over-dosing as they are to under-dosing.
3. It is less likely that ineffective studies in cells with lower numbers of mitochondria will be due to over-dosing.
4. The fluence delivered is more important in determining the success or failure of an *in vitro* study than the irradiance employed.
5. Tissues with higher numbers of mitochondria tend to be deeper within the body than tissues with lower numbers of mitochondria, therefore, over-dosing is less likely.
6. Ineffective studies *in vivo* are more likely to be due to under-dosing regardless of the number of mitochondria.

Disclosures

M.R.H. is on the Scientific Advisory Boards of the following companies: Transdermal Cap Inc., Cleveland, Ohio; Photothera Inc., Carlsbad, California; BeWell Global Inc., Wan Chai, Hong Kong; Hologenix Inc., Santa Monica, California; LumiThera Inc., Poulsbo, Washington; Vielight, Toronto, Canada; Bright Photomedicine, Sao Paulo, Brazil; Quantum Dynamics LLC, Cambridge, Massachusetts; Global Photon Inc., Bee Cave, Texas, Medical Coherence, Boston, Massachusetts; NeuroThera, Newark DE JOOVV Inc., Minneapolis-St. Paul, Minnesota; Illumiheal & Petthera, Shoreline, Washington; MB Lasertherapy, Houston, Texas and has consulted for: USHIO Corp,. Japan; Merck KGaA, Darmstadt, Germany; Philips Electronics Nederland B.V.; Johnson & Johnson Inc., Philadelphia, Pennsylvania; UVLRx Therapeutics, Oldsmar, Florida; Ultralux UV Inc., Lansing MI; AIRx Medical, Pleasanton, California; FIR Industries, Inc., Ramsey, New Jersey.

Acknowledgments

M.R.H. was supported by US NIH Grant Nos. R01AI050875 and R21AI121700.

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HHS Public Access

Author manuscript

MOJ Orthop Rheumatol. Author manuscript; available in PMC 2016 June 09.

Published in final edited form as:

MOJ Orthop Rheumatol. 2015 ; 2(5): . doi:10.15406/mojor.2015.02.00068.

The Use of Low Level Laser Therapy (LLLT) For Musculoskeletal Pain

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Abstract

Pain is the most common reason for physician consultation in the United States. One out of three Americans is affected by chronic pain annually. The number one reason for missed work or school days is musculoskeletal pain. Currently accepted therapies consist of non-steroidal anti-inflammatory drugs, steroid injections, opiate pain medications and surgery, each of which carries their own specific risk profiles. What is needed are effective treatments for pain which have an acceptably low risk-profile. For over forty years, low level laser (light) therapy (LLLT) and LED (light emitting diode) therapy (also known as photobiomodulation) has been shown to reduce inflammation and edema, induce analgesia, and promote healing in a range of musculoskeletal pathologies. The purpose of this paper is to review the use of LLLT for pain, the biochemical mechanisms of action, the dose response curves, and how LLLT may be employed by orthopedic surgeons to improve outcomes and reduce adverse events.

With the predicted epidemic of chronic pain in developed countries, it is imperative to validate cost-effective and safe techniques for managing painful conditions which would allow people to live active and productive lives. Moreover the acceptance of LLLT (which is currently being used by many specialties around the world) into the armamentarium of the American health care provider would allow for additional treatment options for patients. A new cost-effective therapy for pain could elevate quality of life while reducing financial strains.

Keywords

Musculoskeletal; Pain; Low level laser therapy; Photobiomodulation; Injury repair

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Introduction

Musculoskeletal pain affects 116 million Americans annually at a cost of $635 billion a year in medical bills, lost productivity and missed work or school [1,2]. All therapeutic treatments have their benefits, but also possess different side effects, risks and or complications. The current treatment for musculoskeletal pain includes modalities, immobilization, medications, chiropractic care, physical therapy, behavioral management, injections and/or surgery. These standard therapies have their particular associated risks/side effect profiles including peptic ulcers/gastric bleeding [3], systemic effects (cardiovascular) [4], infections (including epidural abscess) [5], narcotic dependency/addiction [6], deformities, neurologic deficits, and surgical complications [7]. The natural history of chronic pain is one of increasing dysfunction, impairment and possible disability.

The definition of pain by the “International Association for the Study of Pain” states: “Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [8]”. Withdrawal of the painful stimulus usually resolves pain promptly. Sometimes however, pain persists in spite of removal of the stimulus and even after healing of the body. Pain can also arise in the absence of any stimulus, disease or injury. Acute pain is considered to last less than thirty days, while chronic pain is of more than six months duration or as “pain that extends beyond the expected period of healing”. There are three different types of pain; nociceptive, neuropathic and central. The current medical treatment of pain or analgesics is directed at various steps of the pain pathways (Figure 1). Clinically, low level laser therapy (LLLT) can treat nociceptive [9] and neuropathic pain [10], while central pain has not yet been proven to be responsive to LLLT.

What is LLLT?

Low Level Laser Therapy (LLLT) sometimes known as Low Level Light Therapy or Photobiomodulation (PBM) is a low intensity light therapy. The effect is photochemical not thermal. The light triggers biochemical changes within cells and can be compared to the process of photosynthesis in plants, where the photons are absorbed by cellular photoreceptors and triggers chemical changes.

History of LLLT

In 1903, Dr. Nils Finsen was awarded a Nobel Prize for his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation [11]. In 1960, Professor Maiman TH [12] built the first working red ruby laser [12], but it was not until 1967 when Mester E et al. [13,14] was able to demonstrate the phenomenon of “laser bio stimulation” [13,14]. In 1999, Whelan H et al. [15] presented his work on the medical applications of light emitting diodes (LED) for use on the NASA space station [15]. Subsequently over 400 Phase III randomized, double-blind, placebo-controlled trials have been published, with over 4000 laboratory studies of LLLT. (Pubmed.gov)

A laser is a device that generates light through a process of optical amplification based on the stimulated emission of electromagnetic radiation. There are four main classes of lasers as

defined by the International Engineering Consortium (IEC standard 60825.) These classes indicate potential danger the radiation is to the eye.

a. Class 1/1M – CD player

b. Class 2/2M – laser pointer

c. Class 3R/3B – LLLT and CD and DVD writers

d. Class 4 – Surgical laser

LLLT is the application of light (usually a low powered laser or LED typically power range of (10mW–500mW). Light with a wavelength in the red to near infrared region of the spectrum (660nm–905nm), is generally employed because these wavelengths have the ability to penetrate skin, and soft/hard tissues (Figure 2) and are proven in clinical trials to have a good effect on pain, inflammation and tissue repair. The power density (irradiance) is usually between 5W/cm^2 and is applied to an injury or to a painful site for 30–60 seconds a few times a week for several weeks. The result is a reduction of inflammation, pain relief and accelerated tissue regeneration. In most cases the lasers/LEDs used for LLLT emit a divergent beam (not focused or collimated) because collimation is lost in tissue, but as a consequence ocular risks are also diminished over distance.

Mechanisms of LLLT (Figure 3)

For low-power visible or near-infrared light to have an effect on a biologic system, the photon must be absorbed by electronic absorption bands belonging to a photon acceptor or chromophore (first law of photobiology) [16]. A chromophore is a molecule (or portion of a molecule) which imparts a color to a compound (e.g. chlorophyll, hemoglobin, myoglobin, cytochrome c oxidase, other cytochromes, flavin, flavoproteins or porphyrins) [17]. The “optical window” in a tissue describes a range of wavelengths where the penetration of light into tissue is maximized by employing red and near-infrared wavelengths [18]. The optimum wavelength has been estimated to be around 810 nm. Mitochondria are “the cellular power plants” in our cells and as such they convert food molecules and oxygen into energy (ATP) by oxidative phosphorylation. It has been proposed that cytochrome c oxidase (COX) is the primary photo-acceptor for the red-NIR wavelength range in mammalian cells [19]. Nitric oxide (NO) produced in mitochondria can inhibit respiration by binding to COX and displace oxygen especially in injured or hypoxic cells [20]. It is proposed that LLLT can photo-dissociate NO from COX and reverse the mitochondrial inhibition of respiration due to excessive NO binding [21]. The process of light mediated vasodilation was first described by RF Furchgott [22] in 1968, and his research on the biological properties of nitric oxide eventually led to the award of a Nobel Prize in 1998 [23]. LLLT is able to produce a shift in the overall cell redox potential in the direction of greater oxidation by increasing reactive oxygen species (ROS) and decreasing reactive nitrogen species (RNS) [24–30]. The long-term effects of LLLT are thought to be due to the activation of various transcription factors by the immediate chemical signaling molecules produce from mitochondrial stimulation by LLLT. The most important of these signaling molecules are thought to be ATP, cyclic-AMP, NO and ROS [16].

LLLT at low doses has been shown to enhance cell proliferation of fibroblasts [31–34], keratinocytes [35], endothelial cells [36] and lymphocytes [37,38]. The mechanism of proliferation is thought to result from photo-stimulation of the mitochondria leading to activation of signaling pathways and up regulation of transcription factors eventually giving rise to increases in growth factors [31,39–42]. LLLT can enhance neovascularization, promote angiogenesis and increase collagen synthesis to aid in the healing of acute [43] and chronic wounds [44–46]. It has been observed in many studies, that LLLT exhibits a biphasic dose response curve [47,48], where by lower doses of light are more effective than much higher doses. These low doses of light have demonstrated the ability to heal skin, nerves, tendons, cartilage and bones. This biphasic dose response curve may have important implications for LLLT for pain relief for the following reasons. Low-intensity LLLT stimulates mitochondria and raises mitochondrial membrane potential [49–51] and might be supposed to be more likely to increase metabolism and transport of action potentials in neurons rather than decrease it. However, much higher intensity LLLT produced by a focused laser spot acting on a nerve has the opposite effect, inhibiting mitochondrial metabolism in c-fibers and a-delta fibers and reducing mitochondrial membrane potential, thereby inducing a nerve blockade (see below).

LLLT in the treatment of pain

Acute orthopedic conditions such as sprains [52,53], strains, post-surgical pain, a whiplash injury [54], muscular back pain, cervical or lumbar radiculopathy [55,56], tendinitis [57,58] and chronic conditions such as osteoarthritis [59–64], rheumatoid arthritis, frozen shoulder [65], neck and back pain [56], epicondylitis [66], carpal tunnel syndrome [67,68], tendinopathy [69], fibromyalgia [70], plantar fasciitis [70], post tibial fracture surgery [9] and chronic regional pain syndrome are amenable to LLLT. Dental conditions producing pain such as orthodontic procedures [71], dentine hypersensitivity [72], and third molar surgery [73] respond well to treatment with LLLT. Neuropathic pain conditions can also be treated such as post herpetic neuralgia [74], trigeminal neuralgia (10), and diabetic neuropathy [75]. Due to the wide spectrum of conditions one would surmise that multiple mechanisms can operate to achieve pain relief.

The peripheral nerve endings of nociceptors, consisting of the thinly myelinated Aϑ and unmyelinated, slow-conducting C fibers, lie within the epidermis. This complex network transduces noxious stimuli into action potentials. Moreover these nerve endings are very superficial in nature and thus are easily within the penetration depths of the wavelengths used in LLLT (Figure 4). The cell bodies of neurons lie within the dorsal nerve root ganglion, but the elongated cytoplasm (axons) of the neurons extends from the cell body to the bare nerve endings in the surface of the skin. The direct effect of LLLT are initially at the level of the epidermal neural network, but the effects move to nerves in subcutaneous tissues, sympathetic ganglia, and the neuromuscular junctions within muscles and nerve trunks.

LLLT applied with a sufficient level of intensity causes an inhibition of action potentials where there is an approximately 30% neural blockade within 10 to 20 minutes of application, and which is reversed within about 24 hours [76]. The laser application to a

peripheral nerve does have a cascade effect whereby there is suppressed synaptic activity in second order neurons so that cortical areas of the pain matrix would not be activated.

Adenosine triphosphate (ATP) is the source of energy for all cells, and in neurons this ATP is synthesized by mitochondria while they are located in the dorsal root ganglion. These mitochondria are then transported along the cytoskeleton of the nerve by a monorail system of molecular motors. LLLT acts like an anesthetic agent, in that both LLLT and anesthetics have been shown to temporally disrupt the cytoskeleton for a matter of hours as evidenced by formation of reversible varicosities or beading along the axons, which in turn cause mitochondria to "pile up" where the cytoskeleton is disrupted [77]. The exact mechanism for this effect is unknown but it is not a thermal action. It has been shown that LLLT at the correct dose decreases mitochondrial membrane potential (MMP) in DRG neurons and that ATP production is then reduced [78] so perhaps the lack of ATP could be cause of this neural blockade. The most immediate effect of nociceptor blockade is pain relief which occurs in a few minutes and has been shown by the timed onset of a conduction blockade in somatosensory-evoked potentials (SSEPs) [76]. This inhibition of peripheral sensitization not only lowers the activation threshold of nerves but also decreases the release of pro inflammatory neuropeptides (i.e. substance P and CGRP). In persistent pain disorders this reduction of tonic input to activated nociceptors and their synaptic connections, leads to a long-term down-regulation of second-order neurons [78]. The modulation of neurotransmitters is a further possible mechanism of pain relief, as serotonin and endorphin levels have been shown to increase in animal models [79,80] and following laser treatment of myofascial pain in patients [81]. Thus LLLT can have short, medium and long term effects. Fast acting pain relief occurs within minutes of application, which is a result of a neural blockade of the peripheral and sympathetic nerves and the release of neuromuscular contractions leading to in a reduction of muscle spasms [82,83].

In the medium term there is a decrease of local edema and a reduction of inflammation within hours to days [84]. The action of LLLT in reducing swelling and inflammation has been well established in animal models as well as in clinical trials. The numbers of inflammatory cells has been shown to be reduced in joints injected with protease [85], in collagen-induced rheumatoid arthritis [86], and in acute pulmonary inflammation [87]. The expression levels of pro-inflammatory cytokines have been shown to be reduced by LLLT in burn wounds [88], in muscle cryo lesions [89] and in delayed type hypersensitivity [90]. The long term effects of LLLT occur within a week or two and can last for months and sometimes years as a result of improved tissue healing.

LLLT parameters

For LLLT to be effective, the irradiation parameters (wavelength, power, power density, pulse parameters, energy density, total energy and time) need to be within certain ranges. The best penetrating wavelengths in the range of 760–850nm and may achieve a light density of 5mW/cm^2 at 5cm deep when the beam power is 1Watt and surface density is 5W/cm^2. There are four clinical targets for LLLT:

a. The site of injury to promote healing, remodeling and reduce inflammation.

b. Lymph nodes to reduce edema and inflammation.

c. Nerves to induce analgesia.

d. Trigger points to reduce tenderness and relax contracted muscle fibers.

Treatment times per point are in the range of 30 seconds to 1 minute. As little as one point may be treated in simple cases, but as many as 10 to 15 points may be treated for more complex dysfunction such as cervical or lumbar radiculopathy.

The potential hazards are mostly ocular, as some LLLT devices are lasers, though increasingly LLLT devices have become LEDs. In most cases, LLLT devices emit divergent beams (not focused or collimated), so the ocular risk diminishes over distance. Manufacturers are obliged to provide the nominal ocular hazard distance (NOHD) within their user instructions. ANSI 2 136.3 (2011) is the current definitive USA document on laser safety in healthcare environments (www.ansi.org) and IEC60825 is the International Standard. Part 8 provides guidelines for the safe use of laser beams on humans (www.iec.ch).

The North American Association for Laser Therapy conference in 2010 held a consensus meeting on safety and contraindications. Their main recommendations were:

I. Eyes - Do not aim laser beams into the eyes and everyone present should wear appropriate safety spectacles.

II. Cancer - Do not treat over the site of any known primary carcinoma or secondary metastasis unless the patient is undergoing chemotherapy when LLLT can be used to reduce side effects such as mucositis. LLLT however can be considered in terminally- ill cancer patients for palliative relief.

III. Pregnancy- Do not treat directly over the developing fetus.

IV. Epileptics - Be aware that low frequency pulsed visible light (<30Hz) might trigger a seizure in photosensitive, epileptic patients.

The adverse effects of LLLT have been reported to be no different from those reported by patients exposed to placebo devices in trials.

Orthopedic outcomes

According to the more than 4000 studies on pub.med.gov, it can be concluded that the majority of laboratory and clinical studies have demonstrated that LLLT has a positive effect on acute and chronic musculoskeletal pain. Due to the heterogeneity of populations, interventions and comparison groups, this diversity means that every single study has not been positive. Pain is a very complex condition which presents in different forms with an interplay of mechanical, biochemical, psychological and socioeconomic factors. It is extremely challenging to compare LLLT to other treatments, and LLLT regimens are complicated by different lengths of treatment, all without standardization of wavelengths and dosages. Currently, there have been no long-term (greater than 2 year follow up) human clinical studies that have evaluated LLLT. The overall positive short term clinical studies in addition to strong laboratory studies should give the clinical confidence that LLLT may be beneficial for many individuals suffering from musculoskeletal pain, regardless of the cause.

Consideration of evidence based treatment studies for LLLT has led to the determination that LLLT is classified as experimental/investigational by insurance companies (BCBSKS 2013), while the American Academy of Orthopedic Surgeons has no recommendations for or against its use. With FDA approval for temporary relief of muscle and joint pain, this underlines the need for further well-designed clinical studies.

Conclusion

One has to be realistic about the therapeutic use of LLLT. The previous discussion has shown that LLLT is beneficial for pain relief and can accelerate the body's ability to heal itself. LLLT has a long history and strong basic science evidence, which supports its use in pain management. It has few side effects and is well tolerated by the elderly. A laser or LED does not correct situations involving structural deficits or instabilities whether in bone or in soft tissue. Also, LLLT should only be used as an adjuvant therapy for pain relief in patients with neuropathic pain and neurologic deficits. Successful outcomes, like all medical management, depend on good clinical skills linked with an understanding of the nature of injury, inflammation, repair, pain, and the mechanism of laser and LED effects.

Acknowledgments

Michael R Hamblin was supported by US NIH grant R01AI050875.

Abbreviations

LED	Light Emitting Diodes
LLLT	Low Level Laser Therapy
PBM	Photobiomodulation
NO	Nitric Oxide
ATP	Adenosine Triphosphate
ROS	Reactive Oxygen Species
MMP	Membrane Potential

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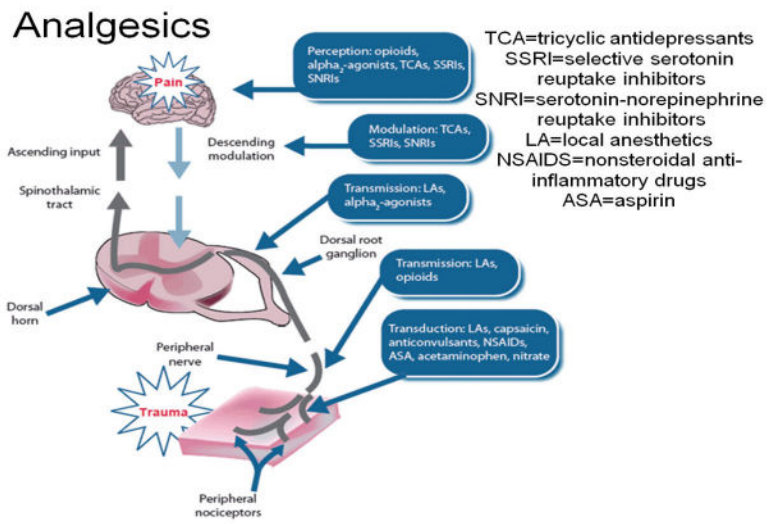

Figure 1.
Site of analgesic action on the pain pathway.

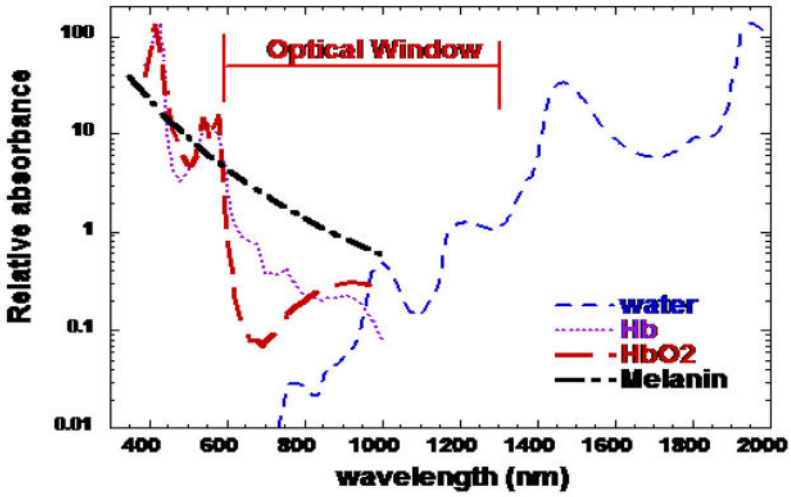

Figure 2.
Tissue optical window.

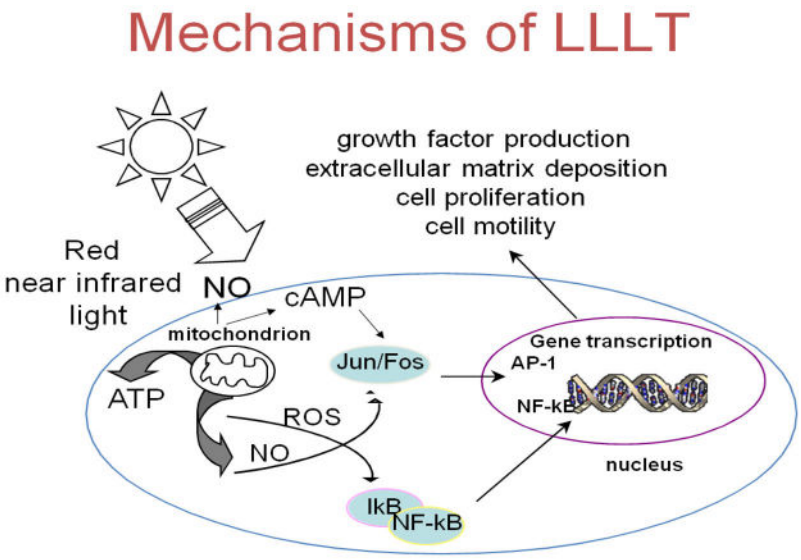

Figure 3.
Mechanisms of LLLT.

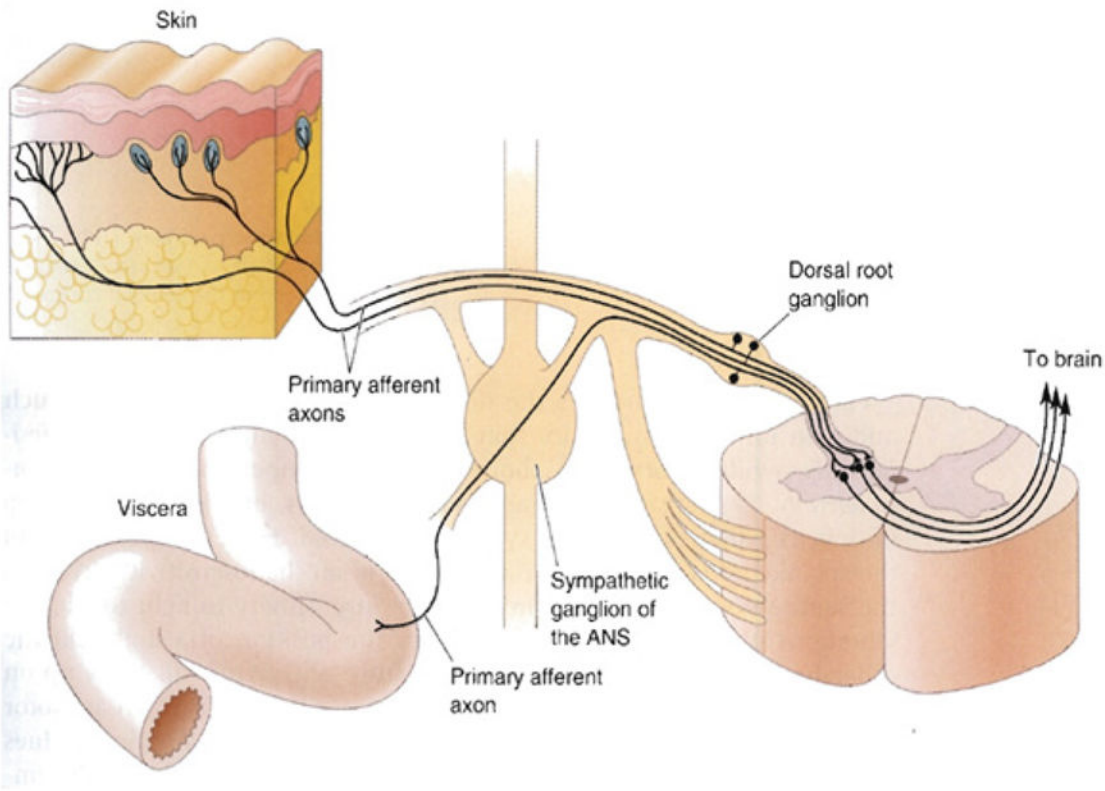

Figure 4.
Afferent nerves.

Published in final edited form as:

IEEE J Sel Top Quantum Electron. 2016 ; 22(3): . doi:10.1109/JSTQE.2016.2561201.

Proposed Mechanisms of Photobiomodulation or Low-Level Light Therapy

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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

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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 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 vital 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 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 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].

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.

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.

PPARy—Peroxisome proliferator-activated receptors (PPAR) are mostly 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. 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.

Acknowledgments

MRH was supported by US NIH grant R01AI050875.

Lucas Freitas de Freitas was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP.

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Biography

Michael R Hamblin Ph.D. is a Principal Investigator at the Wellman Center for Photomedicine at Massachusetts General Hospital, an Associate Professor of Dermatology at Harvard Medical School and is a member of the affiliated faculty of the Harvard-MIT Division of Health Science and Technology. He was trained as a synthetic organic chemist and received his PhD from Trent University in England. His research interests lie in the areas of photodynamic therapy (PDT) for infections, cancer, and stimulation of the immune system, and in low-level light therapy (LLLT) for wound healing, arthritis, traumatic brain injury, neurodegenerative diseases and psychiatric disorders. He directs a laboratory of around a dozen post-doctoral fellows, visiting scientists and graduate students. His research program is supported by NIH, CDMRP, USAFOSR and CIMIT among other funding agencies. He has published over 320 peer-reviewed articles, over 150 conference proceedings, book chapters and International abstracts and holds 10 patents. He is Associate Editor for 7 journals, on the editorial board of a further 30 journals and serves on NIH Study Sections. For the past 11 years Dr Hamblin has chaired an annual conference at SPIE Photonics West entitled "Mechanisms for low level light therapy" and he has edited the 11 proceedings volumes together with four other major textbooks on PDT and photomedicine. He has several other book projects in progress at various stages of completion. In 2011 Dr Hamblin was honored by election as a Fellow of SPIE. He is a Visiting Professor at universities in China, South Africa and Northern Ireland.

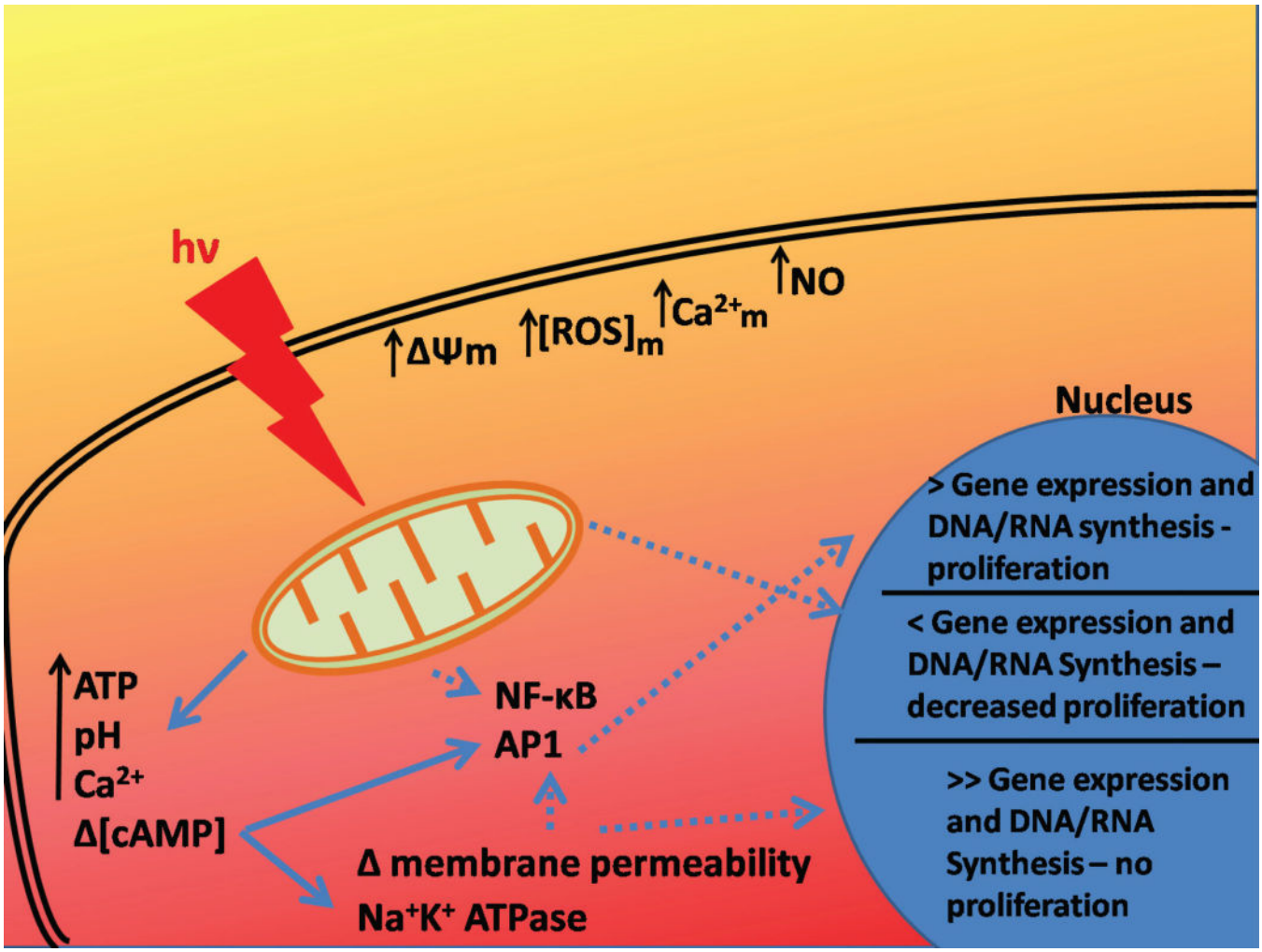

Figure 1.
Scheme of mitochondrial retrograde signaling pathways as proposed by Karu. The main pathway is represented by continuous arrows, and the complementary ones are represented by segmented arrows.

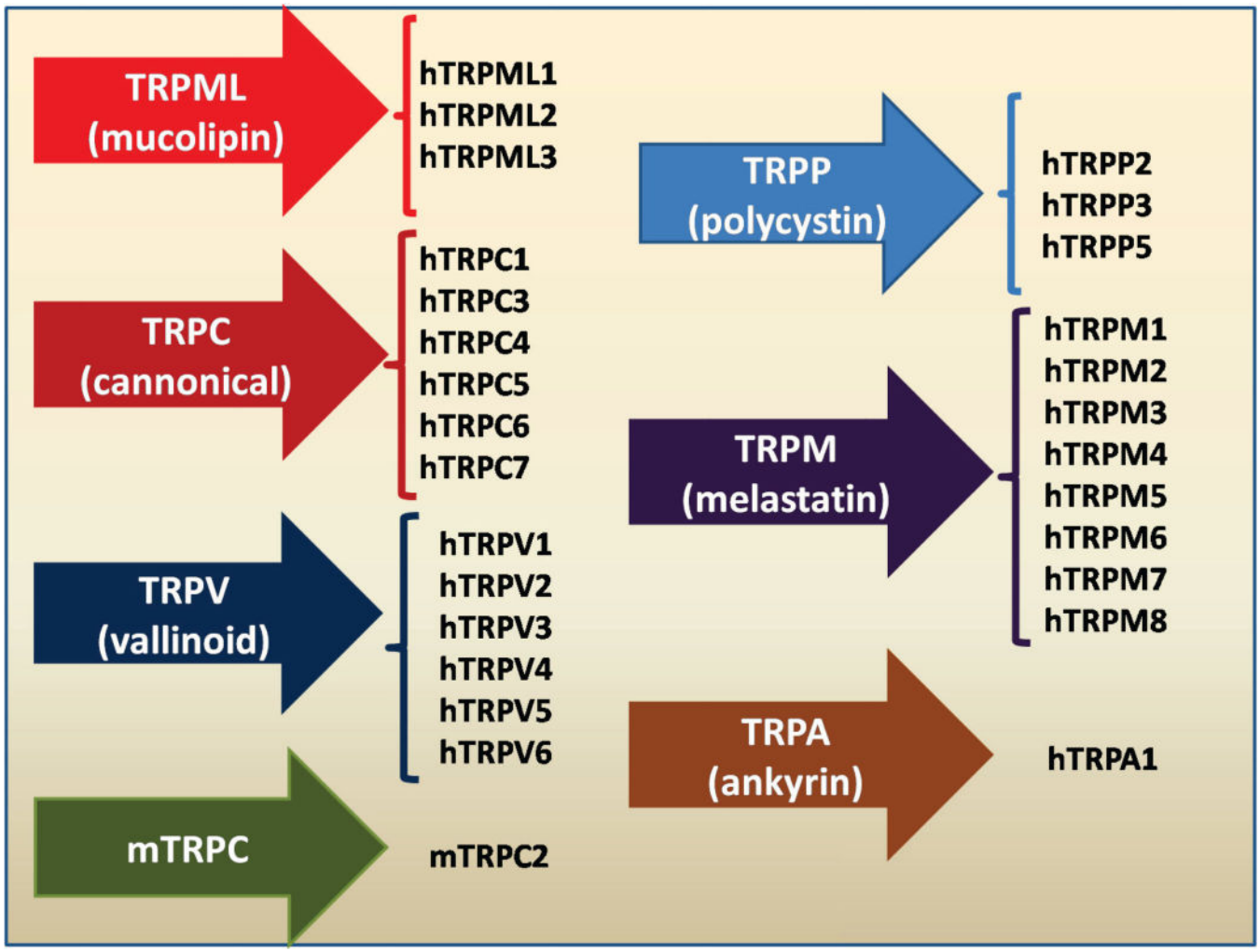

Figure 2.
All the seven subfamilies of Transient Receptor Potential Channels (TRP).

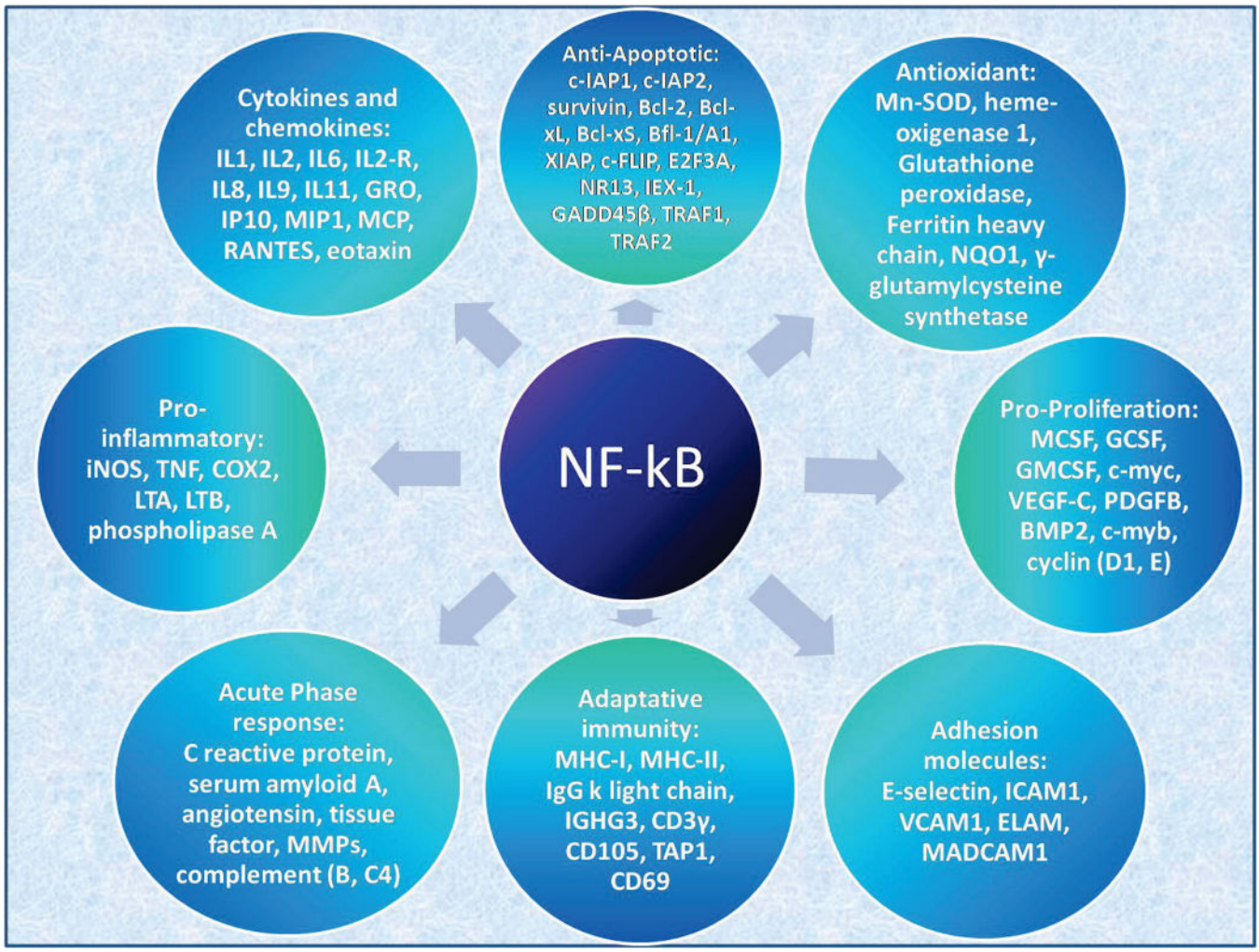

Figure 3.

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

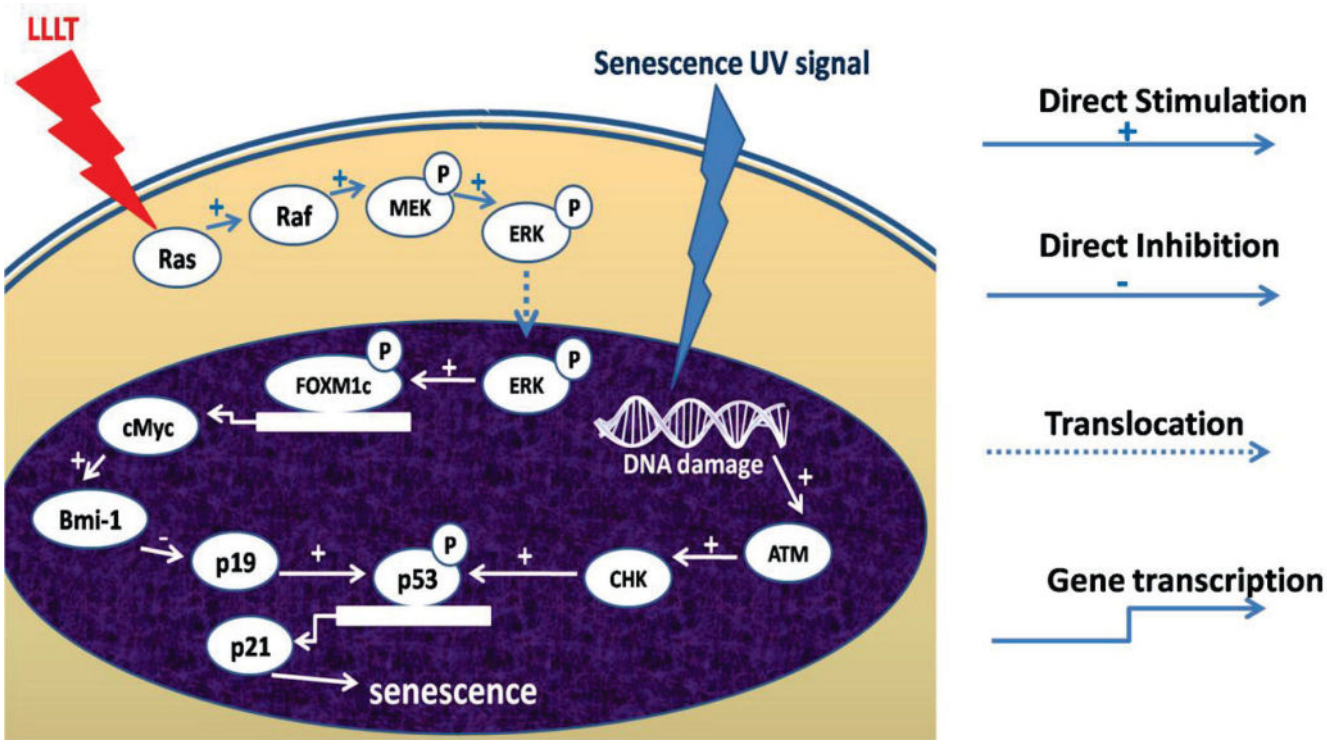

Figure 4.
A model of the signaling pathways for LLLT protecting cell from UVB-induced senescence.

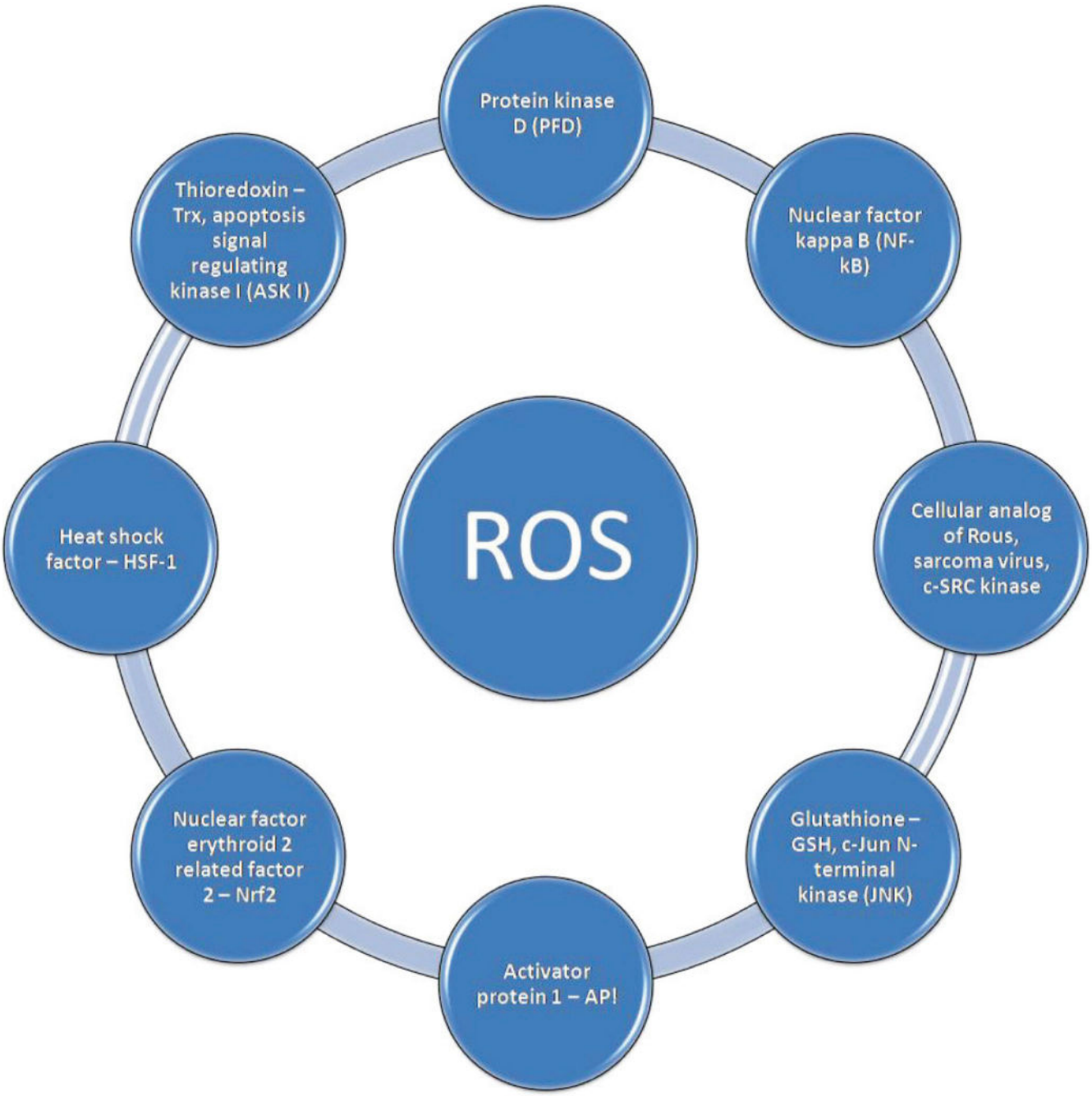

Figure 5.
Reactive Oxygen Species sensors and signaling

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.

Table 2

Description of the light dose parameters.

LIGHT DOSE PARAMETERS		
Irradiation Parameter	Measurement unit	Description
Energy	Joules (J)	It cannot be mistook 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 n 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 the real "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.

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Author manuscript

J Biophotonics. Author manuscript; available in PMC 2017 December 01.

Published in final edited form as:

J Biophotonics. 2016 December ; 9(11-12): 1273–1299. doi:10.1002/jbio.201600176.

Photobiomodulation in human muscle tissue: an advantage in sports performance?

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Abstract

Photobiomodulation (PBM) describes the use of red or near-infrared (NIR) light to vstimulate, heal, and regenerate damaged tissue. Both pre-conditioning (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

photobiomodulation; LLLT; LEDT; fatigue; creatine kinase; delayed onset muscle soreness

INTRODUCTION

Photobiomodulation (PBM) is also called low-level laser therapy (LLLT) and light-emitting diode therapy (LEDT). PBM can provide several benefits to muscle tissue as evidenced by a plethora of studies that have been carried out *in vitro*, *in vivo* and in clinical trials. This review will cover these effects that have been reported up to now in the current literature,

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concentrating on: i) prevention of muscle damage after exercise, including delayed onset muscle soreness (DOMS); and ii) increasing capacity for muscle workload, improving fatigue resistance, functional and athletic activity, and hastening recovery after exercise. We summarize studies with different levels of evidence such as randomized clinical trials (level 1b) and case-control studies (level 3b) published up to September 2016 that have aimed to increase muscle performance, recovery after exercise or prevent muscle damage by photobiomodulation using LLLT and/or LEDT applied to the upper limbs (*biceps brachii*) and lower limbs (*quadriceps femoris* muscles, hamstrings and *triceps surae*) in healthy volunteers/athletes.

The rationale for using PBM on muscles relies on the well-known stimulation of mitochondrial activity that occurs after red or near-infrared photons delivered to the tissue have been absorbed by cytochrome c oxidase. Muscles rely heavily on adenosine tri-phosphate (ATP), which is the biological source of energy needed for muscle work, and therefore robust increased ATP levels are the most popular hypothesis to explain the extraordinary effects that PBM appears to exert on muscle tissue. Moreover, there are several mechanisms of action to explain the effects of PBM on muscle tissue, and consequently improvement in sports performance. Several of these mechanisms have been described previously [1]: i) increases in energy metabolism and ATP synthesis; ii) stimulation of defenses against oxidative stress; iii) prevention and repair of muscle damage; iv) modulation of gene expression by activation of transcription factors; v) possible increase in the excitability of muscle fibers. For more details, see the review [1].

The use of PBM to prevent muscle damage was first demonstrated in animal models, usually by irradiating skeletal muscles before a bout of intense exercise (known as muscular pre-conditioning) and by assessing the severity of muscle damage by measuring creatine kinase (CK) levels in the bloodstream. To the best of our knowledge, the first study that used photobiomodulation to prevent muscle damage in animal models was carried out by Lopes-Martins et al. [2] in rats. These authors investigated the effects of different doses of light (wavelength 655 nm, 0.5 J/cm^2; 1.0 J/cm^2 and 2.5 J/cm^2) to prevent muscle fatigue and muscle damage (CK) induced by neuromuscular electrical stimulation. This study reported a dose response with the delivered fluence and its ability to decrease CK levels.

Another experimental study used training of rats on a treadmill running in decline (downhill running) and measured inhibition of inflammation, reduction of CK activity and lowering of oxidative stress. They also found increases in defense against oxidative stress (increased activity of superoxide dismutase - SOD) 24 h and 48 h after exercise [3]. These previous studies and other similar ones [2–7] were important to establish a scientific bridge to clinical trials for prevention of muscle damage in humans by photobiomodulation.

Regarding improved muscle performance and exercise recovery in clinical trials, the first published studies were looking at delayed onset muscle soreness (DOMS) [8–11] and resistance to muscle fatigue [12,13] during one or only a few bouts of exercise; and measuring muscle strength [14] and resistance to muscle fatigue [15] after exercise training programs combined with PBM.

The primary objective of the present review was to determine the effects of PBM in the form of LLLT or LEDT on muscle tissue in clinical trials that enrolled both untrained and trained healthy subjects, or athletes who were treated with muscular pre-conditioning (PBM before bouts of exercises), or PBM irradiation delivered after exercise/training sessions. The outcomes assessed in this review aimed to identify the effects (no effects, or positive effects) on muscle performance/recovery in upper and lower limbs when effective PBM was compared with control groups and/or placebo (sham) PBM therapy in humans regarding: i) number of repetitions, ii) torque, iii) torque in maximum voluntary contraction (MVC) or maximum voluntary isometric contraction (MVIC), iv) 1-repetition maximum test (1-RM), v) fatigue by surface electromyography analysis or time to exhaustion, vi) prevention of muscle damage (creatine kinase-CK), vii) reduction of DOMS, viii) blood lactate, and ix) muscle recovery. Finally, as a secondary objective, this review aimed to cover all the parameters used in the included studies in order to show which parameters "worked" (produced positive effects) and which did not "work" (produced no effects) when PBM was combined with exercise for upper and lower limbs. However, this review did not aim to conduct comparative (statistical) analyses (meta-analysis) regarding all outcomes of interest.

MATERIAL AND METHODS

Data Source and Searches

The effects of photobiomodulation (PBM) using low-level laser therapy (LLLT) and/or light-emitting diode therapy (LEDT) on muscle tissue in humans were determined through a review following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) [16] recommendations. However, risk of bias, quality of evidence (GRADE or PEDro approach), and comparative (statistical) analysis (mean differences, 95 % confidence intervals, effect sizes - meta-analysis) regarding all outcomes assessed from all included studies were not evaluated in this review.

The strategy to identify the studies to be included in the present review was accomplished by searching the current literature on MEDLINE (via Pubmed). The time window used for the search was from January 1960 to September 2016. All retrieved studies were analyzed to determine their eligibility according to the inclusion criteria. Potential studies were assessed by two authors that screened independently all titles and abstracts. Disagreements between review authors (CF and YYH) were resolved with decision from the third review author (MRH). We used only full-text papers, written in English, to determine the final inclusion in the review. There were no restrictions regarding gender, age, physical training condition of the healthy participants (athletes or untrained subjects) in each study included.

Randomized controlled trials (RCTs) and crossover trials (level of evidence 1b), as well as case control studies (level 3b), were eligible to be included in this review. Inclusion criteria were that the effective PBM, or placebo (sham) PBM should be applied before (muscular pre-conditioning) a bout of exercise or training programs, and/or after a bout of exercise or training programs in RCTs and/or case-control studies. Control groups were required as a mandatory criteria for study inclusion excluding those where PBM had no other kind of control therapy such as placebo (sham). Studies assessing PBM effects on exercises for upper or lower limbs, as well as exercises performed on treadmills were also considered

eligible. All terms used to search all studies were described in Table 1. Filters for article types, text availability, publication dates or species were not applied during search procedures in MEDLINE via Pubmed.

Data extraction (PBM parameters and outcomes) and quality assessment

Two review authors (CF and YYH) extracted study characteristics, PBM parameters and outcomes measured. They also qualified PBM effects as positive (providing an advantage) or without effect (no effect) according to the statistical results and statements in each included study. All studies were classified as acute (≤7 exercise sessions repeated with or without washout period plus PBM), and chronic (>7 consecutive exercise sessions without a washout period plus PBM). Calculations were done when PBM parameters were not fully or correctly provided in the study. A flowchart of this review is presented in Figure 1. All PBM parameters and outcomes from each study are summarized in tables 2, 3 and 4 and 5.

RESULTS

The database search retrieved 993 studies. After further selection based on the title and abstract, a total of 50 studies were screened and considered eligible to be included in this review taking into account the objectives and inclusion criteria. One study was excluded due to lack of a control group [17] and three studies [18–20] were excluded due to PBM being applied during exercise. Therefore, this review included in total 46 studies [Figure 1], comprising 1045 participants enrolled in the included trials. Risk of bias and comparative (statistical) analysis (meta-analysis) were not conducted in this review.

All 46 studies included in this review were allocated into 4 subsections according to their similarity regarding the number of exercise sessions (acute or chronic), combined or not with PBM, exercise or test used, and muscle or muscle groups subjected to such tests or exercises:

- **A.** Acute photobiomodulation (PBM) response in exercise using upper limb muscles;
- **B.** Acute photobiomodulation (PBM) responses in exercise using lower limb muscles;
- **C.** Acute photobiomodulation (PBM) responses in exercise on a treadmill;
- **D.** Chronic responses in clinical trials and case control studies.

A. Acute photobiomodulation (PBM) response to exercise using upper limb muscles

To our best of knowledge, the first randomized clinical trial (RCT) was conducted by Craig et al. [10] (level 1b). The authors investigated the efficacy of three different frequencies (2.5 Hz, 5 Hz, 20 Hz) of pulsed PBM using low-level laser therapy (LLLT) at 660 nm and 950 nm to mitigate DOMS induced by repeated eccentric contractions of the elbow flexor muscles with free weights until exhaustion in a randomized, placebo-controlled trial. The subjects received LLLT on the *biceps brachii* exercised for 3 days. However, DOMS increased for all groups treated with different LLLT pulse frequencies, as well as for the control and placebo groups. Range of motion was impaired and pain increased for all

groups. The authors reported no effect of PBM in DOMS assessed by visual analogue scale (VAS) and mechanical pain threshold/tenderness (MPT).

Years later, the same research group [11] (level 1b) conducted a very similar study to investigate the effectiveness of LLLT on DOMS during a treatment period of 7 days in a randomized, placebo-controlled trial. DOMS induction, as well as LLLT device used were similar to previous study [10], consisting of elbow flexion exercises with free weights and laser diodes at (660 nm and 950 nm, 73 Hz), respectively. After complete DOMS induction, subjects were treated com placebo, or LLLT, or control (rest in supine position) on 1 point over the *biceps brachii* for 5 consecutive days, plus 2 days in the following week. The authors reported no effect of LLLT to mitigate DOMS assessed by VAS and MPT.

Vinck et al. [9] (level 1b) investigated the effects of LEDT (cluster of 32 LEDs, 950 nm) on DOMS after induction of fatigue in the *biceps brachii* muscle using an isokinetic dynamometer. The authors reported no significant difference between the LEDT group and the placebo group regarding elbow flexor peak torque exerted and pain in this randomized, single-blind, placebo-controlled trial. However, using similar methodology, Douris et al. [8] (level 1b) in a randomized, double-blind placebo-controlled trial applied LEDT (cluster of 36 LEDs, 880 nm and 660 nm) on the *biceps brachii* muscles after elbow flexion/extension bearing free weights, and found a significant reduction in DOMS after 48 h when comparing LEDT to the control and placebo groups.

Using a muscular pre-conditioning protocol, Leal Junior et al. [13] (level 1b) applied LLLT (655 nm) on the *biceps brachii* muscle before elbow flexion/extension until exhaustion on a Scott bench in a randomized, double-blind, placebo-controlled trial. The PBM was applied on 4 points located on the muscle, and then maximum voluntary contractions (MVC) were applied with a load of 75%. The number of repetitions and total time to accomplish the exercise increased significantly in all the volunteers that received muscular pre-conditioning PBM compared to the placebo group. However the results of blood lactate were not significantly different. Moreover, similar studies have been conducted by the same research group, investigating a muscular pre-conditioning regimen with LLLT (830 nm) applied on 4 points of the *biceps brachii* [21] (8/10, level 1b). They also compared red (660 nm) and near-infrared (830 nm) wavelengths applied on the *biceps brachii* muscle before MVC on a Scott bench [22] (level 1b). These authors found significant differences between the placebo group and both groups irradiated with LLLT, but without significant difference between the two different wavelengths regarding the mean and peak force developed by each group irradiated.

Applying a muscular pre-conditioning protocol on 2 sites on the *biceps brachii* during 30 sec, Leal Junior et al. [23] (level 1b) in a randomized, double-blind, crossover, placebo-controlled trial reported an increased number of repetitions could be achieved, and found decreased lactate levels in blood as well as lower CK and C-reactive protein. The PBM used in this study was delivered by a cluster of 5 laser diodes (810 nm). Similar results were reported by the same research group [24] (level 1b) using a cluster of 69 LEDs (850 nm and 660 nm) applied on 1 site of the *biceps brachii* muscle in a muscular pre-conditioning protocol using elbow flexion/extension with a load of 75% of MVC on a Scott bench. The

LEDT group increased the number of repetitions and the duration of exercise compared to placebo group in this randomized, double-blind, crossover, placebo-controlled trial. LEDT also decreased blood levels of lactate, CK and C-reactive protein compared to placebo group.

Investigating the effects of PBM by LEDT (630 nm) applied after an exercise protocol to induce muscle damage by eccentric elbow flexion/extension, Borges et al. [25] (level 1b) reported a reduction in DOMS, and a slower decay in the isometric force able to be exerted by the LEDT group when compared to the placebo group in a randomized, double-blind, placebo-controlled trial. The authors also reported less restriction or a smaller decrease in the range of motion possible in the LEDT group 24 h, 48 h, 72 h and 96 h after induction of muscle damage. In a similar study Felismino et al. [26] (level 1b) applied LLLT (808 nm) on the *biceps brachii* muscle between 10 sets of 10 repetitions with a load corresponding to 50% of the 1-repetition maximum test (1-RM) during exercise consisting of elbow flexion/extension on a Scott bench. The results of this randomized, double-blind, placebo-controlled trial pointed to a significant reduction in muscle damage (measured by CK) only at 72 h after the exercise test compared to the placebo group. However, there was no significant difference in muscle performance (load) between groups.

Rossato et al. [27] (level 1b) compared the effects of large cluster probe (33 diodes; 30.2 cm^2) *versus* small cluster probe (9 diodes; 7.5 cm^2) on elbow flexor muscle fatigue in a randomized, crossover, double-blind, placebo-controlled trial. The authors reported an increased time to exhaustion when active PBM by LEDT with large and small cluster probes were used as muscular pre-conditioning to increase fatigue resistance assessed in an isokinetic dynamometer through maximum voluntary isometric contraction (MVIC). Both cluster probes induced an increase in time to exhaustion compared with respective placebo therapies, but there was no difference between both active cluster probes. Moreover, there were no significant differences among placebo, large and small cluster probes groups regarding maximum peak torque and electromyography analysis in the *biceps brachii*.

Some other studies did not report significant benefits of PBM on the *biceps brachii*, such as that by Higashi et al. [28] (level 1b) who used an exercise protocol on a Scott bench. These authors used a muscular pre-conditioning protocol with LLLT (808 nm) on 8 sites of irradiation on the *biceps brachii*. There was no significant increase in the number of repetitions and reduction in lactate levels in the blood, or reduction in fatigue assessed by surface electromyography in this randomized, triple-blind, crossover, placebo-controlled trial. Finally Larkin-Kaiser et al. [29] (level 1b) in a randomized, double-blind, crossover, placebo-controlled trial enrolling both men and women, applied a muscular pre-conditioning protocol with LLLT (800 nm and 970 nm) on the *biceps brachii* muscle at 15 points of irradiation. Although only a very small difference was found compared with placebo group, the LLLT group had an improvement in MVIC assessed by isokinetic dynamometer, and no significant effect on muscle point tenderness.

Figure 2 shows an example of the sites of irradiation on the muscles and exercise test for upper arms. Table 2 presents all parameters of photobiomodulation used for testing acute response in exercises with upper arm muscles.

B. Acute photobiomodulation (PBM) responses in exercise using lower limb muscles

One of the first studies published in this area was conducted by Gorgey et al. [12] (6/10, level 1b). The authors performed a muscular pre-conditioning protocol applying photobiomodulation (PBM) by LLLT (808 nm) for 5 minutes (scanning mode) comparing low energy (3 J) with high energy (7 J) on the *quadriceps femoris* muscles before induction of fatigue by neuromuscular electrical stimulation. Although apparently less fatigue in the LLLT groups compared to control, there was no significant difference among all groups.

Leal Junior et al. [30] (level 1b) in randomized, double-blind, crossover, placebo-controlled trial irradiated the *rectus femoris* muscle before Wingate tests with a muscular pre-conditioning protocol. These authors reported no significant effects of the LLLT (830 nm) on muscle performance, but the levels of CK and lactate in bloodstream were decreased when compared to the placebo group. Leal Junior et al. [31] (level 1b) conducted another randomized, double-blind, crossover, placebo-controlled trial using the Wingate test and compared the effects of LLLT (single diode, 810 nm) to LEDT (cluster of 69 LEDs, 850 nm and 660 nm) on the muscle performance of athletes. LLLT or LEDT was applied on 2 sites on the *rectus femoris* muscle in a muscular pre-conditioning protocol. LEDT decreased CK levels in blood compared to placebo and LLLT groups. However, there was no improvement in muscle performance or reduction in lactate levels in the bloodstream of the LEDT group compared to placebo and/or LLLT groups. Similar results were reported by Denis et al. [32] (level 1b) who also did not find positive results when LEDT (cluster of 69 LEDs, 950 nm and 660 nm) was applied during the rest intervals of the Wingate tests. These authors reported no significant differences in muscle peak power, fatigue index and blood lactate levels when compared to the placebo group in this randomized, single-blind, crossover, placebo-controlled trial.

In another randomized, double-blind, crossover, placebo-controlled trial using Wingate test, Leal Junior et al. [33] (level 1b) also compared the effects of photobiomodulation by LEDT (cluster of 69 LEDs, 850 nm and 660 nm) to the use of cold-water immersion (cryotherapy) in order to promote muscle recovery. Six athletes performed 3 Wingate tests on non-consecutive days and received either LEDT or cold-water immersion of the lower limbs (5°C for 5 minutes) as a therapy. LEDT was applied on 2 sites on the *quadriceps femoris* muscles, 2 sites on the hamstrings and 1 site on the *triceps surae*. Comparing both groups, LEDT significantly decreased levels of CK and lactate in the blood but was not able to increase muscle work in the Wingate test and did not reduce C-reactive protein.

In a similar study, de Paiva et al. [34] (level 1b) investigated the effects of cryotherapy on DOMS and muscle damage (CK) as a single treatment, or combined with PBM by a cluster with 1 infrared laser diode (905 nm), 4 red LEDs (640 nm) and 4 infrared LEDs (875 nm) in a randomized, double-blinded, placebo-controlled trial. The authors induced muscle damage in subjects with eccentric contractions of knee extensor muscles in an isokinetic dynamometer. Three minutes after, the subjects received either placebo, or PBM, or cryotherapy, or PBM + cryotherapy, or cryotherapy + PBM. Post-exercise assessments consisted of measuring CK in blood, visual analogue scale for pain (VAS) and maximum voluntary isometric contraction (MVIC) at 1 h, 24 h, 48 h, 72 h and 96 h. Treatments were repeated at 24 h, 48 h and 72 h. PBM improved MVIC, decreased DOMS and CK from 24 h

to 9 h when compared to placebo, cryotherapy and cryotherapy + PBM. The combination of PBM + cryotherapy reduced the efficacy of PBM. Finally, cryotherapy as a single treatment, and cryotherapy + PBM were similar to placebo, showing the superiority of PBM as a single treatment.

Assessing muscle performance by an exercise protocol using eccentric exercise in an isokinetic dynamometer, Baroni et al. [35] (level 1b) used a muscular pre-conditioning protocol with LLLT (cluster of 5 laser diodes, 810 nm) applied on 6 sites of the *quadriceps femoris* muscles in a randomized, double-blind, placebo-controlled trial. The authors reported an improved MVIC immediately and 24 h after the exercise protocol, and increased lactate dehydrogenase (LDH) activity at 48 h after exercise, and the CK levels in blood decreased after 24 h and 48 h when compared to the placebo group. However, the DOMS was not improved by LLLT. In addition, this same research group [36] (level 1b) also investigated the effects of the same LEDT cluster (69 LEDs, 850 nm and 660 nm) applied on 3 sites of the *quadriceps femoris* muscles before a fatigue protocol performed in an isokinetic dynamometer for knee flexion/extension. The authors reported that LEDT was able to decrease the decay of the knee extensor peak torque exerted compared to placebo group in this randomized, double-blind, crossover, placebo-controlled trial.

With a similar protocol of exercise in isokinetic dynamometer of previous study [36],Antonialli et al. [37] (level 1b) used laser diodes (905 nm) and LEDs (640 nm and 875 nm) assembled in the same device to stimulate muscle recovery when applied as a muscular pre-conditioning protocol on the *quadriceps femoris* muscles before a fatigue protocol with eccentric contractions and MVIC in an isokinetic dynamometer. Muscular pre-conditioning using lasers and LEDs was performed 3 minutes before the exercise protocol in a randomized, double-blind, placebo-controlled trial. The authors reported that light energies of 10 J, 30 J and 50 J per site of irradiation increased the percentage of knee extensor peak torque of maximum voluntary isometric contraction (MVIC) immediately and the effect lasted until 96 h after the muscular pre-conditioning. DOMS was reduced significantly using light energies of 30 J and 50 J when compared with the placebo group. Finally, this study reported a significant reduction of CK with light energies (doses) of 10 J, 30 J and 50 J.

On the other hand, the same aforementioned research group conducted another randomized, double-blind, placebo-controlled trial with soccer athletes and assessed also the effects of PBM using LLLT on the knee extensor peak torque of MVIC, DOMS, CK and interleukin-6 expression [38] (level 1b). This study applied LLLT on 6 sites of irradiation on the *quadriceps femoris* muscles similar to Antonialli et al. [37], but interestingly found different effects for light energies (doses) of 10 J, 30 J and 50 J. Light energies (doses) of 10 J and 50 J per site of irradiation were more effective to decrease CK levels in blood and interleukin 6 (IL-6), with better results in favor of 50 J. Moreover, MVIC was improved immediately after exercise up to 24 h after exercise with 50 J, and from 24 h to 96 h with 10 J. However, 30 J per site of radiation had no effect on MVIC, CK, IL-6 and DOMS. The main difference from this study [38] and the previous study [37] was the device used for photobiomodulation. While Antonialli et al. [37] used a cluster of lasers and LEDs, this study [38] used a cluster of 5 lasers diodes (810 nm) similar to used in previous study [35] that found positive effects with 30 J per site of irradiation.

In another study looking at dose-response, Hemmings et al. [39] (level 1b) developed a randomized, double-blind, crossover, placebo-controlled trial with 34 subjects to investigate the effects of PBM with LEDT on *quadriceps femoris* muscle performance in an isokinetic dynamometer. LEDT was applied as muscular pre-conditioning with light energies of 41.7 J, 83.4 J, 166.8 J, or placebo. Compared to placebo therapy, the authors reported a higher number of repetitions when LEDT was applied 83.4 J and 166.8 J per site of irradiation. However, there was no effect on blood lactate levels or torque exerted by knee extensor muscles in MVIC.

Toma et al. [40] (level 1b) in a randomized, triple-blind, crossover, placebo-controlled trial used a muscular pre-conditioning protocol with LLLT (808 nm) on the *quadriceps femoris* muscles and assessed muscle fatigue by surface electromyography during 60 seconds of leg extension exercise, also measuring the maximum number of repetitions. The authors reported an increased number of repetitions in the LLLT group compared to the placebo, but surface electromyography showed no statistical difference. In a similar study, Dos Santos Maciel et al. [41] (level 1b) used a muscular pre-conditioning protocol with LLLT (780 nm) applied on the *tiabialis anterior* muscle and investigated fatigue in an isokinetic dynamometer combined with surface electromyography analysis. This double-blind controlled trial reported an increased torque exerted by the *tiabialis anterior* muscle after muscular pre-conditioning, but muscle fatigue analyzed by surface electromyography was not reduced, and lactate levels in blood were not significantly different between groups.

On the other hand, de Brito Vieira et al. [42] (level 1b) in a randomized, double-blind, crossover, placebo-controlled trial applied a photobiomodulation by LLLT (808 nm) on 5 sites of the *quadriceps femoris* muscles between 3 sets of 20 maximum voluntary repetitions in an isokinetic dynamometer at a single training session. Two days after the training session, all volunteers were evalutated through the number of maximum repetitions of knee flexion/extension in the isokinetic dynamometer in conjunction with surface electromyography to measure muscle fatigue. The authors reported increased number of repetitions in the LLLT group and less muscle fatigue compared to the placebo group. Similarly, aiming to investigate the responsivess of elderly woman to photobiomodulation, Vassao et al. [43] (level 1b) applied LLLT (808 nm) on 8 sites of the *rectus femoris* before a fatigue protocol in an isokinetic dynamometer in conjunction with surface electromyography and blood lactate analysis in a randomized, double-blind, crossover, placebo-controlled trial. The LLLT group had decreased blood levels of lactate, and reduced muscle fatigue assessed by surface electromyography, but were not able to improve their muscle performance in an isokinetic dynamometer.

da Silva Alves et al. [44] (level 1b) investigated the effects of PBM on cardiopulmonary exercise testing in conjunction with electromyography analysis performed on a cycle ergometer in a randomized, double-blind, crossover, placebo-controlled trial. The authors applied a muscular pre-conditioning protocol with a cluster of 7 laser diodes (850 nm) on three sites of the *quadriceps femoris* muscles (1 *vastus lateralis*, 1 *vastus medialis*, 1 *rectus femoris*) and 1 site on the gastrocnemius muscles. Compared to placebo, LLLT increased the peak O_2 uptake, cardiovascular efficiency, but had no effect on time of running, and fatigue in electromyography analysis.

Zagatto et al. [45] recently developed a randomized, double-blind, placebo controlled-trial to investigated the effects of PBM by LLLT on performance, muscle damage and inflammatory markers in water polo players. LLLT (810 nm) was applied on 8 points of the adductor muscle region immediately after each one of the 5 training days. The authors reported a moderate improvement in the 30-s crossbar jump test (performance) in the group treated with LLLT compared to placebo, but without further improvements in performance (200 m maximal swim). In addition, there was no significant differences between groups regarding muscle damage measuring lactate dehydrogenase (LDH) and CK, or inflammatory markers measuring interleukin 1 beta (IL-1β), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α).

In an attempt to find the best time point to apply PBM on muscles, Dos Reis et al. [46] (level 1b) compared the effectiveness of LLLT (830 nm) applied on the *quadriceps femoris* muscles as muscular pre-conditioning, or after an exercise protocol using leg extension with 75% of 1-RM until muscle fatigue in a randomized, double-blind, placebo-controlled trial. The authors reported that there was no significant difference regarding the number of maximum repetitions performed by all groups. However, lactate and CK levels in blood were lower in the group that was treated with LLLT after the exercise protocol when compared to placebo and LLLT delivered before the exercise.

Fritsch et al. [47] (level 1b) investigated the effects of photobiomodulation by LLLT (810 nm) on plyometric exercises, muscle damage (ultrasonography), soreness (visual analogue scale - VAS), torque in maximum voluntary contraction (MVC), as well as the best moment of irradiation: muscular pre-conditioning *versus* irradiation after a bout of exercise. This randomized, double-blind, placebo-controlled trial reported that both irradiation time points (muscular pre-conditioning and after exercise) promoted significant reduction in muscle damage compared to placebo (contralateral limb), but did not produce positive effects on VAS and MVC.

Recently Pinto et al. [48] (level 1b) investigated the effects of photobiomodulation using laser diodes and LEDs on muscle performance of rugby athletes in a randomized, crossover, double-blinded, placebo-controlled trial. These athletes received a muscular pre-conditioning protocol with laser diodes (905 nm) and LEDs (640 nm and 875 nm) assembled in the same device, or placebo therapy, before assessment in the Bangsbo Sprint Test at the training field, and blood lactate collection at 3, 10, 30 and 60 minutes after the Bangsbo Sprint Test. Compared to placebo therapy, the authors reported an improved average time for the sprints, lower fatigue index and lower percentage of blood lactate when athletes received the muscular pre-conditioning. However, PBM was not able to improve the best time for all the seven sprints performed in the Bangsbo Sprint Test.

Regarding the use of photobiomodulation in the sports field in real life competition, and trying to translate laboratory findings to clinical practice, Ferraresi et al. [49] (level 1b) used an array of 200 LEDs (100 LEDs at 850 nm and 100 LEDs at 630 nm) to test the effectiveness of PBM by LEDT for prevention of muscle damage in a randomized, double-blind placebo- controlled trial. The authors applied muscular pre-conditioning with LEDT on the *quadriceps femoris*, hamstrings and *triceps surae* muscles of professional volleyball

players before each official match during a national championship to prevent muscle damage measured by CK. In addition, this study tested 4 light energies (doses): 105 J, 210 J and 315 J and placebo. The authors reported that the effective light energies (210 J and 315 J) could prevent a statistically significant rise in CK while 105 J and placebo allowed significant increases in CK to occur in the blood 24 h after each official match.

Table 3 presents all parameters of PBM used in acute response to exercises with lower limbs. Figure 3 shows an example of the sites of irradiation on the *quadriceps femoris* muscles and an exercise test for lower limbs in isokinetic dynamometer in conjunction with surface electromyography analysis.

C. Acute photobiomodulation (PBM) responses in exercise on a treadmill

There have been several studies in the literature looking at the acute effects of PBM on muscles when used in muscular pre-conditioning regimens, and testing exercise performed on a treadmill. De Marchi et al. [50] (level 1b) in a randomized, double-blind, crossover, placebo-controlled trial used a cluster of 5 laser diodes (810 nm) applied on the *quadriceps femoris* muscles, hamstrings and *triceps surae* before a protocol of progressive and maximum effort on a treadmill. This study showed that LLLT increased both the relative and absolute oxygen uptake as well as the maximum time of running on the treadmill when compared with the placebo group. In addition, LDH, CK and lipid peroxidation (TBARS - thiobarbituric acid reactive substances) were significantly higher in the placebo group showing the PBM had protected against muscle damage. Finally, superoxide dismutase enzyme activity was lower in the placebo group after the exercise compared to the PBM group sugesting that PBM can protect aginst oxidative stress.

Analyzing the kinetics of oxygen uptake, Ferraresi et al. [51] (level 3b) conducted a randomized, double-blind, crossover, placebo-controlled trial with a single elite runner athlete. The authors applied a muscular pre-conditioning protocol on the lower and upper limbs and the trunk muscles using an array of 50 LEDs (850 nm) before high-intensity constant workload running exercise on a treadmill. Compared to placebo therapy, LEDT improved the speed of muscular VO_2 adaptation, decreased O_2 deficit, increased the VO_2 from the slow component phase, and increased the time limit of exercise. LEDT also decreased CK in blood (muscle damage) and reduced other markers of muscle damage and fatigue, which were alanine and lactate levels in the urine analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H NMR).

Miranda et al. [52] (level 1b) also conducted a randomized, double-blind, crossover, placebo-controlled trial to investigate the effects of PBM using a cluster probe with laser diodes (905 nm) and LEDs (640 nm and 875 nm) on cardiopulmonary exercise tests. The authors reported increases in the distance covered, time to exhaustion, ventilatory rate and less dyspnea when active PBM was applied on 9 sites of the *quadricesps femoris* muscles, 2 sites of the hamstrings and 2 sites on *gastrocnemius* muscles immediately before (muscular pre-conditioning) the cardiopulmonary exercise test.

However, recently a double-blind, crossover, placebo-controlled trial conducted by Malta et al. [53] (level 1b) investigated the effects of LEDT applied as muscular pre-conditioning to

improve high-intensity running effort in health subjects. This study used a cluster with 56 red (660 nm) and 48 infrared (850 nm) LEDs applied on the *quadriceps femoris* and *biceps femoris* muscles and between *soleus* and *gastrocnemius* region. The authors reported no effect of LEDT on maximal accumulated oxygen deficit, or contributions of the three different types of muscle metabolism (aerobic or glycolytic or phosphagen), time to exhaustion, peak lactate concentration, exhaustion perceived and heart rate at exhaustion. Moreover, the authors reported a possible negative effect of LEDT on maximal accumulated oxygen deficit and lactate based on the magnitude inference of effect size.

Table 4 presents all parameters of PBM used in acute response to exercises performed on treadmill. Figure 4 shows an example of the sites of irradiation on the *quadriceps femoris* muscles and a cardiopulmonary exercise testing on treadmill.

D. Chronic responses in clinical trials and case control studies

Ferraresi et al. [14] (level 1b) in a randomized clinical trial studied the effects of photobiomodulation (PBM) by LLLT (cluster with 6 laser diodes, 808 nm) on volunteers undertaking a strength training program using a load of 80% of 1-RM, twice a week during 12 weeks. Immediately after each training session, the LLLT was applied on 7 regions of the *quadriceps femoris* muscles. The authors reported an increased load in the 1-RM test achieved by the LLLT group compared to the control group and the groups undergoing training without any LLLT. In addition, only the LLLT group increased the knee peak torque extensor assessed by an isokinetic dynamometer.

Investigating resistance to muscle fatigue, Vieira et al. [15] (level 1b) in a randomized clinical trial measured the effects of LLLT (cluster with 6 laser diodes, 808 nm) on moderate training using a cycle ergometer performed 3 days per week during 9 weeks. LLLT was applied on 5 sites of the *quadriceps femoris* muscles immediately after each training session. The authors reported that only the LLLT group showed a reduced fatigue index of the knee extensor muscles in an isokinetic dynamometer.

Ferrerasi et al [54] (level 3b) recently conducted a randomized, double-blind, placebo controlled study in which a pair of identical twins were treated with a flexible 850 nm LED array (real or placebo delivered to different twins) applied on the *quadriceps femoris* muscles immediately after each strength training session (3X/week for 12 weeks). Real PBM (compared to placebo) increased the maximal load, muscle hypertrophy, expression of genes for hypertrophy and defense against oxidative stress; and decreased fatigue, markers of muscle damage and DOMS, expression of genes related to inflammation and muscle atrophy in muscle biopsies.

Baroni et al. [55] (level 1b) in a randomized clinical trial assessed the effects of LLLT (cluster with 5 laser diodes, 810 nm) applied as muscular pre-conditioning on the *quadriceps femoris* muscles during an eccentric training program in an isokinetic dynamometer, twice a week for 8 weeks. The thickness of the knee extensor muscle increased in the LLLT group compared to placebo assessed by ultrasonography, as well as significant increases in isometric knee extensor peak torque and eccentric knee extensor peak torque in an isokinetic dynamomenter.

Recently other studies have investigated the effects of training programs combined with PBM. Kakihata et al. [56] (level 1b) assessed the effects of LLLT (single probe, 660 nm) on vertical jump performance and delayed onset muscle soreness in healthy subjects during 2 weeks. Vertical jump performance was assessed at the first, fifth, eighth, twelfth and fifteenth days. Group 1 (control group) did not receive LLLT irradiation on the *triceps surae* before or after vertical jump assessments; group 2 received active LLLT between or before two vertical jump assessments and two days after for muscle recovery (6 sessions/days of LLLT); and group 3 received LLLT between or before two vertical jump assessments and six days after for muscle recovery (10 sessions/days of LLLT). The authors reported no improvement in vertical jump performance (muscle power and fatigue) and decay in delayed onset muscle soreness among all the groups.

Toma et al. [57] (level 1b) conducted a randomized, double-blind, placebo controlled trial with elderly women submitted to a strength training program in a leg extension chair twice a week for 8 weeks, combined or not with PBM by LLLT (single probe, 808 nm). The irradiation was applied on the *quadriceps femoris muscles* immediately after each training session. The authors reported that PBM increased work, peak torque and power in an isokinetic dynamometer in the training + LLLT group compared to the control group; there were no changes in fatigue index, lactate concentration, 6-min walk test and surface electromyography among all the groups (training + LLLT, training, control) as well as further differences between training + LLLT and training group.

Finally, a comparison between the effects of PBM on muscle performance when applied as muscular pre-conditioning and/or after a bout of exercise [46] or training program [58] was also investigated by Vanin et al. [59] (level 1b). The authors used laser diodes (905 nm) and LEDs (640 nm and 875 nm) assembled in the same device, such as used by the same research group in previous study [37]. The strength training program was based on their previous study [14], but adding leg extension exercises [54]. This study measured the peak torque in the maximum voluntary isometric contraction (MVIC), load in a 1-repetition maximum test (1-RM) and also measured thigh circumference (perimetry). The authors reported significant increases in MVIC, 1-RM in leg press and in 1-RM in leg extension when PBM was applied before (muscular pre-conditioning) each training session. PBM before (pre-conditioning) and after, or only PBM after each training session added no effect to muscle performance. Thigh perimetry was not increased with any type of PBM.

Figure 5 shows an exercise training program and an example of the sites of irradiation applied to the muscles of lower limbs after each training session. Table 5 presents all parameters of PBM in chronic response studies with exercise of lower limb muscles.

Discussion

The foregoing summary of the clinical results that have been obtained with PBM of muscles, will raise some interesting questions that remain to be answered by further research.

1. What is (are) the best wavelength(s) to use?

2. When is the best time to apply PBM on muscles? Before or after exercise? If before or after exercise, how long should the time interval between light and exercise be?
3. What are the best PBM parameters (irradiance, fluence, pulse structure)?
4. How many points or sites of irradiation should be used on each muscle group?
5. How exactly does PBM interact with muscle tissue on a biochemical level to increase sports performance?
6. Does the well-known biphasic dose response that is typical of PBM apply to muscles? In other words is it possible to use too much light?

There are several questions, such as those raised above, that a health professional or a sports physiologist will certainly require to be answered before PBM is widely adopted to improve sports performance. Based on the present literature review, the wavelengths employed have been mainly in the red (630–660 nm) and near-infrared (808–950 nm) spectral regions. However, although a previous study did not find any significant difference between red and NIR LLLT for improved muscle performance in upper limbs [22], there appears to be a preference for NIR wavelengths in published studies, possibly due to its better penetration into muscle tissue. Possibly motivated by the desire to “get the best of both worlds” there has been an increased use of mixed red and NIR wavelengths recently made possible by newly developed clusters and arrays of laser diodes or LEDs for PBM. See tables 2, 3, 4 and 5.

It is important highlight the scietific rationale for the use of red and NIR wavelengths at the same time. Our research group already reported previously [58,60] that irradiations with red and NIR wavelengths at the same time possibly offer advantages based on the absorption bands of the chromophores in the cells that absorb light [61–64], in special cytochrome c oxidase in the mitochondrial electric transport chain, resulting in even more synthesis of ATP than either red or NIR used alone [65,66].

One of the key questions is what is the appropriate time point to irradiate muscle tissue? The current literature shows there are two main strategies concerning the use of PBM to increase muscle performance and exercise recovery in clinical trials aiming at sports performance. The first strategy is a muscular pre-conditioning protocol, i.e., irradiation of muscle tissue usually about 3–5 minutes before the bout of exercise. There are several pieces of scientific evidence in favor of a muscular pre-conditioning protocol [1,67,68], when the purpose is to increase sports performance, reduce muscle damage and prevent pain developing after a single bout of exercise. This strategy deals with acute response simulating an athletic competition. This strategy generally seems to be effective for this purpose; however recent studies have suggested that in fact short times before the exercise such as 5 minutes may not in fact be the best time. Therefore, a recent study conducted by our research group reported a wide time window (time-response) for PBM to produce highly significant increases in mitochondrial metabolism and synthesis of ATP, with a best time occurring between 3–6 hours after irradiation [60].

Another study also conducted by our research group corroborated these aforementioned results, Ferraresi et al. [69] applied a muscular pre-conditioning protocol with PBM in mice, and measured the performance (ladder climbing carrying a load) after different time intervals. They found that the muscle performance increased more than 300–600% (compared to sham) after waiting for 3–6 hours. However a group that received PBM only 5 minutes before exercise did not show any significant difference to the sham group. In this context, other studies also reported a time-response for PBM to increase cytochrome c oxidase activity in rats with red and/or NIR wavelengths [70,71].

The effects of time-response with PBM on biological tissues is not new [72,65], but requires further investigation *in vitro*, *in vivo* and in clinical trials. In this perspective, a muscular pre-conditioning protocol delivered to professional volleyball players, 40–60 minutes before official matches was able to prevent statistically significant muscle damage measured by CK levels in the bloodstream [49], showing that muscular pre-conditioning applied 3–5 minutes before a bout of exercise may not actually be the best time point. Moreover, it is very important to note that several studies (see tables 2, 3 and 4) applied a muscular pre-conditioning PBM protocol using LLLT or LEDT, or both together, and in general showed positive effects in preventing muscle damage and increase muscle performance from 1 h until 72–96 h. These results reinforce previous results *in vitro* [60,72,65] and *in vivo* [69–71] regarding the best time window or time-response for PBM to maintain its beneficial effects on biological tissues, may also apply to skeletal muscles in humans.

The second strategy is to apply PBM using LLLT or LEDT immediately after each bout of exercise in order to accelerate muscle recovery [1,46]. This strategy appears to be especially effective when used in combination with regular exercise training programs that can last for days or even weeks [58,14]. In addition, the use of PBM after each session training of exercise training programs also seems to increase the potential gains of performance, including defense against oxidative stress, muscle cell proliferation, energy muscle content (glycogen and ATP) and mitochondrial metabolism [58], in addition to several other effects reported previously (see review [1]). However, this issue is not completely clear in the literature, since a recent study reported better results in favor of muscular pre-conditioning in training programs [59]. We believe that further investigations to answer this question in are necessary.

The parameters of PBM reported in the literature show a large degree of variation. The total power of the device, time of irradiation, total energy of the light delivered to muscles, the energy density or fluence (J/cm^2), and the power density or irradiance (mW/cm^2) have not been standardized, and wide variations are apparent between the parameters used in different studies. One of the main factors that contributes to this variability is the wide diversity of different devices available, that can either be custom-made or commercially available, and exactly how they are used by the researchers. However, based on the present review, it is possible to establish a range for the aforementioned PBM parameters, mainly light dose (J), that produced positive effects in muscular pre-conditioning, or irradiations after a bout of exercise or training program.

We could identify a therapeutic window, or PBM window, suggesting a biphasic dose-response [73,74] for total energy applied on the *biceps brachii* (20–80 J), regardless whether PBM was applied as a muscular pre-conditioning protocol or after exercise. Parameters within the therapeutic window increased the number of repetitions, time of contractions, and decreased delayed onset muscle soreness (DOMS) in elbow flexion exercises. However, some studies presented ambiguous results (either positive or no effects) regarding these outcomes [21,27]. It is important to highlight that 41.7 J was the maximum energy per site of irradiation using cluster of LEDs, while 5 J was the maximum energy per point of irradiation using laser probes. Studies that applied total energy inside the identified range (20–80 J), but applied more than 41.7 J per site of irradiation, or more than 5 J per point of irradiation did not achieve positive effects [9,28] [Figure 6]. Finally, the light power and total time of irradiation ranged from 50 mW to 1500 mW and 30 seconds to 720 seconds, respectively.

For *quadriceps femoris* muscles, PBM used acutely also produced a biphasic dose-response [73,74]. to increase fatigue resistance or increase number of repetitions, increase muscle force or work, and decrease CK or related markers of muscle damage [12,30–40,42,43,46–49]. The range in total energy (J) identified to produce the most positive effects was 56–315 J, regardless if PBM was applied as muscular pre-conditioning or after exercise. It is important to note that only energy applied on the *quadriceps femoris* muscles of the references [33,48,49] were considered in the years 2011, 2015 and 2016, respectively [Figure 7]. The light power and total time of irradiation ranged from 60 mW to 2500 mW and 60 seconds to 3876 seconds, respectively.

A possible explanation for the negative or ambiguous effects of PBM parameters that were within the range of light dose (J) identified, could be attributed to: a) the type of test used to assess muscle performance and/or induce muscle damage (Wingate tests) [31–33], b) differences between cluster of lasers/LEDs as well as single laser probes, c) diferences between the populatons treated or enrolled in each study [38,35]. We suggest further investigations are needed to clarify these results.

Regarding exercise performed on a treadmill, the PBM effects on oxygen uptake or ventilatory responses, time of running, and muscle damage (CK) were more pronounced with light doses (J) ranging from 360–510 J applied on the body (mainly the lower limbs [50,52], plus trunk and upper limbs [51]) [Figure 8]. The light power and total time of irradiation ranged from 131.25 mW to 2500 mW and 360 seconds to 3876 seconds, respectively. Regarding the study conducted by Malta et al. [53], the authors applied 60 J per site of irradiation. As discussed for the *biceps brachii*, possibly 60 J per site of irradiation may be excessive.

PBM in conjunction with exercise training programs have demonstrated positive effects on *quadriceps femoris* muscles regarding 1-RM, torque, fatigue resistance [14,15,54,55,57,59], and ambiguous effects on hypertrophy and muscle work [14,15,57,59]. We could identify a large range in light dose (J) applied on *quadriceps femoris* muscles in chronic response (18–240 J) [Figure 9]. The light power and total time of irradiation ranged from 100 mW to 5000 mW and 15 seconds to 1368 seconds, respectively. These results require further

investigation, mainly regarding the best time to apply PBM (muscular pre-conditioning *versus* after exercise).

Regarding the number of points or the arrangement of sites of irradiation on the muscles, the reports show a larger number on the lower limbs, where the muscles are naturally larger than the muscles of the arms. It appears logical to irradiate as much of the muscle area as possible [14,1], but avoiding excessive light doses (energy - J) per muscle group.

We believe that it will be necessary to arrive at a standardization of the parameters of light irradiation [power of the light (mW), energy (J), fluence (J/cm^2), irradiance (mW/cm^2), number of irradiation points or irradiated area] as well as devices for PBM in sports/exercise settings. Although there are several important positive results already achieved in laboratory settings, some results of PBM are contradictory between studies both within and between research groups. This issue is evident after a thorough analysis of all parameters of PBM and results summarized in each of the tables in this review. While some studies report effects in favor of PBM (positive effects), other studies report no effects using the same or similar light parameters. Some examples: references [13] and [21] disagree about the total time of contractions (table 2); references [23] and [28] disagree about the total number of repetitions (table 2); references [35] and [38] disagree about the light dose of 30 J per site of irradiation (table 3); references [50] and [53] disagree regarding time to exhaustion (table 4). However, there are more positive effects in favor of PBM than there are conflicting results or negative results. In addition if all the positive results achieved in laboratory settings go on to demonstrate comparable improvements in sports performance in the real world, PBM will become very popular mainly amongst high level athletes.

It must be noted if the use of PBM becomes widespread in high level sports competitions, especially before major National or International competitions, or during athletic training, it is possible that the World Anti-Doping Agency (WADA) or the International Olympic Committee (IOC) will need to discuss the position they will take on whether PBM will be allowed or not. If they decide it should not be allowed, they will be faced with the tricky problem of how to forensically detect if muscles have been exposed to light? We cannot envisage a biochemical test that could be conducted on blood or urine that would conclusively detect whether muscles had been exposed to the “banned light” used in PBM. However we believe that discussions about this issue will happen soon.

CONCLUSION

After an extensive search of the literature, with considerable selection and analysis, all the studies included in this review matched our primary objective: to determine the effects of photobiomodulation (PBM) in clinical trials that enrolled both untrained and trained healthy subjects, or athletes. Moreover, all parameters have been assessed and summarized in tables in the present review and these allowed us to identify ranges in PBM parameters that promoted positive effects (“worked”), gave no effects (did not “work”) and some studies had ambiguous effects (both positive and no effects). However, the identified ranges in light dose (J) are not designed to be recommendations, or prescriptions of the best light dose to be used in future studies or clinical practice at present. We are aware of several factors that affect

light penetration and absorption by human tissues, and the existence of the biphasic dose-response that is typical of PBM. All these considerations may preclude a final prescription of the exact doses of light for general use. Finally, we suggest caution be exercised in generalization of the findings.

LIMITATIONS

This review was conducted searching only one database (MEDLINE via Pubmed) and did not assess the risk of bias and quality of evidence by the GRADE or PEDRo approach. In addition, this review did not perform a comparative analysis (meta-analysis). However, to our best knowledge, such analysis could not be easily accomplished due to the lack of consistency in PBM parameters, and the lack of uniformity in PBM devices found in current literature.

Acknowledgments

MRH was funded by US NIH grant AI050875. CF was supported by CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico (Scholarship 202313/2014-0).

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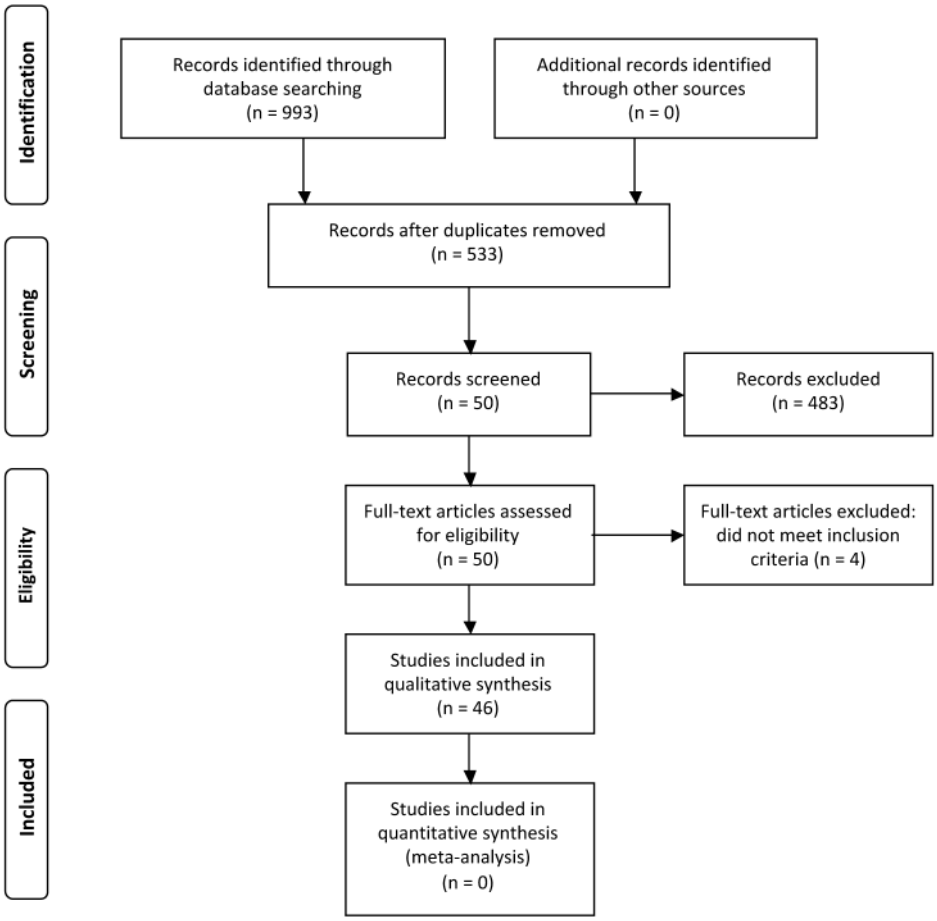

Figure 1.
Flow diagram of the systematic review recommended by PRISMA [15]

Figure 2.

A) Example of muscular pre-conditioning irradiating multiple sites or points on the *biceps brachii*. **B)** Exercise test on a Scott bench.

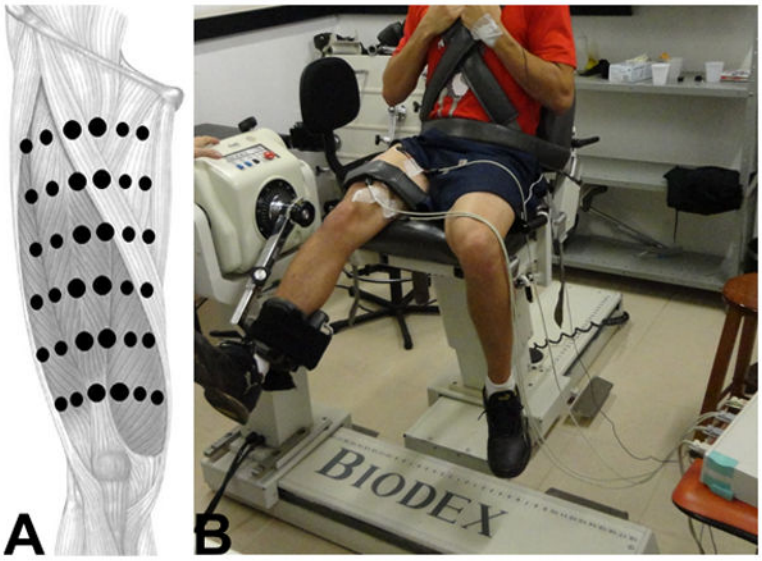

Figure 3.

A) Muscular pre-conditioning irradiating multiple sites or points of the *quadriceps femoris* muscles in order to cover the whole muscle group. **B)** Exercise testing in an isokinetic dynamometer in conjunction with surface electromyography analysis.

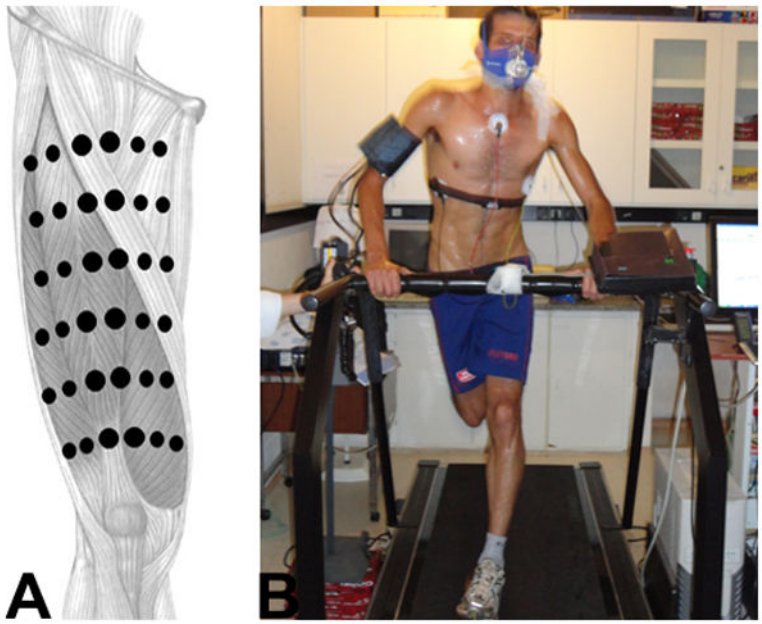

Figure 4.

A) Muscular pre-conditioning irradiating multiple sites or points of the *quadriceps femoris* muscles in order to cover all muscle group. **B)** Cardiopulmonary exercise testing on a treadmill.

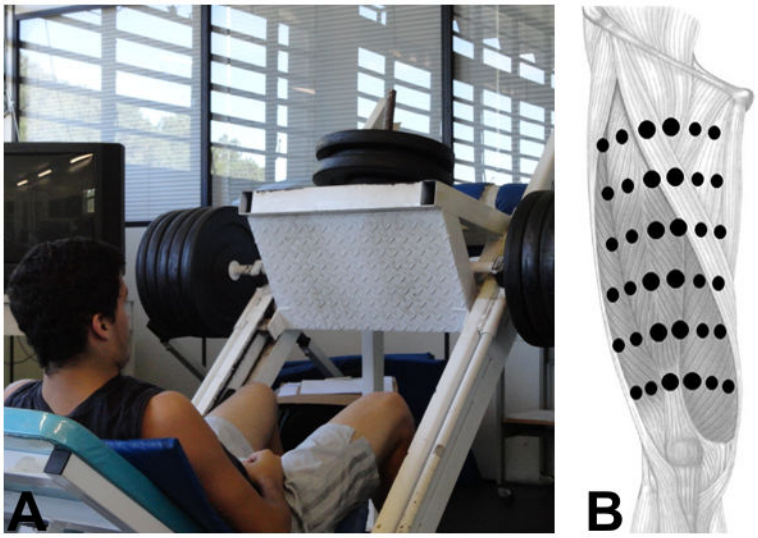

Figure 5.

A) Exercise training program in a leg press. **B)** Photobiomodulation irradiating multiple sites or points of the *quadriceps femoris* muscles in order to cover all the muscle group applied after each training session.

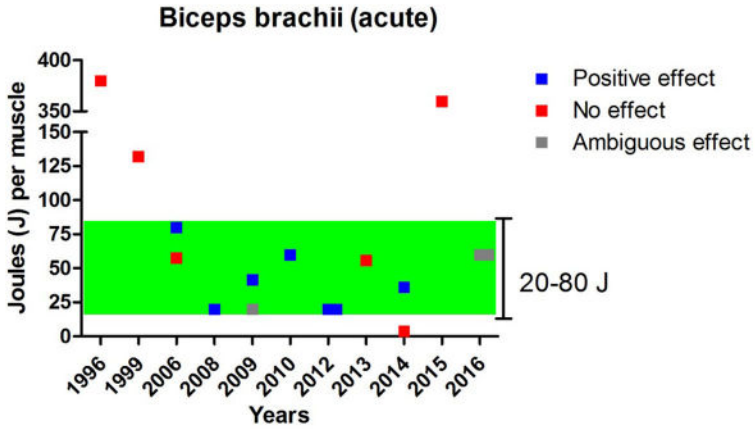

Figure 6.

Total energy (Joules - J) applied on *biceps brachii* that produced positive effects, no effects, and ambiguous effects (positive and no effects) on the following outcomes: fatigue resistance or number of repetitions, time of contraction and delayed onset muscle soreness (DOMS) in studies included in this review.

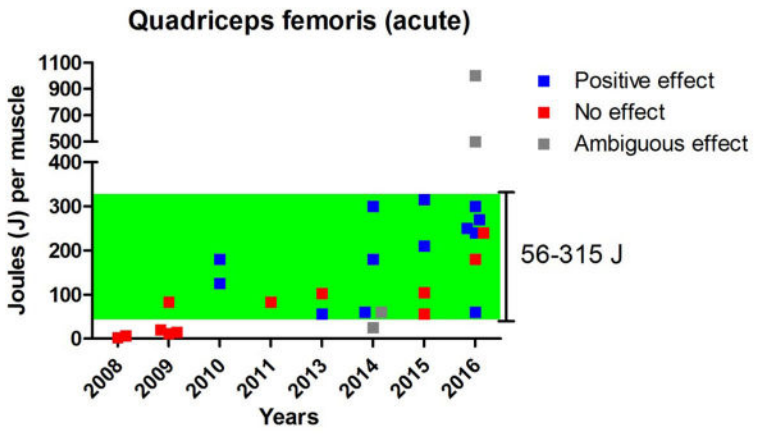

Figure 7.

Total energy (Joules - J) applied on *quadriceps femoris* muscles that produced positive effects, no effects, and ambiguous effects (positive and no effects) on the following outcomes: fatigue resistance or number of repetitions, muscle force or work, and creatine kinase (CK) or a related marker of muscle damage in studies included in this review.

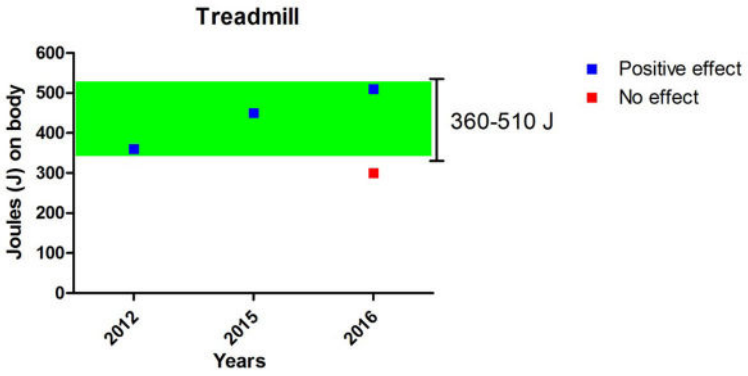

Figure 8.

Total energy (Joules - J) applied on body muscles that produced positive effects or no effects on the following outcomes: oxygen uptake or ventilatory responses, time of running, and muscle damage (CK) in studies included in this review.

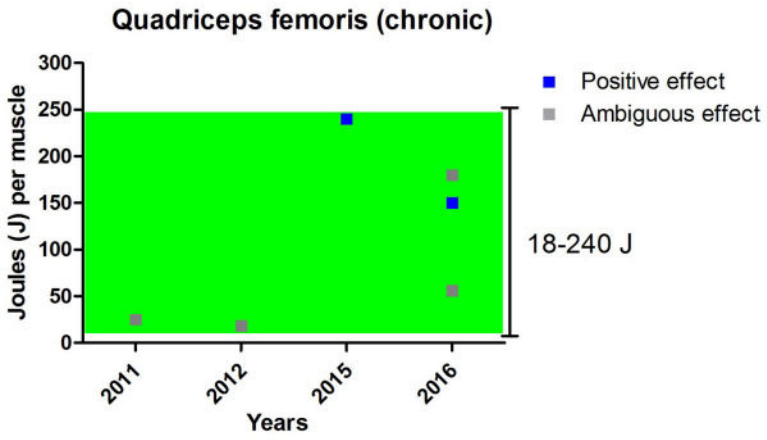

Figure 9.

Total energy (Joules - J) applied on *quadriceps femoris* muscles during training programs that produced positive effects or ambiguous effects on the following outcomes: 1-RM, torque, fatigue resistance, hypertrophy and muscle work in studies included in this review.

Table 1

Search strategy via Pubmed and number of identified studies

Term	# studies retrieved
#1 LLLT AND muscle damage	43
#2 LEDT AND muscle damage	6
#3 Phototherapy AND muscle damage	119
#4 LLLT AND CK	28
#5 LEDT AND CK	5
#6 Phototherapy AND CK	41
#7 LLLT AND fatigue	60
#8 LEDT AND fatigue	13
#9 Phototherapy AND fatigue	152
#10 LLLT AND time to exhaustion	9
#11 LEDT AND time to exhaustion	4
#12 Phototherapy AND time to exhaustion	11
#13 LLLT AND lactate	46
#14 LEDT AND lactate	7
#15 Phototherapy AND lactate	186
#16 LLLT AND DOMS	3
#17 LEDT AND DOMS	0
#18 Phototherapy AND DOMS	7
#19 LLLT an AND d torque	17
#20 LEDT AND torque	1
#21 Phototherapy AND torque	20
#22 LLLT AND 1RM	2
#23 LEDT AND 1RM	0
#24 Phototherapy AND 1RM	2
#25 LLLT AND repetition	44
#26 LEDT AND repetition	1
#27 Phototherapy AND repetition	63
#28 LLLT AND MVC	10
#29 LEDT AND MVC	2
#30 Phototherapy AND MVC	63
#31 LLLT AND MVIC	0
#32 LEDT AND MVIC	1
#33 Phototherapy AND MVIC	1
#34 LLLT AND treadmill	12
#35 LEDT AND treadmill	1
#36 Phototherapy AND treadmill	13

Table 2

Clinical trials of photobiomodulation (PBM) in acute responses in exercises with upper limb muscles

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
[10]	Cluster with 31 laser diodes; 660 nm; 950 nm; Cluster area 12 cm^2; 527.77 mW (calculated) 31.7 J/cm^2 (720 s) Pulsed frequencies (Hz): 2.5; 5; 20; 1 site of irradiation per limb Total delivered: 380 J (calculated)	Contact After exercise	1 site of irradiation on *biceps brachii* Elbow flexion (free weights)	RCT with 60 subjects (12 control; 12 placebo; 36 LLLT) No effect of PBM in DOMS No effect of PBM in mechanical pain threshold No effect of PBM in MPT, VAS and McGill questionnaire
[11]	Cluster with 31 laser diodes; 660 nm; 950 nm; Cluster area 12 cm^2; 534 mW 11 J/cm^2 (240 s) Pulsed frequency (Hz): 73 1 site of irradiation per limb Total delivered: 132 J (calculated)	Contact After exercise	1 site of irradiation on *biceps brachii* Elbow flexion (free weights)	RCT with 36 subjects (12 control, 12 placebo, 12 LLLT) No effect of PBM in DOMS No effect of PBM in mechanical pain threshold No effect of PBM in MPT, VAS and McGill questionnaire
[9]	Cluster with 32 LEDs; 950 nm; Cluster area 18 cm^2; 160 mW; 8.8 mW/cm^2 (calculated) 3.2 J/cm^2 (360 s); 1 site of irradiation per limb Total delivered: 57.6 J (calculated)	Contact After exercise	1 site of irradiation on *biceps brachii* Elbow flexion (isokinetic dynamometer)	RCT with 32 subjects (16 placebo; 16 LEDT) No effect of PBM in DOMS No effect of PBM in mechanical pain threshold No effect of PBM in isokinetic peak torque
[8]	Cluster with 36 LEDs; 32 LEDs 880 nm; 4 LEDs 660 nm; Cluster area 5 cm^2; 500 mW (calculated); 100 mW/cm^2; 40 J per site of irradiation (80 s); 8 J/cm^2; 2 sites of irradiation per limb Total delivered: 80 J	Contact After exercise	2 sites of irradiation on *biceps brachii* Elbow flexion (Scott bench)	RCT with 27 subjects (9 Control; 9 Placebo; 9 LEDT) PBM decreased DOMS after 48 h No effect of PBM in girth No effect of PBM in resting extension angle
[13]	Laser diode 655 nm; Diode area 0.1 cm^2; 50 mW; 500 J/cm^2; 5 W/cm^2; 5 J per diode (100 s); 4 sites of irradiation per limb Total delivered: 20 J	Contact Muscular pre-conditioning	4 sites of irradiation on *biceps brachii* Elbow flexion (Scott bench) Maximum voluntary contraction until exhaustion	RCT with 12 volleyball players (6 Placebo; 6 LLLT) PBM increased number of repetitions PBM increased total time of contractions No effect of PBM in blood lactate
[21]	Laser diode 830 nm; Diode area 0.0028 cm^2; 100 mW; 35.7 W/cm^2; 5 J (50 s), 1,785 J/cm^2; 4 sites of irradiation per limb Total delivered: 20 J	Contact Muscular pre-conditioning	4 sites of irradiation on *biceps brachii* Elbow flexion (Scott bench) Maximum voluntary contraction until exhaustion	RCT with 10 volleyball players (crossover study) PBM increased number of repetitions No effect of PBM in blood lactate No effect of PBM in total time of contractions
[22]	Laser diode 660 nm; Diode area 0.0028 cm^2; 50 mW; 17.85 W/cm^2; 5 J (100 s); 1,785 J/cm^2; 4 sites of irradiation per limb Total delivered: 20 J *versus* Laser diode 830 nm; Diode area 0.0028 cm^2; 50 mW; 17.85W/cm^2; 5 J (100 s), 1,785 J/cm^2; 4 sites of irradiation per limb	Contact Muscular pre-conditioning	4 sites of irradiation on *biceps brachii* Elbow flexion (Scott bench) Maximum voluntary contraction (60 s)	RCT with 10 subjects (crossover study) PBM 630 nm increased mean force PBM 630 nm increased peak force PBM 830 nm increased mean force PBM 830 nm increased peak force

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
	Total delivered: 20 J			
[23]	Cluster with 5 laser diodes 810 nm; Diode area 0.0364 cm^2; 200 mW; 5.495 W/cm^2; 6 J per diode (30 s); 164.85 J/cm^2; 30 J per site of irradiation: 5 × 6 J Total delivered: 60 J	Contact Muscular pre-conditioning	2 sites of irradiation *on biceps brachii* Elbow flexion (Scott bench) Maximum voluntary contraction until exhaustion	RCT with 9 volleyball players (crossover study) PBM increased number of repetitions PBM increased total time of contractions PBM decreased blood lactate 5 min post-exercise PBM decreased CK in blood post-exercise PBM decreased C-reactive protein post-exercise
[24]	Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 850 nm; 30 mW; 150 mW/cm^2; 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.9 J LED 850 nm (30 s), 4.5 J/cm^2; 41.7 J per site of irradiation; 1 site of irradiation per limb Total delivered: 41.7 J	Contact Muscular pre-conditioning	1 site of irradiation *on biceps brachii* Elbow flexion (Scott bench) Maximum voluntary contraction until exhaustion	RCT with 10 volleyball players (crossover study) PBM increased number of repetitions PBM decreased blood lactate post-exercise PBM decreased CK in blood post-exercise PBM decreased C-reactive protein post-exercise
[25]	LED 630 nm; LED area 1.77 cm^2; 300 mW; 169.49 mW/cm^2; 9 J (30 s); 5.1 J/cm^2; 4 sites of irradiation per limb Total delivered: 36 J	Contact Muscular pre-conditioning	4 sites of irradiation on *biceps brachii* Elbow flexion (Scott bench) 30 eccentric contraction	RCT with 17 subjects (9 placebo; 8 LEDT) PBM decreased DOMS from 48 h to 96 h post-exercise PBM decreased isometric force loss from 24 h to 96 h PBM improved range of motion from 24 h to 96 h
[26]	Laser diode 808 nm; Diode area 0.0028 cm^2; 100 mW; 35.71 W/cm^2; 1 J (10 s), 357.14 J/cm^2 4 sites of irradiation per limb Total delivered: 4 J	Contact Between sets of exercise	4 sites of irradiation on *biceps brachii* Elbow flexion (biceps curl) 10 sets of 10 repetitions	RCT with 22 subjects (11 placebo; 11 LLLT) No effect of PBM in 1-repetition maximum (1-RM) PBM decreased CK in blood after 72 h post-exercise
[27]	Large cluster with 33 diodes: 5 laser diodes; 850 nm; 100 mW; 0.06 cm^2; 1,666.6 mW/cm^2; 3.2 J (32 s); 53.3 J/cm^2 each; 12 LEDs; 670 nm; 10 mW; 1.92 cm^2; 5.20 mW/cm^2; 0.3 J (32 s); 0.15 J/cm^2 each; 8 LEDs; 980 nm; 25 mW; 1.28 cm^2; 19.53 mW/cm^2; 0.8 J (32 s); 0.62 J/cm^2 each 8 LEDs; 950 nm; 15 mW; 1.28 cm^2; 11.71 mW/cm^2; 0.5 J (32 s); 0.39 J/cm^2 each 2 sites of irradiation per limb Total delivered: 60 J *versus* Small cluster with 9 diodes: 5 laser diodes; 850 nm; 100 mW; 0.06 cm^2; 1,666.6 mW/cm^2; 5.6 J (56 s); 93.3 J/cm^2 each; 4 LEDs; 670 nm; 10 mW; 0.64 cm^2; 15.62 mW/cm^2; 0.56 J (56 s); 0.87 J/cm^2 each 2 sites of irradiation per limb Total delivered: 60 J	Contact Muscular pre-conditioning	2 sites of irradiation on *biceps brachii* Maximum isometric voluntary contraction until exhaustion (isokinetic dynamometer)	RCT with 10 subjects (crossover study) PBM increased time to exhaustion No effect of PBM on peak torque No effect of PBM on electromyography analyses
[28]	Laser diode 808 nm; Diode area 0.0028 cm^2; 100 mW; 35.7 W/cm^2; 7 J (70 s), 2,500 J/cm^2;	Contact Muscular pre-conditioning	8 sites of irradiation on *biceps brachii* Elbow flexion	RCT with 20 subjects (crossover study) No effect of PBM in number of repetitions No effect of PBM in blood lactate post-exercise

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
	8 sites of irradiation per limb Total delivered: 56 J			No effect of PBM on fatigue by surface electromyography
[29]	Cluster with 2 laser diodes 800 nm and 970 nm; Diode area not provided; 3 W (50% duty cycle); average power output 1.5 W; 24 J point of irradiation (16 s -calculated); 15 sites of irradiation per limb Total delivered: 360 J	Contact Muscular pre-conditioning	15 sites of irradiation on *biceps brachii* Elbow flexion (isokinetic dynamometer)	RCT with 39 subjects (crossover study) PBM decreased discreetly maximum voluntary isometric contraction No effect of PBM on muscle tenderness No effect of PBM on muscle strength

Table 3

Clinical trials of photobiomodulation (PBM) in acute responses in exercises using lower limb muscles

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
[12]	Cluster with 4 laser diodes 808 nm; 500 mW; 8.3 mW/cm^2; Groups: 7 J (10 min); 3 J (5 min); 1 site of irradiation per limb Total delivered: 7 J/3 J	Scanning Muscular pre-conditioning	1 site of radiation on *quadriceps femoris* Maximum voluntary contraction	RCT with 5 subjects (crossover study) No effect of PBM on muscle fatigue
[30]	Laser diode 830 nm; Diode area 0.0028 cm^2; 100 mW; 35.714 W/cm^2; Volleyball athletes: 4 J (40 s); 1,428.57 J/cm^2 Soccer Athletes: 3 J (30 s); 1,071.43 J/cm^2 5 sites of irradiation per limb Total delivered: 20 J (volleyball athletes)/15 J (soccer athletes)	Contact Muscular pre-conditioning	5 sites of irradiation *on rectus femoris* Wingate test	RCT with 20 athletes (crossover study) PBM decreased blood lactate 15 min post-exercise PBM decreased CK in blood 3 min post-exercise No effect of PBM on muscle work
[31]	Laser diode 810 nm; Diode area 0.036 cm^2; 200 mW; 5.5 W/cm^2; 6 J (30 s); 164.84 J/cm^2; 2 sites of irradiation per limb Total delivered: 12 J *Versus* Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 850 nm; 30 mW; 150 mW/cm^2; 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.9 J LED 850 nm (30 s), 4.5 J/cm^2; 41.7 J per site of irradiation; 2 sites of irradiation per limb Total delivered: 83.4 J	Contact Muscular pre-conditioning	2 sites of radiation on *rectus femoris* Wingate test	RCT with 8 athletes (crossover study) PBM (LEDT) decreased CK in blood 3 min post-exercise No effect of PBM (LLLT) on muscle peak and mean power No effect of PBM (LEDT) on muscle peak and mean power No effect of PBM (LLLT) in CK 3 min post-exercise No effect of PBM (LLLT) in blood lactate post-exercise No effect of (LEDT) in blood lactate post-exercise
[32]	Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 950 nm; 15 mW; 75 mW/cm^2; 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.4 J LED 950 nm (30 s), 2.2 J/cm^2; 25.9 J per site of irradiation; 4 sites of irradiation per limb Total delivered: 103 J	Contact Between 2° and 3° Wingate	2 sites of radiation on *rectus femoris* 1 site on *vastus lateralis* 1 site on *vastus medialis* Wingate test	RCT with 18 athletes (crossover study) No effect of PBM on muscle peak power No effect of PBM on fatigue index No effect of PBM in blood lactate levels
[33]	Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 850 nm; 30 mW; 150 mW/cm^2; 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.9 J LED 850 nm (30 s), 4.5 J/cm^2; 41.7 J per site of irradiation 5 sites of irradiation per limb	Contact After exercise	2 sites of irradiation on *quadriceps femoris* 2 sites on hamstrings 1 site on gastrocnemius 3 Wingate tests	RCT with 6 athletes (crossover study) PBM decreased CK in blood post-exercise PBM decreased blood lactate post-exercise No effect of PBM on muscle peak power No effect of PBM on mean muscle power No effect of PBM in C-reactive protein

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
	Total delivered: 208.5 J *Versus* Cryotherapy (water immersion for 5 minutes at 5 ° C)			
[34]	Cluster with: 1 laser diode 905 nm; Diode area 0.44 cm^2; 1.25 mW; 2.84 mW/cm^2; 4 LEDs 875 nm; Diode area 0.9 cm^2; 17.5 mW; 19.44 mW/cm^2; 4 LEDs 640 nm; Diode area 0.9 cm^2; 15 mW; 16.67 mW/cm^2; 39.37 J per site of irradiation: 300 s 6 sites of irradiation per limb Total delivered: 240 J	Contact After exercise	2 sites of irradiation on *vastus medialis* 2 sites on *vastus laterallis* 2 sites on *rectus femoris* Maximum voluntary isometric contraction (isokinetic dynamometer)	RCT with 50 subjects (10 placebo; 10 cryotherapy, 10 PBM; 10 PBM + cryotherapy; 10 cryotherapy + PBM) PBM decreased CK post-exercise from 24 h to 96 h PBM decreased DOMS from 24 h to 96 h PBM improved MIVC PBM+cryotherapy reduced efficacy of PBM Cryotherapy+PBM and cryotherapy were similar to placebo
[35]	Cluster with 5 laser diodes 810 nm; Diode area 0.029 cm^2; 200 mW; 6.89 W/cm^2; 6 J per diode (30 s); 206.89 J/cm^2; 30 J per site of irradiation: 5 × 6 J 6 sites of irradiation per limb Total delivered: 180 J	Contact Muscular pre-conditioning	2 sites of irradiation on *vastus medialis* 2 sites on *vastus laterallis* 2 sites on *rectus femoris* Maximum voluntary isometric contraction (isokinetic dynamometer)	RCT with 36 subjects (18 placebo; 18 LLLT) PBM increased maximum voluntary contraction PBM increased LDH activity 48 h post-exercise PBM decreased blood lactate 24 h and 48 h post-exercise PBM decreased CK in blood 24 h and 48 h post-exercise No PBM effect in DOMS
[36]	Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 850 nm; 30 mW; 150 mW/cm^2; 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.9 J LED 850 nm (30 s), 4.5 J/cm^2; 41.7 J per site of irradiation (30 s) 3 sites of irradiation per limb Total delivered: 125.1 J	Contact Muscular pre-conditioning	1 site of radiation on *rectus femoris* 1 site on *vastus medialis* 1 site on *vastus lateralis* Maximum voluntary isometric contraction (isokinetic dynamometer)	RCT with 17 subjects (crossover study) PBM decreased the decay of the knee extensor peak torque after a fatigue exercise protocol
[37]	Cluster with: 4 laser diodes 905 nm; Diode area 0.44 cm^2; 0.3125 mW; 0.71 mW/cm^2; 4 LEDs 875 nm; Diode area 0.9 cm^2; 17.5 mW; 19.44 mW/cm^2; 4 LEDs 640 nm; Diode area 0.9 cm^2; 15 mW; 16.66 mW/cm^2; Groups: 10 J per site of irradiation: 76 s 30 J per site of irradiation: 228 s 50 J per site of irradiation: 381 s 6 sites of irradiation per limb Total delivered: 60 J/180 J/300 J	Contact Muscular pre-conditioning	2 sites of irradiation on *vastus medialis,* 2 sites on *vastus lateralis* 2 sites on *rectus femoris* Eccentric contractions and maximum voluntary isometric contraction (isokinetic dynamometer)	RCT with 40 subjects (10 placebo; 30 PBM) PBM 10 J, 30 J and 50 J increased the percentage of the knee extensor peak torque immediately after and until 96 h after eccentric exercise protocol PBM 30 J and 50 J decreased DOMS PBM 10 J, 30 J and 50 J decreased CK in blood from 1 h to 96 h
[38]	Cluster with 5 laser diodes 810 nm; Diode area 0.036 cm^2; 200 mW; 5.495 W/cm^2; 2 J per diode (10 s); 54.95 J/cm^2; 6 J per diode (30 s); 164.84 J/cm^2; 10 J per diode (50 s); 274.73 J/cm^2;	Contact Muscular pre-conditioning	2 sites of irradiation on *vastus medialis* 2 sites on *vastus laterallis* 2 sites on *rectus femoris*	RCT with 28 athletes (7 placebo; 21 LLLT) PBM 50 J increased knee extensor peak torque immediately after and until 96 h after eccentric exercise protocol

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
	Groups: 10 J per site of irradiation: 5 × 2 J 30 J per site of irradiation: 5 × 6 J 50 J per site of irradiation: 5 × 10 J 6 sites of irradiation per limb: Total delivered: 60 J/180 J/300 J		Maximum voluntary isometric contraction (isokinetic dynamometer)	PBM 10 J increased knee extensor peak torque after 24 h until 96 h after eccentric exercise protocol PBM 10 J and 50 J decreased CK and IL-6 from 24 h to 96 h PBM (10 J, 30 J and 50 J) had no effect on DOMS PBM 30 J had any effect in all assessments
[39]	Cluster with 69 LEDs; LED area 0.2 cm^2; 34 LEDs 660 nm; 10 mW; 50 mW/cm^2; 35 LEDs 850 nm; 30 mW; 150 mW/cm^2; Groups: 0.3 J LED 660 nm (30 s), 1.5 J/cm^2; 0.9 J LED 850 nm (30 s), 4.5 J/cm^2; 41.7 J per site of irradiation (30 s) 0.6 J LED 660 nm (60 s), 3.0 J/cm^2; 1.8 J LED 850 nm (60 s), 9.0 J/cm^2; 83.4 J per site of irradiation (60 s); 1.2 J LED 660 nm (120 s), 6.0 J/cm^2; 3.6 J LED 850 nm (120 s), 18 J/cm^2; 166.8 J per site of irradiation (120 s); 6 sites of irradiation per limb Total delivered: 250.2 J/500.4 J/1,000.8 J	Contact Muscular pre-conditioning	2 site of radiation on *rectus femoris* 2 sites of irradiation on *vastus medialis* 2 sites of irradiation on *vastus lateralis* Maximum voluntary isometric contraction and number of repetitions (isokinetic dynamometer)	RCT with 34 subjects (crossover study) PBM (83.4 J) increased number of repetition (knee extension) PBM (166.8 J) increased number of repetition (knee extension) No effects of PBM (41.7 J) in number of repetition (knee extension), torque or blood lactate levels. No effects of PBM (all doses) on blood lactate levels and torque.
[40]	Laser diode 808 nm; Diode area 0.00785 cm^2; 100 mW; 12.7 W/cm^2; 7 J (70 s), 892 J/cm^2; 8 sites of irradiation per limb Total delivered: 56 J	Contact Muscular pre-conditioning	8 sites of irradiation on *rectus femoris* Knee extension (extensor chair)	RCT with 24 subjects (crossover study) PBM increased number of repetitions No effect of PBM on surface electromyography
[41]	Laser diode 780 nm Diode area 0.2 cm^2; 30 mW; 0.15 W/cm^2; 0.81 J (27 s), 4 J/cm^2; 29 sites of irradiation per limb Total delivered: 23.49 J	Contact Muscular pre-conditioning	29 sites of irradiation on *tibialis anterior* Ankle dorsiflexion (isokinetic dynamometer)	Controlled trial with 12 subjects (crossover study) PBM increased ankle dorsiflexion peak torque in strength test No effect of PBM on surface electromyography No effect of PBM in blood lactate
[42]	Laser diode 808 nm; Diode area 0.0028 cm^2; 100 mW; 35.71 W/cm^2; 4 J (40 s); 1,428.57 J/cm^2; 5 sites of irradiation per limb Total delivered: 20 J × 3 times = 60 J	Contact Between sets of exercise	3 sites of irradiation on *rectus femoris* 1 site on *vastus lateralis* 1 site on *vastus medialis* Knee extension (isokinetic dynamometer)	RCT with 7 subjects (crossover study) PBM increased number of repetitions PBM decreased fatigue index in surface electromyography
[43]	Laser diode 808 nm; Diode area 0.028 cm^2; 100 mW; 3.57 W/cm^2; 7 J (70 s), 250 J/cm^2; 8 sites of irradiation per limb Total delivered: 56 J	Contact Muscular pre-conditioning	8 sites of irradiation on *rectus femoris* Knee extension (isokinetic dynamometer)	RCT with 30 subjects (crossover study) PBM decreased blood lactate 6 min post fatigue exercise protocol PBM decreased muscle fatigue by surface electromyography No effect of PBM on muscle peak torque No effect of PBM on muscle average power No effect of PBM on muscle total work

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
				No effect of PBM on muscle fatigue index
[44]	Cluster with 7 laser diodes 850 nm; Diode area 0.05 cm^2; 100 mW; 2 W/cm^2; 2 J per diode (20 s), 40 J/cm^2; 14 J per site of irradiation: 7 × 2 J 4 sites of irradiation per limb: Total delivered: 56 J	Contact Muscular pre-conditioning	1 site of irradiation on *vastus medialis,* 1 site on *vastus lateralis* 1 site on *rectus femoris* 1 site on gastrocnemius Running on treadmill until exhaustion	RCT with 18 subjects (crossover study) PBM increased the peak oxygen uptake PBM increased cardiovascular efficiency No effect of PBM on muscle fatigue in surface electromyography
[45]	Laser diode 810 nm; Diode area 0.028 cm^2; 100 mW; 3.57 W/cm^2; 3 J (30 s), 107.14 J/cm^2; 8 sites of irradiation per limb Total delivered: 24 J	Contact After exercise	8 sites of irradiation on adductor muscles 200 m maximal free style swim, 30-s crossbar jump	RCT with 20 subjects (10 placebo; 10 PBM) PBM improved moderately 30-s crossbar jump test No effect of PBM on 200 m maximal swim No effect of PBM on CK and LDH No effect of PBM on IL-1β, IL-10, TNF-α
[46]	Cluster with 6 laser diodes 830 nm; Diode area 0.0028cm^2; 60 mW; 21.42W/cm^2 0.6 J per diode (10 s), 214.28 J/cm^2 3.6 J per site of irradiation: 6 × 0.6 J 7 sites of irradiation per limb Total delivered: 25.2 J	Contact Muscular pre-conditioning/after exercise	7 sites of irradiation on *quadriceps femoris* Knee extension	RCT with 27 subjects (9 placebo; 9 PBM pre-conditioning; 9 PBM after exercise) PBM applied after exercise decreased blood lactate PBM applied after exercise decreased CK in blood No effects of PBM (pre-conditioning) in number of maximum repetitions No effects of PBM (pre-conditioning) on time to fatigue
[47]	Cluster with 5 laser diodes 810 nm; Diode area 0.029cm^2; 200 mW; 6.9 W/cm^2 6 J per diode (30 s), 206.9 J/cm^2 30 J per site of irradiation: 5 × 6 J 8 sites of irradiation per limb Total delivered: 240 J	Contact Muscular pre-conditioning/after exercise	3 sites on *quadriceps femoris* 2 sites *on vastus medialis* 3 sites *on vastus lateralis* Knee extension/flexion (plyometric exercise)	RCT with 24 subjects (12 PBM pre-conditioning; 12 PBM after exercise) - placebo therapy applied on contralateral limb PBM (pre-conditioning or after exercise) promoted smaller muscle damage No effects of PBM (pre-conditioning or after exercise) in MVC No effects of PBM (pre-condtioning or after exercise) on VAS
[48]	Cluster with: 4 laser diodes 905 nm; Diode area 0.44 cm^2; 0.3125 mW; 0.71 mW/cm^2; 4 LEDs 875 nm; Diode area 0.9 cm^2; 17.5 mW; 19.44 mW/cm^2; 4 LEDs 640 nm; Diode area 0.9 cm^2; 15 mW; 16.66 mW/cm^2; 30 J per site of irradiation: 228 s 17 sites of irradiation per limb Total delivered: 510 J	Contact Muscular pre-conditioning	3 sites of irradiation on *vastus medialis,* 3 sites of irradiation on *vastus lateralis* 3 sites of irradiation on *rectus femoris* 3 sites of irradiation on *biceps femoris* 3 sites of irradiation on *semitendinosus* and *semimembranosus* Bangsbo Sprint Test	RCT with 12 athletes (crossover study) PBM improved average time in the sprints PBM decreased fatigue index PBM promoted lower percentage of increment in blood lactate No effects of PBM in the best time of sprint
[49]	Array with 200 LEDs; 100 LEDs 850 nm arranged in 25 clusters of 4 LEDs, 130 mW, 185.74 mW/cm^2; 100 LEDs 630 nm arranged in 25 clusters of 4 LEDs, 80 mW, 114.28 mW/cm^2;	Contact Muscular pre-conditioning	Whole *quadriceps femoris*, Whole hamstrings Whole *triceps surae* Before official volleyball matches	RCT with 6 athletes (crossover study) PBM 210 J and 315 J could prevent significant increases in CK in blood 24 h after professional volleyball matches

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
	Groups: 105 J (20 s), total delivered 315 J 210 J (40 s), total delivered 630 J 315 J (60 s), total delivered 945 J Placebo - 0 J (30 s) Sites of irradiation per limb: Whole *quadriceps femoris*, hamstrings and *triceps surae* Total delivered: 315 J/630 J/945 J			

Table 4

Clinical trials of photobiomodulation (PBM) in acute responses (≤5 sessions) in exercises on treadmill

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
[50]	Cluster with 5 laser diodes 810 nm; Diode area 0.0364 cm^2; 200 mW; 5.495 W/cm^2; 6 J per diode (30 s), 164.85 J/cm^2; 30 J per site of irradiation: 5 × 6 J 12 sites of irradiation Total delivered: 360 J	Contact Muscular pre-conditioning	2 sites of irradiaton on *vastus medialis* 2 sites on *vastus lateralis* 2 sites on *rectus femoris* 4 sites on hamstrings 2 sites on gastrocnemius Running on treadmill until exhaustion	RCT with 22 subjects (crossover study) PBM increased oxygen uptake PBM increased time of running PBM could prevent increases in LDH PBM could prevent increases in CK in blood PBM could prevent increases in lipid peroxidation
[51]	Array with 50 LEDs 850 nm; Diode area 0.2 cm^2; 50 mW; 250 mW/cm^2; 0.75 J per diode (15 s); 3.75 J/cm^2; 37.5 J applied per muscle group: 50 × 0.75 J Sites of irradiation per limb *Biceps brachii* + *triceps brachii* External oblique + latissimus dorsi *Quadríceps femoris*, Hamstrings *Tibialis anterior* + *peroneus longus* Gastrocnemius + soleus Total delivered: 450 J	Contact Muscular pre-conditioning	Irradiation on whole *biceps brachii* and *triceps brachii* Whole external oblique and *latissimus dorsi* Whole *quadríceps femoris* Whole hamstrings Whole *tibialis anterior* Whole *peroneus longus*, gastrocnemius and soleus Running on treadmill until exhaustion	Case-control with 1 athlete (crossover study) PBM improved speed of muscular VO_2 adaptation PBM decreased O_2 deficit PBM increased the VO_2 PBM increased time of running PBM decreased CK in blood PBM decreased lactate in urine PBM decreased alanine in urine
[52]	Cluster with: 4 laser diodes 905 nm; Diode area 0.44 cm^2; 0.3125 mW; 0.71 mW/cm^2; 4 LEDs 875 nm; Diode area 0.9 cm^2; 17.5 mW; 19.44 mW/cm^2; 4 LEDs 640 nm; Diode area 0.9 cm^2; 15 mW; 16.66 mW/cm^2; 30 J per site of irradiation: 228 s 17 sites of irradiation per limb Total delivered: 510 J	Contact Muscular pre-conditioning	3 sites of irradiation on *vastus medialis* 3 sites of irradiation on *rectus femoris* 3 sites of irradiation on *vastus lateralis* 3 sites of irradiation on *biceps femoris* 3 sites of irradiation on *semitendinosus* and *semimembranosus* 2 sites of irradiation on *gastrocnemius* Running on treadmill until exhaustion	20 subjects (crossover study) PBM increased distance covered PBM increased time to exhaustion PBM increased ventilatory rate PBM decreased dyspnea
[53]	Cluster with 104 LEDs 56 LEDs 660 nm; 10 mW; 50 mW/cm^2; 1.5 J/cm^2; 0.2 cm^2 48 LEDs 850 nm; 30 mW; 150 mW/cm^2; 4.5 J/cm^2; 0.2 cm^2 60 J per site of irradiation: 30 s 5 sites of irradiation per limb Total delivered: 300 J	Contact Muscular pre-conditioning	2 sites of irradiation on *quadriceps femoris* 2 sites of irradiation on *biceps femoris* 1 site of irradiation between *gastrocnemius* and *soleus* Running on treadmill until exhaustion	15 subjects (crossover study) No effect of PBM on maximal accumulated oxygen deficit No effect of PBM on aerobic metabolism No effect of PBM on glycolysis metabolism No effect of PBM on phosphagen metabolism No effect of PBM on time to exhaustion No effect of PBM on lactate levels No effect of PBM on perceived exertion No effect of PBM on heart rate

Table 5

Clinical trials of photobiomodulation (PBM) in chronic responses in exercises with lower limb muscles

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
[14]	Cluster with 6 laser diodes 808 nm; Diode area 0.0028cm^2; 60 mW; 21.42W/cm^2 0.6 J per diode (10 s), 214.28 J/cm^2 3.6 J per site of irradiation: 6 × 0.6 J 7 sites of irradiation per limb Total delivered: 25.2 J	Contact After exercise	7 sites of irradiation on *quadriceps femoris* Leg press training and isokinetic dynamometer	RCT with 36 subjects (12 control; 12 training; 12 training+ PBM) PBM increased load in maximum repetition (1-RM) PBM increased knee extensor peak torque No effect of PBM on thigh perimetry
[15]	Cluster with 6 laser diodes 808 nm Diode area 0.0028cm^2; 60 mW; 21.42W/cm^2 0.6 J per diode (10 s), 214.28 J/cm^2 3.6 J per site of irradiation: 6 × 0.6 J 5 sites of irradiation per limb Total delivered: 18 J	Contact After exercise	5 sites of irradiation on *quadriceps femoris* Cycle ergometer training and isokinetic dynamometer	RCT with 45 subjects (15 control; 15 training; 15 training+PBM) PBM decreased fatigue index of knee extensor muscles No effect of PBM in total knee extensor work
[54]	Array with 50 LEDs 850 nm; Diode area 0.2 cm^2; 100 mW; 500 mW/cm^2; 1.5 J per diode (15 s); 7.5 J/cm^2; 75 J applied per muscle group: 50 × 1.5 J Sites of irradiation per limb *Quadríceps femoris* Total delivered: 150 J	Contact After exercise	Irradiation on whole q*uadriceps femoris* Leg press training Leg extension training	Case-control with 2 subjects monozigotic twins (1 placebo; 1 PBM) PBM increased maximal load PBM increased muscle hypertrophy PBM decreased CK in blood PBM decreased fatigue PBM increased gene expression of muscle hypertrophy and defense against oxidative stress PBM decreased gene expression of inflammation and muscle atrophy
[55]	Cluster with 5 laser diodes 810 nm Diode area 0.029 cm^2; 200 mW; 6.89 W/cm^2 6 J per diode (30 s); 206.89 J/cm^2 30 J per site of irradiation: 5 × 6 J 8 sites of irradiation per limb Total delivered: 240 J	Contact Muscular pre-conditioning	2 sites of irradiation on *vastus medialis* 3 sites on *vastus lateralis* 3 sites on *rectus femoris* Eccentric training in isokinetic dynamometer	RCT with 30 subjects (10 control; 10 training; 10 training+PBM) PBM increased muscle thickness PBM increased isometric knee extensor peak torque PBM increased eccentric knee extensor peak torque
[56]	Laser diode 660 nm; Diode area 0.06 cm^2; 30 mW; 0.5 W/cm^2; 0.24 J (8 s), 4 J/cm^2; 8 sites of irradiation per limb Total delivered: 1.92 J	Contact Muscular pre-conditioning and after exercise	8 sites on *triceps surae* Vertical jumps	RCT with 22 subjects (8 control; 7 LLLT-6 days; 7 LLLT-10 days) No effect of PBM on vertical jump performance (power and fatigue) No effect of PBM on delayed onset muscle soreness
[57]	Laser diode 808 nm; Diode area 0.028 cm^2; 100 mW; 35.71 W/cm^2; 7 J (70 s), 250 J/cm^2; 8 sites of irradiation per limb Total delivered: 56 J	Contact After exercise	8 sites of irradiation on *quadriceps femoris* Leg extension training	RCT with 48 subjects (15 control; 17 training+LLLT; 16 training) PBM increased work compared to control PBM increased peak torque compared to control PBM increased power compared to control No effect of PBM on fatigue index No effect of PBM on lactate levels No effect of PBM on 6-min walk test No effect of PBM on surface electromyography No effect of PBM in 1 maximum repetition (1-RM)

Reference	PBM parameters	Mode of irradiation	Muscle/exercise	Main findings
[59]	Cluster with: 4 laser diodes 905 nm; Diode area 0.44 cm^2; 0.3125 mW; 0.71 mW/cm^2; 4 LEDs 875 nm; Diode area 0.9 cm^2; 17.5 mW; 19.44 mW/cm^2; 4 LEDs 640 nm; Diode area 0.9 cm^2; 15 mW; 16.66 mW/cm^2; 30 J per site of irradiation: 228 s 6 sites of irradiation per limb Total delivered: 180 J	Contact Muscular pre-conditioning/ after exercise/pre-conditioning and after exercise	2 sites of irradiation on *vastus medialis*, 2 sites on *vastus lateralis* 2 sites on *rectus femoris* Leg press training Leg extension training	RCT with 48 subjects (12 placebo; 36 PBM) PBM (pre-conditioning only-12 subjects) increased 1-RM in leg press PBM (pre-conditioning only-12 subjects) increased 1-RM in leg extension PBM (pre-conditioning only-12 subjects) increased peak torque in MVC PBM (pre-conditioning and after-12 subjects) added no effect PBM (after only-12 subjects) added no effect PBM (all modalities-36 subjects) did not increase thigh perimetry

MECHANISMS OF LOW LEVEL LIGHT THERAPY

photobiology.info/Hamblin.html

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The use of low levels of visible or near-infrared (NIR) light for reducing pain, inflammation and edema, promoting healing of wounds, deeper tissues and nerves, and preventing tissue damage has been known for almost forty years since the invention of lasers. Originally thought to be a peculiar property of laser light (soft or cold lasers), the subject has now broadened to include photobiomodulation and photobiostimulation using non-coherent light. Despite many reports of positive findings from experiments conducted in vitro, in animal models and in randomized controlled clinical trials, LLLT remains controversial. This likely is due to two main reasons; firstly, the biochemical mechanisms underlying the positive effects are incompletely understood, and secondly, the complexity of rationally choosing amongst a large number of illumination parameters such as wavelength, fluence, power density, pulse structure and treatment timing has led to the publication of a number of negative studies as well as many positive ones. In particular, a biphasic dose response has been frequently observed where low levels of light have a much better effect than higher levels.

This introductory review will cover some of the proposed cellular chromophores responsible for the effect of visible light on mammalian cells, including cytochrome c oxidase (with absorption peaks in the NIR), and photoactive porphyrins. Mitochondria are thought to be a likely site for the initial effects of light, leading to increased ATP production, modulation of reactive oxygen species, and induction of transcription factors. These effects in turn lead to increased cell proliferation and migration (particularly by fibroblasts), modulation in levels of cytokines, growth factors and inflammatory mediators, and increased tissue oxygenation. The results of these biochemical and cellular changes in animals and patients include such benefits as increased healing of chronic wounds,

improvements in sports injuries and carpal tunnel syndrome, pain reduction in arthritis and neuropathies, and amelioration of damage after heart attacks, stroke, nerve injury, and retinal toxicity.

That is why in some cases testosterone can cause a stroke when they get into the blood, for example, manufacturers of clomid warn drug buyers about possible side effects.

1. HISTORY

In 1967, a few years after the first working laser was invented, Endre Mester in Semmelweis University, Budapest, Hungary wanted to test if laser radiation might cause cancer in mice [1]. He shaved the dorsal hair, divided them into two groups and gave a laser treatment with a low powered ruby laser (694 nm) to one group. They did not get cancer, and to his surprise the hair on the treated group grew back more quickly than the untreated group. This was the first demonstration of "laser biostimulation". Since then, medical treatment with coherent-light sources (lasers) or noncoherent light (light-emitting diodes, LEDs) has passed through its childhood and adolescence. Currently, low-level laser (or light) therapy (LLLT), also known as "cold laser", "soft laser", "biostimulation" or "photobiomodulation" is practiced as part of physical therapy in many parts of the world. In fact, light therapy is one of the oldest therapeutic methods used by humans (historically as solar therapy by Egyptians, later as UV therapy for which Nils Finsen won the Nobel prize in 1904 [2]). The use of lasers and LEDs as light sources was the next step in the technological development of light therapy, which is now applied to many thousands of people worldwide each day. In LLLT, the question is no longer whether light has biological effects, but rather how energy from therapeutic lasers and LEDs work at the cellular and organism levels, and what are the optimal light parameters for different uses of these light sources.

One important point that has been demonstrated by multiple studies in cell culture [3], animal models [4] and in clinical studies is the concept of a biphasic dose response when the outcome is compared with the total delivered light energy density (fluence). It has been found that there exists an optimal dose of light for any particular application, and doses lower than this optimum value, or more significantly, larger than the optimum value will have a diminished therapeutic outcome, or for high doses of light a negative outcome may even result. Evidence suggests that both energy density and power density are key biological parameters for the effectiveness of laser therapy, and they may both operate with thresholds (i.e., a lower and an upper threshold for both parameters between which laser therapy is effective, and outside of which laser therapy is too weak to have any effect or so intense that the tissue is inhibited) [5].

The reason why the technique is termed LOW-level is that the optimum levels of energy density delivered are low when compared to other forms of laser therapy as practiced for ablation, cutting, and thermally coagulating tissue. In general, the power densities used for LLLT are lower than those needed to produce heating of tissue, i.e., less than 100 mW/cm2, depending on wavelength and tissue type.

2. PHYSICAL MECHANISMS

According to quantum mechanical theory, light energy is composed of photons or discrete packets of electromagnetic energy. The energy of an individual photon depends only on the wavelength. Therefore, the energy of a "dose" of light depends only on the number of photons and on their wavelength or color (blue photons have more energy than green photons, that have more energy than red, that have more energy than NIR, etc). Photons that are delivered into living tissue can either be absorbed or scattered. Scattered photons will eventually be absorbed or will escape from the tissue in the form of diffuse reflection. The photons that are absorbed interact with an organic molecule or chromophore located within the tissue. Because these photons have wavelengths in the red or NIR regions of the spectrum, the chromophores that absorb these photons tend to have delocalized electrons in molecular orbitals that can be excited from the ground state to the first excited state by the quantum of energy delivered by the photon. According to the first law of thermodynamics, the energy delivered to the tissue must be conserved, and three possible pathways exist to account for what happens to the delivered light energy when low level laser therapy is delivered into tissue.

The commonest pathway that occurs when light is absorbed by living tissue is called internal conversion. This happens when the first excited singlet state of the chromophore undergoes a transition from a higher to a lower electronic state. It is sometimes called "radiationless de-excitation", because no photons are emitted. It differs from intersystem crossing in that, while both are radiationless methods of de-excitation, the molecular spin state for internal conversion remains the same, whereas it changes for intersystem crossing. The energy of the electronically excited state is given off to vibrational modes of the molecule, in other words, the excitation energy is transformed into heat.

The second pathway that can occur is fluorescence. Fluorescence is a luminescence or re-emission of light, in which the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. The energy difference between the absorbed and emitted photons ends up as molecular vibrations or heat. The wavelengths involved depend on the absorbance curve and Stokes shift of the particular fluorophore.

The third pathway that can occur after the absorption of light by a tissue chromophore, represents a number of processes broadly grouped under an umbrella category of photochemistry. Because of the energy of the photons involved, covalent bonds cannot be broken. However, the energy is sufficient for the first excited singlet state to be formed, and this can undergo intersystem crossing to the long-lived triplet state of the chromophore. The long life of this species allows reactions to occur, such as energy transfer to ground state molecular oxygen (a triplet) to form the reactive species, singlet oxygen. Alternatively the chromophore triplet state may undergo electron transfer (probably reduction) to form the radical anion that can then transfer an electron to oxygen to form superoxide. Electron transfer reactions are highly important in the mitochondrial respiratory chain, where the principal chromophores involved in laser therapy are thought to be situated. A third photochemistry pathway that can occur after the absorption of a red or NIR photon is the dissociation of a non-covalently bound ligand from a binding site on

a metal containing cofactor in an enzyme. The most likely candidate for this pathway is the binding of nitric oxide to the iron-containing and copper-containing redox centers in unit IV of the mitochondrial respiratory chain, known as cytochrome c oxidase (see below).

It should be mentioned that there is another mechanism that has been proposed to account for low level laser effects on tissue. This explanation relies on the phenomenon of laser speckle, which is peculiar to laser light. The speckle effect is a result of the interference of many waves, having different phases, which add together to give a resultant wave whose amplitude, and therefore intensity, varies randomly. Each point on illuminated tissue acts as a source of secondary spherical waves. The light at any point in the scattered light field is made up of waves that have been scattered from each point on the illuminated surface.If the surface is rough enough to create path-length differences exceeding one wavelength, giving rise to phase changes greater than 2 π , the amplitude (and hence the intensity) of the resultant light varies randomly. It is proposed that the variation in intensity between speckle spots that are about 1 micron apart can give rise to small but steep temperature gradients within subcellular organelles such as mitochondria without causing photochemistry. These temperature gradients are proposed to cause some unspecified changes in mitochondrial metabolism

3. BIOCHEMICAL MECHANISMS

There are perhaps three main areas of medicine and veterinary practice where LLT has a major role to play (Figure 1). These are (i) wound healing, tissue repair and prevention of tissue death; (ii) relief of inflammation in chronic diseases and injuries with its associated pain and edema; (iii) relief of neurogenic pain and some neurological problems. The proposed pathways to explain the mechanisms of LLLT should ideally be applicable to all these conditions.

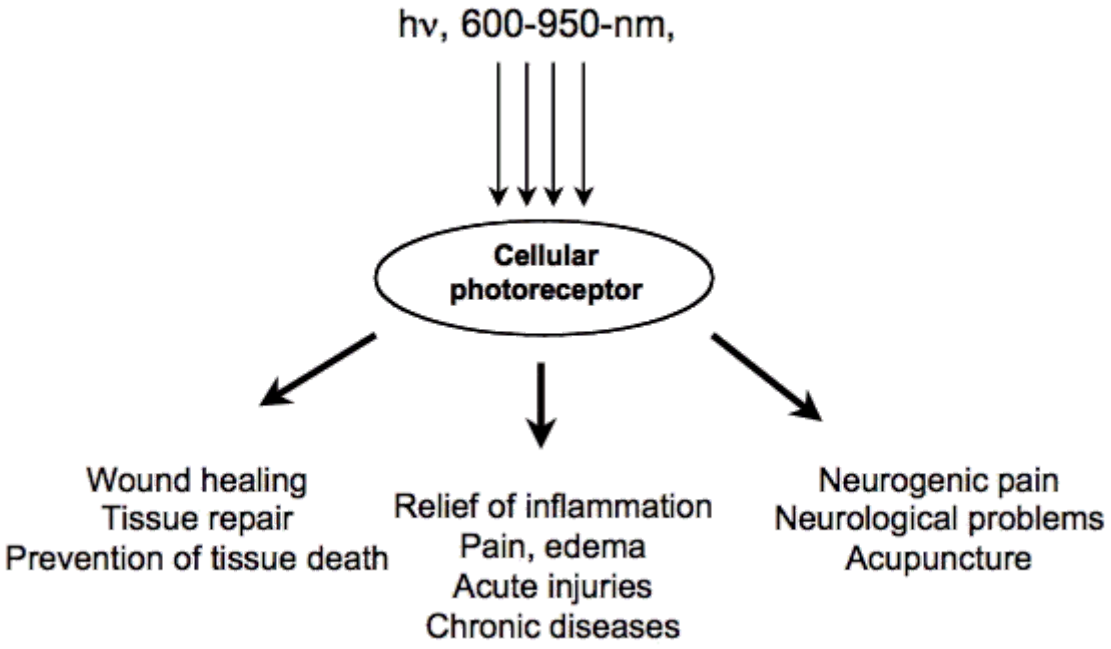

Figure 1. Schematic representation of the main areas of application of LLLT.

3.1 Tissue photobiology. The first law of photobiology states that for low power visible light to have any effect on a living biological system, the photons must be absorbed by electronic absorption bands belonging to some molecular chromophore or photoacceptor [6]. One approach to finding the identity of this chromophore is to carry out action spectra. This is a graph representing biological photoresponse as a function of wavelength, wave number, frequency, or photon energy, and should resemble the absorption spectrum of the photoacceptor molecule. The fact that a structured action spectrum can be constructed supports the hypothesis of the existence of cellular photoacceptors and signaling pathways stimulated by light.

The second important consideration involves the optical properties of tissue. Both the absorption and scattering of light in tissue are wavelength dependent (both much higher in the blue region of the spectrum than the red), and the principal tissue chromophore (hemoglobin) has high absorption bands at wavelengths shorter than 600 nm. For these reasons, there is a so-called "optical window". The second important consideration involves the optical properties of tissue. Both the absorption and scattering of light in tissue are wavelength dependent (both much higher in the blue region of the spectrum than the red), and the principal tissue chromophores (hemoglobin and melanin) have high absorption bands at wavelengths shorter than 600 nm. Water begins to absorb significantly at wavelengths greater than 1150 nm. For these reasons, there is a so-called "optical window" in tissue covering the red and NIR wavelengths, where the effective tissue penetration of light is maximized (Figure 2). Therefore, although blue, green and yellow light may have significant effects on cells growing in optically transparent culture medium, the use of LLLT in animals and patients almost exclusively involves red and NIR light (600 - 950 nm).

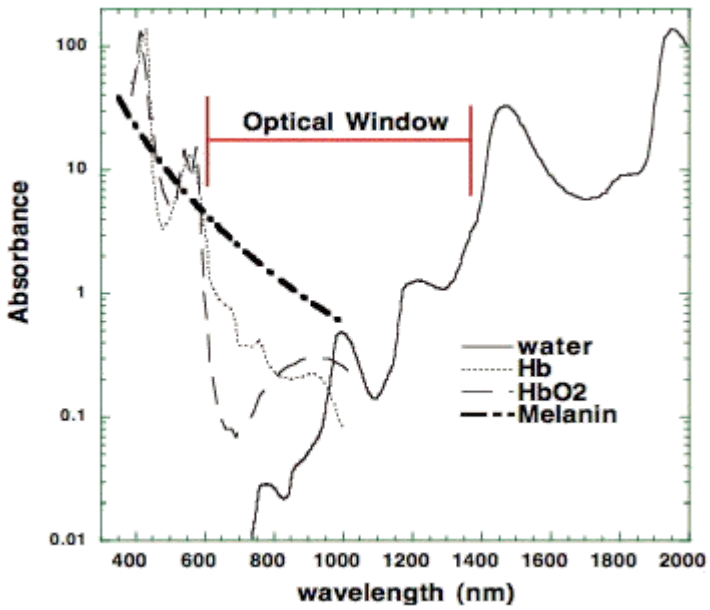

Figure 2. Optical window in tissue due to reduced absorption of red and NIR wavelengths (600-1200 nm) by tissue chromophores.

3.2 Action spectra. It was suggested in 1989 that the mechanism of LLLT at the cellular level was based on the absorption of monochromatic visible and NIR radiation by components of the cellular respiratory chain [7]. The inner mitochondrial membrane contains 5 complexes of integral membrane proteins: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV), ATP synthase (Complex V), and two freely diffusible molecules, ubiquinone and cytochrome c, which shuttle electrons from one complex to the next (Figure 3). The respiratory chain accomplishes the stepwise transfer of electrons from NADH and FADH2 (produced in the citric acid or Krebs cycle) to oxygen molecules to form (with the aid of protons) water molecules harnessing the energy released by this transfer to the pumping of protons (H+) from the matrix to the intermembrane space. The gradient of protons formed across the inner membrane by this process of active transport forms a miniature battery. The protons can flow back down this gradient, re-entering the matrix, only through another complex of integral proteins in the inner membrane, the ATP synthase complex.

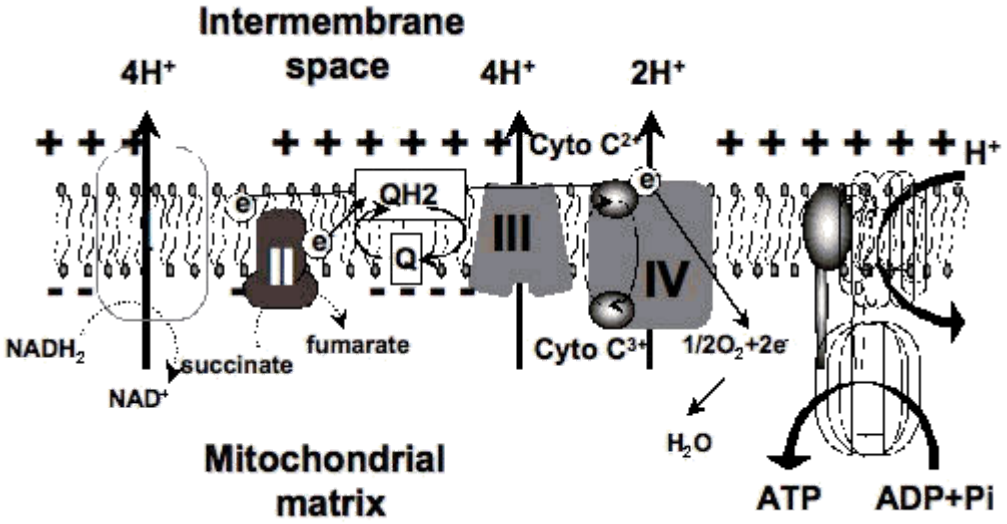

Figure 3. Structure of the mitochondrial respiratory chain.

Absorption spectra obtained for cytochrome c oxidase in different oxidation states were recorded and found to be very similar to the action spectra for biological responses to light. Therefore, it was proposed that cytochrome c oxidase (Cox) is the primary photoacceptor for the red-NIR range in mammalian cells [8] (Figure 4). The single most important molecule in cells and tissue that absorbs light between 630 and 900 nm is Cox (responsible for more than 50% of the absorption greater than 800 nm. Cytochrome C oxidase contains two iron centers, haem a and haem a3 (also referred to as cytochromes a and a3), and two copper centers, CuA and CuB [9] . Fully oxidized cytochrome c oxidase has both iron atoms in the Fe(III) oxidation state and both copper atoms in the Cu(II) oxidation state, while fully reduced cytochrome c oxidase has the iron in Fe(II) and copper in Cu(I) oxidation states. There are many intermediate mixed-valence forms of the enzyme and other coordinate ligands such as CO, CN, and formate can be involved. All

the many individual oxidation states of the enzyme have different absorption spectra [10], thus probably accounting for slight differences in action spectra of LLLT that have been reported. A recent paper from Karu's group [11] gave the following wavelength ranges for four peaks in the LLLT action spectrum: 1) 613.5-623.5 nm, 2) 667.5-683.7 nm, 3) 750.7-772.3 nm, 4) 812.5-846.0 nm.

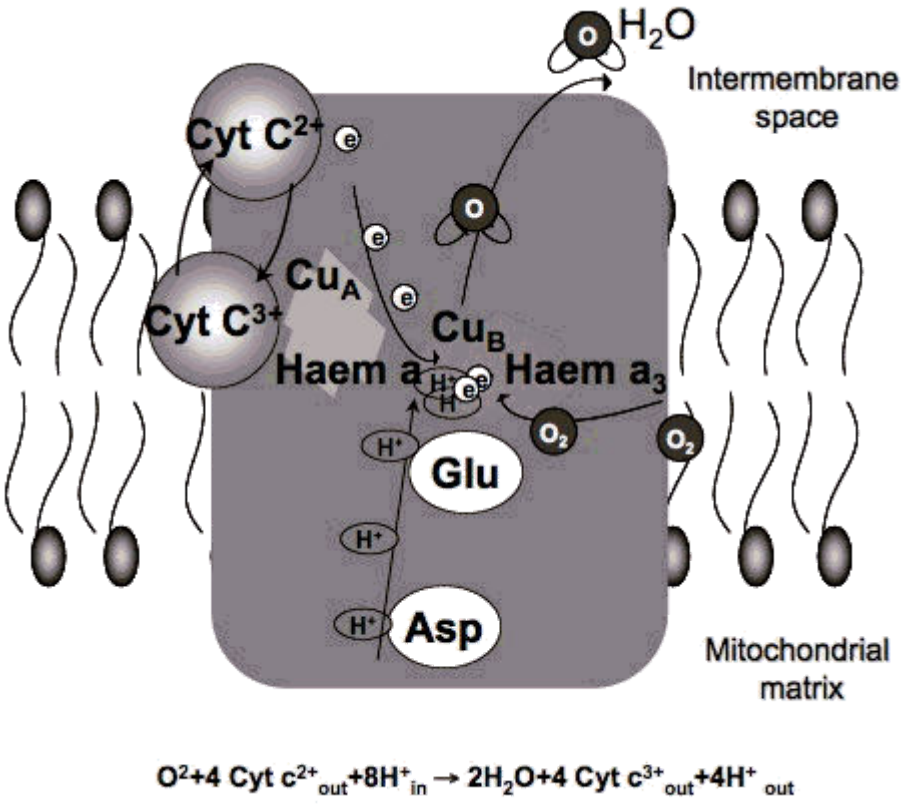

Figure 4. Structure and mode of action of cytochrome c oxidase.

A study from Pastore et al. [12] examined the effect of He-Ne laser illumination (632.8 nm) on the purified cytochrome c oxidase enzyme, and found increased oxidation of cytochrome c and increased electron transfer. Artyukhov and colleagues found [13] increased enzyme activity of catalase after He-Ne laser illumination.

The absorption of photons by molecules leads to electronically excited states, and consequently can lead to an acceleration of electron transfer reactions [14]. More electron transport necessarily leads to the increased production of ATP [15]. The light-induced increase in ATP synthesis and increased proton gradient leads to an increasing activity of the Na+/H+ and Ca2+/Na+ antiporters, and of all the ATP driven carriers for ions, such as Na+/K+ ATPase and Ca2+ pumps. ATP is the substrate for adenyl cyclase, and therefore the ATP level controls the level of cAMP. Both Ca2+ and cAMP are very important second messengers. Ca2+ regulates almost every process in the human body (muscle contraction, blood coagulation, signal transfer in nerves, gene expression, etc.).

3.3 Nitric oxide and LLLT. Light mediated vasodilation was first described in 1968 by Furchgott, in his nitric oxide research that lead to his receipt of a Nobel Prize thirty years later in 1998 [16]. Later studies conducted by other researchers confirmed and extended Furchgott's early work, and demonstrate the ability of light to influence the localized production or release of NO, and to stimulate vasodilation through the effect NO on cGMP. This finding suggests that properly designed illumination devices may be effective, noninvasive therapeutic agents for patients who would benefit from increased localized NO availability. However, the wavelengths that are most effective on this light mediated release of NO are different from those used in LLLT, being in the UV-A (320-400 nm) and blue range [17].

Some wavelengths of light are absorbed by hemoglobin, and that illumination can release the NO from hemoglobin (specifically from the nitrosothiols in the beta chain of the hemoglobin molecule) in red blood cells (RBCs) [18-20] Since RBCs are continuously delivered to the area of treatment, there is a natural supply of NO that can be released from each new RBC that passes under the light source, and is exposed to the appropriate wavelength of photo energy. Since the half life of the NO released under the area of illumination is only 2 to 3 seconds, NO release is very local, preventing the effect of increased NO from being manifested in other portions of the body. Vasodilation from NO is based on its effect on the enzyme guanylate cyclase (GC), which forms cGMP to phosphorylate myosin and relax smooth muscle cells in the vascular system. Once available levels of GC are saturated with NO, or once maximum levels of cGMP are achieved, further vasodilation through illumination will not occur until these biologic compounds return to their pre-illumination status. Again, the wavelengths that have been shown to mediate this effect tend to be in the UV-A and blue ranges, not the red and NIR wavelength ranges that are mainly used for LLLT [21].

The activity of cytochrome c oxidase is inhibited by nitric oxide (NO) [22, 23]. This surprising discovery that the body could poison one of its own enzymes was initially shrugged off as an imperfection [24], but a few years later, several groups reported that mitochondria produced an enzyme that synthesizes NO [25], that was identified as the neuronal isoforms of NO synthase [26]. It was proposed that evolution crafted cytochrome c oxidase to bind not only oxygen, but also NO. The effect of slowing respiration in some locations was to divert oxygen elsewhere in cells and tissues, for instance, NO blocks respiration in the endothelial cells lining blood vessels, and this helps to transfer oxygen into smooth muscle cells in these vessels [27].

This inhibition of mitochondrial respiration by NO can be explained by a direct competition between NO and O2 for the reduced binuclear center CuB/a3 of cytochrome c oxidase, and is reversible [28]. It was proposed that laser irradiation could reverse the inhibition of cytochrome c oxidase by NO by photodissociating NO from its binding sites [24, 29]. Because this coordinate binding is much weaker than a covalent bond, this dissociation is possible by visible and NIR light that has insufficient energy to break covalent bonds. The dissociation of NO from Cox will thus increase the respiration rate ("NO hypothesis") [29]. Light can indeed reverse the inhibition caused by NO binding to cytochrome oxidase,

both in isolated mitochondria and in whole cells [30]. Light can also protect cells against NO-induced cell death. These experiments used light in the visible spectrum, with wavelengths from 600 to 630 nm. NIR also seems to have effects on cytochrome oxidase in conditions where NO is unlikely to be present.

Tiina Karu provided experimental evidence [29] that NO was involved in the mechanism of the cellular response to LLLT in the red region of the spectrum. A suspension of HeLa cells was irradiated with 600-860 nm, or with a diode laser at 820 nm, and the number of cells attached to a glass matrix was counted after a 30 minute incubation. The NO donors, sodium nitroprusside (SNP), glyceryl trinitrate (GTN), or sodium nitrite (NaNO2), were added to the cellular suspension before or after irradiation. Treating the cellular suspension with SNP before irradiation significantly modifies the action spectrum for the enhancement of the cell attachment property, and eliminates the light-induced increase in the number of cells attached to the glass matrix, supposedly by way of binding NO to cytochrome c oxidase. Other in vivo studies on the use of 780 nm light for stimulating bone healing in rats [31], the use of 804 nm laser to decrease damage inflicted in rat hearts after creation of heart attacks [32], have shown significant increases of NO in illuminated tissues after LLLT. On the other hand, studies have been reported on the use of red and NIR LLLT to treat mice with arthritis caused by intra-articular injection of zymosan [33], and studies with 660 nm laser for strokes created in rats [34]. have both shown a reduction of NO in the tissues. These authors explained this observation by proposing that LLLT inhibited inducible nitric oxide synthase (iNOS).

In addition to the cytochrome c oxidase mediated increase in ATP production, other mechanisms may be operating in LLLT. The first of these we will consider is the "singlet-oxygen hypothesis." Certain molecules with visible absorption bands, like porphyrins lacking transition metal coordination centers [35] and some flavoproteins [36], can be converted into a long-lived triplet state after photon absorption. This triplet state can interact with ground-state oxygen with energy transfer leading to production of a reactive species, singlet oxygen. This is the same molecule utilized in photodynamic therapy (PDT) to kill cancer cells, destroy blood vessels, and kill microbes. Researchers in PDT have known for a long time that very low doses of PDT can cause cell proliferation and tissue stimulation, instead of the killing observed at high doses [37].

The next mechanism proposed was the "redox properties alteration hypothesis" [38]. Alteration of mitochondrial metabolism, and the activation of the respiratory chain by illumination would also increase the production of superoxide anions, O2.-. It has been shown that the total cellular production of O2.- depends primarily on the metabolic state of the mitochondria. Other redox chains in cells can also be activated by LLLT. NADPH-oxidase is an enzyme found on activated neutrophils, and is capable of a non-mitochondrial respiratory burst, and production of high amounts of ROS can be induced [39]. These effects depend on the physiological status of the host organism as well as on radiation parameters.

3.4 Cell signaling. The combination of the products of the reduction potential and reducing capacity of the linked redox couples present in cells and tissues represent the redox environment (redox state) of the cell. Redox couples present in the cell include: nicotinamide adenine dinucleotide (oxidized/ reduced forms) NAD/NADH, nicotinamide adenine dinucleotide phosphate NADP/NADPH, glutathione/glutathione disulfide couple GSH/GSSG, and thioredoxin/ thioredoxin disulfide couple Trx(SH)2/TrxSS [40]. Several important regulation pathways are mediated through the cellular redox state. Changes in redox state induce the activation of numerous intracellular signaling pathways, regulate nucleic acid synthesis, protein synthesis, enzyme activation and cell cycle progression [41]. These cytosolic responses in turn induce transcriptional changes. Several transcription factors are regulated by changes in cellular redox state. Among them redox factor-1 (Ref-1)-dependent activator protein-1 (AP-1) (Fos and Jun), nuclear factor (B (NF-(B), p53, activating transcription factor/cAMP-response element-binding protein (ATF/ CREB), hypoxia-inducible factor (HIF)-1 α , an HIF-like factor. Figure 5 illustrates the effect of redox-sensitive transcription factors activated after LLLT in causing the transcription of protective gene products. As a rule, the oxidized form of redox-dependent transcription factors have low DNA-binding activity. Ref-1 is an important factor for the specific reduction of these transcription factors. However, it was also shown that low levels of oxidants appear to stimulate proliferation and differentiation of some type of cells [42-44].

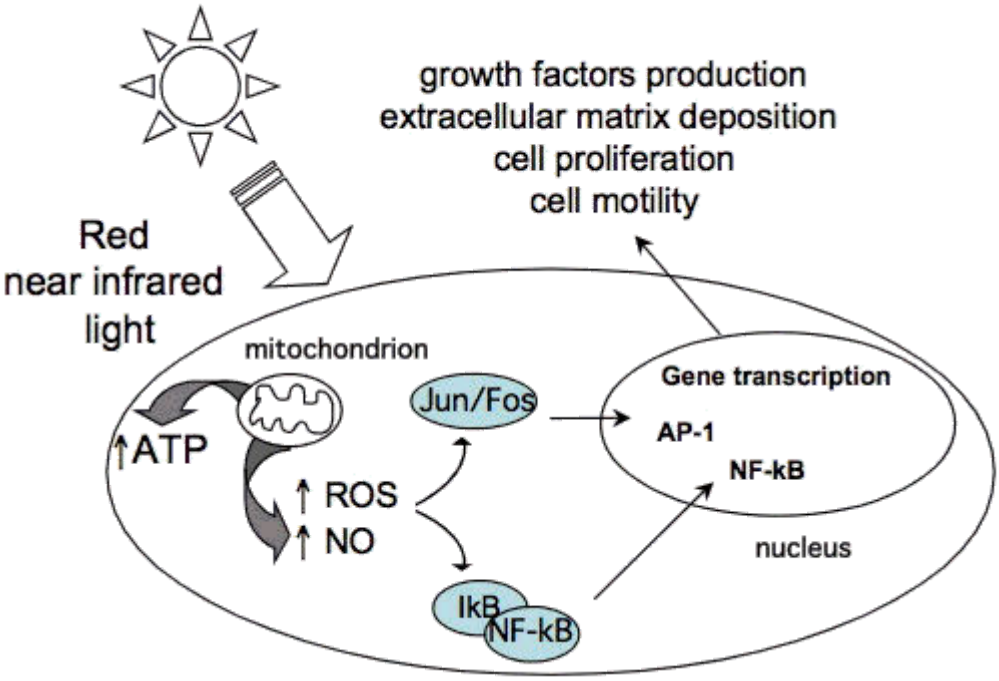

Figure 5. Cell signaling pathways induced by LLLT.

It is proposed that LLLT produces a shift in overall cell redox potential in the direction of greater oxidation [45]. Different cells at a range of growth conditions have distinct redox states. Therefore, the effects of LLLT can vary considerably. Cells being initially at a more reduced state (low intracellular pH) have high potential to respond to LLLT, while cells at the optimal redox state respond weakly or do not respond to treatment with light.

4. *IN VITRO* RESULTS

4.1 Cell types. There is evidence that multiple mammalian and microbial cell types can respond to LLLT. Much of Karu's work has used *Escherichia coli* (a Gram-negative aerobic bacterium) [46] and HeLa cells [47], and a human cervical carcinoma cell line. However, for the clinical applications of LLLT to be validated, it is much more important to study the effects of LLLT on non-malignant cell types likely to be usefully stimulated in order to remedy some disease or injury. For wound healing type studies, these cells are likely to be endothelial cells [48], fibroblasts [49], keratinocytes [50], and possibly some classes of leukocytes. such as macrophages [51] and neutrophils [52]. For pain relief and nerve regrowth studies, these cells will be neurons [53-55] and glial cells [56]. For anti-inflammatory and anti-edema applications, the cell types will be macrophages [51], mast-cells [57], neutrophils [58], lymphocytes [59], etc. There is literature evidence for *in vitro* LLLT effects for most of these cell types.

4.2 Isolated mitochondria. Since the respiratory chain and cytochrome c oxidase are located in mitochondria, several groups have tested the effect of LLLT on preparations of isolated mitochondria. The most popular system to study is the effects of HeNe laser illumination (632.8 nm) of mitochondria isolated from rat liver. Increased proton electrochemical potential and ATP synthesis was found [60]. Increased RNA and protein synthesis was demonstrated after 5 J/cm2 [61]. Pastore et al. [62] found increased activity of cytochrome c oxidase, and an increase in polarographically measured oxygen uptake after 2 J/cm2 of 632.8 nm. A major stimulation in the proton pumping activity, about 55% increase of H+/e- ratio was found in illuminated mitochondria. Yu et al. [14] used 660 nm laser at a power density of 10 mW/cm2 and showed increased oxygen consumption (0.6 J/cm2 and 1.2 J/cm2), increased phosphate potential, and energy charge (1.8 J/cm2 and 2.4 J/cm2), and enhanced activities of NADH, ubiquinone oxidoreductase, ubiquinol, ferricytochrome C oxidoreductase, and ferrocytochrome C, and oxygen oxidoreductase (between 0.6 J/cm2, and 4.8 J/cm2).

4.3 LLLT cellular response. The cellular responses observed in vitro after LLLT can be broadly classed under increases in metabolism, migration, proliferation, and increases in synthesis and secretion of various proteins. Many studies report effects on more than one of these parameters. Yu et al. [50] reported on cultured keratinocytes and fibroblasts that were irradiated with 0.5-1.5 J/cm2 HeNe laser (632.8 nm). They found a significant increase in basic fibroblast growth factor (bFGF) release from both keratinocytes and fibroblasts, and a significant increase in nerve growth factor release from keratinocytes. Medium from laser irradiated keratinocytes stimulated [3H]thymidine uptake, and the proliferation of cultured melanocytes. Furthermore, melanocyte migration was enhanced either directly by HeNe laser or indirectly by the medium derived from HeNe laser (632.8 nm) treated keratinocytes.

The presence of cellular responses to LLLT at molecular level was also demonstrated [63]. Normal human fibroblasts were exposed for 3 days to 0.88J/cm2 of 628 nm light from a light emitting diode. Gene expression profiles upon irradiation were examined using a cDNA microarray containing 9982 human genes. 111 genes were found to be

affected by light. All genes from the antioxidant related category and genes related to energy metabolism and respiratory chain were upregulated. Most of the genes related to cell proliferation were upregulated too. Amongst genes related to apoptosis and stress response, some genes such as JAK binding protein were upregulated, others such as HSP701A, caspase 6 and stress-induced phosphoprotein were downregulated. It was suggested that LLLT stimulates cell growth directly by regulating the expression of specific genes, as well as indirectly by regulating the expression of the genes related to DNA synthesis and repair, and cell metabolism.

5. ANIMAL MODELS

There has been a large number of animal models that have been used to demonstrate LLLT effects on a variety of diseases, injuries, and both chronic and acute conditions. In this review, I will only discuss three particular applications for which there are good literature reports of efficacy.

5.1 Wound healing. The literature on LLLT applied to a stimulation of wound healing in a variety of animal models contains both positive and negative studies. The reasons for the conflicting reports, sometimes in very similar wound models, are probably diverse. It is probable that applications of LLLT in animal models will be more effective if carried out on models that have some intrinsic disease state. Although there have been several reports that processes such as wound healing are accelerated by LLLT in normal rodents [3, 34], an alternative approach is to inhibit healing by inducing some specific disease state. This has been done in the case of diabetes, a disease known to significantly depress wound healing in patients. LLLT significantly improves wound healing in both diabetic rats [35, 36] and diabetic mice [37, 38]. LLLT was also effective in X-radiation impaired wound healing in mice [39]. A study [64] in hairless mice found improvement in the tensile strength of the HeNe laser (632.8 nm)-irradiated wounds at 1 and 2 weeks. Furthermore, the total collagen content was significantly increased at 2 months, when compared with control wounds.

The beneficial effect of LLLT on wound healing can be explained by considering several basic biological mechanisms including the induction of expression cytokines and growth factors known to be responsible for the many phases of wound healing. Firstly, there is a report [65] that HeNe laser (632.8 nm) increased both protein and mRNA levels of IL-1 α and IL-8 in keratinocytes. These are cytokines responsible for the initial inflammatory phase of wound healing. Secondly, there are reports [66] that LLLT can upregulate cytokines responsible for fibroblast proliferation and migration, such as bFGF, HGF and SCF. Thirdly, it has been reported [67] that LLLT can increase growth factors such as VEGF, responsible for the neovascularization necessary for wound healing. Fourthly, TGF-ß is a growth factor responsible for inducing collagen synthesis from fibroblasts, and has been reported to be upregulated by LLLT [68]. Fifthly, there are reports [69, 70] that LLLT can induce fibroblasts to undergo transformation into myofibloblasts, a cell type that expresses smooth muscle α -actin and desmin, and has the phenotype of contractile cells that hasten wound contraction.

5.2 Neuronal toxicity. Studies from Whelan's group have explored the use of 670 nm LEDs in combating neuronal damage caused by neurotoxins. Methanol intoxication is caused by its metabolic conversion to formic acid that produces injury to the retina and optic nerve, resulting in blindness. Using a rat model and the electroretinogram as a sensitive indicator of retinal function, they demonstrated that three brief 670 nm LED treatments (4 J/cm2), delivered at 5, 25, and 50 h of methanol intoxication, attenuated the retinotoxic effects of methanol-derived formate. There was a significant recovery of rod- and cone-mediated function in LED-treated, methanol-intoxicated rats, and histopathologic evidence of retinal protection [71]. A subsequent study [72] explored the effects of an irreversible inhibitor of cytochrome c oxidase, potassium cyanide, in primary cultured neurons. LED treatment partially restored enzyme activity blocked by 10-100 μM KCN. It significantly reduced neuronal cell death induced by 300 μM KCN from 83.6 to 43.5%. LED significantly restored neuronal ATP content only at 10 μM KCN, but not at higher concentrations of KCN tested. In contrast, LED was able to completely reverse the detrimental effect of tetrodotoxin, which only indirectly down-regulated enzyme levels. Among the wavelengths tested (670, 728, 770, 830, and 880 nm), the most effective ones (670 nm and 830 nm) paralleled the NIR absorption spectrum of oxidized cytochrome c oxidase.

5.3 Nerve regeneration. Animal models have been employed to study LLLT effects in nerve repair [73, 74]. Byrnes et al. [56] used 1,600 J/cm2 of 810-nm diode laser to improve healing and functionality in a T9 dorsal hemisection of the spinal cord in rats. Anders et al. [75] studied LLLT for regenerating crushed rat facial nerves; by comparing 361, 457, 514, 633, 720, and 1064 nm, and found the best response with 162.4 J/cm2 of 633 nm HeNe laser.

6. CLINICAL STUDIES

Low-power laser therapy is used by physical therapists to treat a wide variety of acute and chronic musculoskeletal aches and pains, by dentists to treat inflamed oral tissues and to heal diverse ulcerations, by dermatologists to treat edema, non-healing ulcers, burns, and dermatitis, by orthopedists to relieve pain and treat chronic inflammations and autoimmune diseases, and by other specialists, as well as general practitioners. Laser therapy is also widely used in veterinary medicine (especially in racehorse-training centers), and in sports-medicine and rehabilitation clinics (to reduce swelling and hematoma, relieve pain, improve mobility, and treat acute soft-tissue injuries). Lasers and LEDs are applied directly to the respective areas (e.g., wounds, sites of injuries) or to various points on the body (acupuncture points, muscle-trigger points). However, one of the most important limitations to advancing the LLLT field into mainstream medical practice is the lack of appropriately controlled and blind clinical trials. The trials should be prospective, placebo controlled, and double blinded, and contain sufficient subjects to allow statistically valid conclusions to be reached.

Clinical applications of low-power laser therapy are diverse. The field is characterized by a variety of methodologies, and uses of various light sources (lasers, LEDs) with different parameters (wavelength, output power, continuous-wave or pulsed operation modes,

pulse parameters). In recent years, longer wavelengths (~800 to 900 nm) and higher output powers (to 100 mW) have been preferred in therapeutic devices, especially to allow deeper tissue penetration. In 2002, MicroLight Corp received 510K FDA clearance for the ML 830 nm diode laser for the treatment of carpal tunnel syndrome. There were several controlled trials reporting significant improvement in pain, and some improvement in objective outcome measures [76-78]. Since then several light sources have been approved as equivalent to an infrared heating lamp for treating a wide-range of musculoskeletal disorders with no supporting clinical studies.

7. UNRESOLVED QUESTIONS

7.1 Wavelength. This is probably the parameter where there is most agreement in the LLLT community. Wavelengths in the 600-700 nm range are chosen for treating superficial tissue, and wavelengths between 780 and 950 nm are chosen for deeper-seated tissues, due to longer optical penetration distances through tissue. Wavelengths between 700 and 770 nm are not considered to have much activity. Some devices combine a red wavelength with a NIR wavelength on the basis that the combination of two wavelengths can have additive effects, and can also allow the device to be more broadly utilized to treat more diseases. There is of course much more work to be done to define what is the optimum wavelength for the different indications for which LLLT is employed.

7.2 Laser vs non-coherent light. One of the most topical and widely discussed issues in the LLLT clinical community is whether the coherence and monochromatic nature of laser radiation have additional benefits, as compared with more broad-band light from a conventional light source or LED with the same center wavelength and intensity. Two aspects of this problem must be distinguished: the coherence of light itself and the coherence of the interaction of light with matter (biomolecules, tissues). The latter interaction produces the phenomenon known as laser speckle, which has been postulated to play a role in the photobiomodulation interaction with cells and subcellular organelles. It is difficult to design an experiment to directly compare coherent laser light with non-coherent non-laser light for the following reason. Laser light is almost always monochromatic with a bandwidth of 1 nm or less, and it is very difficult to generate light from any other source (even an LED) that has a bandwidth narrower than 10-20 nm, therefore it will be uncertain if observed differences are due to coherent versus non-coherent light, or due to monochromatic versus narrow bandwidth light.

7.3 Dose. Because of the possible existence of a biphasic dose response curve referred to above, choosing the correct dosage of light (in terms of energy density) for any specific medical condition is difficult. In addition there has been some confusion in the literature about the delivered fluence when the light spot is small. If 5J of light is given to a spot of 5 mm2, the fluence is 100 J/cm2, which is nominally the same fluence as 100 J/cm2 delivered to 10 cm2, but the total energy delivered in the latter case is 200 times greater. The dose of light that is used depends on the pathology being treated, and in particular upon how deep the light is thought to need to penetrate into the tissue. Doses that are frequently used in the red wavelengths for fairly superficial diseases tend to be in the

region of 4 J/cm2 with a range of 1-10 J/cm2. Doses of the NIR wavelengths that tend to be employed for deeper-seated disorders can be higher than these values, i.e., in the 10-50 J/cm2 range. The light treatment is usually repeated either every day or every other day, and a course of treatment can last for periods around two weeks.

7.4 Pulsed or CW. There have been some reports that pulse structure is an important factor in LLLT; for instance Ueda et al. [79, 80] found better effects using 1 or 2 Hz pulses than 8 Hz or CW 830 nm laser on rat bone cells, but the underlying mechanism for this effect is unclear.

7.5 Polarization status. There are some claims that polarized light has better effects in LLLT applications than otherwise identical non-polarized light (or even 90-degree rotated polarized light) [81]. However, it is known that polarized light is rapidly scrambled in highly scattering media such as tissue (probably in the first few hundred μm), and it therefore seems highly unlikely that polarization could play a role, except for superficial applications to the upper layers of the skin.

7.6 Systemic effects. Although LLLT is mostly applied to localized diseases and its effect is often considered to be restricted to the irradiated area, there are reports of systemic effects of LLLT acting at a site distant from the illumination [82, 83]. It is well known that UV light can have systemic effects [84], and it has been proposed that red and NIR light can also have systemic effects. These have been proposed to be mediated by soluble mediators such as endorphins and serotonin. There is a whole field known as laser acupuncture [85] in which the stimulation of specific acupuncture points by a focused laser beam is proposed to have similar effects at distant locations to the more well known needle acupuncture techniques.

EDITOR'S NOTE (02/23/13)
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Lasers in Medical Science
https://doi.org/10.1007/s10103-021-03277-2

REVIEW ARTICLE

Mechanistic aspects of photobiomodulation therapy in the nervous system

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Received: 12 June 2020 / Accepted: 15 February 2021

Abstract

Photobiomodulation therapy (PBMT) previously known as low-level laser therapy (LLLT) has been used for over 30 years, to treat neurological diseases. Low-powered lasers are commonly used for clinical applications, although recently LEDs have become popular. Due to the growing application of this type of laser in brain and neural-related diseases, this review focuses on the mechanisms of laser action. The most important points to consider include the photon absorption by intracellular structures; the effect on the oxidative state of cells; and the effect on the expression of proteins involved in oxidative stress, inflammation, pain, and neuronal growth.

Keywords Photobiomodulation therapy · Neuronal cells · Oxidative stress · Low-level laser therapy · Mechanisms

What is photobiomodulation?

"Light amplification by the stimulated emission of radiation" (laser) is based on a theory first described by Einstein in 1917 known as stimulated emission [1, 2]. Theodore Maiman built the first flash lamp-pumped ruby laser in 1960 [3]. Laser produces a beam of high intensity, spatially coherent, parallel light in which all the photons have the same frequency, wavelength, and direction. It is achieved by pumping the active gain medium to a state in which a majority of electrons are in an excited state, known as population inversion. Upon relaxation of these electrons, the stimulated emission yields a coherent beam of light known as a laser. Depending on the gain material, the wavelength of the light can be in any spectral region from the gamma-ray laser to the microwave laser (maser) [4]. Today lasers are widely used in many different aspects of everyday life, as well as in industrial, military, astronomy, and medical applications. Based on the power, medical lasers are categorized into high-powered lasers (class IV) and low-powered lasers (class I-III) [5–8].

The optical spectral range used in LLLT/PBMT is between 600 and 1100 nm, which covers the range from visible red light up to near-infrared light, and the light is generally used with a maximum power density below 500 mW/cm^2, above which tissue heating becomes problematic. The amount of heating produced in the tissue depends to some extent on the wavelength, so that wavelengths absorbed by water tend to produce more heating, but blood flow does automatically increase to remove any excess heat from the tissue. Moreover, pulsed light will produce less tissue heating than CW light, because the dark intervals between pulses will allow some

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Published online: 24 February 2021

heat to be lost. Most commentators agree that the biological effects are not due to the heat produced by light absorption [9–11].

According to optical simulations, the absorption at 1064 nm is similar to that at 700-900nm. In addition, the scattering of 1064 nm is lower than that at 700-900nm. Thus, the penetration depth of 1064 nm is comparable to 600-900 nm. Furthermore, PBMT treatments at this wavelength (1064 nm) have been validated for increasing blood oxygenation, blood flow, concentration of cytochrome c oxidase, and EEG power in vivo in the human brain [12–15].

In this range, the laser does not produce any significant adverse effects from heating the tissue and is known as a cold laser used in LLLT [16, 17]. This type of laser mostly has a stimulatory effect on cell proliferation. Hence, it is often used as a practical and useful method to induce tissue regeneration, wound healing, anti-inflammation, and pain relief and prevent tissue from dying [18–22]. Its applications are progressing in photomedicine and photobiomodulation. Near-infrared (NIR) low-powered lasers have good penetration through the skin and soft/hard tissue and therefore are considered to be suitable physiotherapy devices for pain relief and treatment of orthopedic injuries [17, 23]. Laser light can have three different types of interactions after hitting the skin: absorption, reflection, or dispersion (scattering). Many factors determine the relative importance of these interactions, including the type of tissue and the wavelength of the laser. Studies have shown that tissue absorbs different wavelengths of laser light because various molecular components of cells have absorption peaks matching the laser wavelength [24, 25]. The thickness of the skin and the type of underlying tissue (fat, bone, or muscle) are also determining factors in the absorbance of the light [25–28].

Scattering, transmittance, and reflection of the laser inside the human body are all known to be highly dependent on the wavelength of the light [28, 29]. Lasers with a wavelength of around 500 nm can penetrate to some degree through the chest and the abdomen [29–31]. However, the transmittance significantly increases in the 600-900 nm region. In this case, bone and harder tissues show more transmittance than soft tissues [32]. Lasers in the NIR region are absorbed mostly in the deeper layers rather than the skin because they have penetration depth of 1-5 cm [33, 34].

The benefits of using PBMT in the treatment of many diseases and pathological disorders are becoming more widely appreciated. Using PBMT to normalize cellular function, to maintain or restore tissue homeostasis, to improve the healing of injured tissue and organs, and to reduce pain and inflammations is one of the many applications of PBMT in the clinical arena [21, 22, 35–40].

For the first time in 1986, Shimon Rochkind studied the effect of a low-powered laser on the repair of sciatic nerve injury, thus opening a new window in neuroscience and neurology [41]. During the last 30 years, many studies have investigated the effect of laser on neurodegenerative diseases. In this article, we intend to discuss the mechanism of action of LLLT on the cells and tissues of the nervous system.

Mechanisms of PBM in the central and peripheral nervous system

Central nervous system (CNS) diseases such as stroke, spinal cord injury, neuropathic pain, traumatic brain injury (TBI) neurodegenerative disorders, and multiple sclerosis (MS) can be fatal, are often disabling, and usually cannot be easily treated. PBMT has shown benefits both in animal models and in human studies, such as improving memory and cognitive function, attenuation of depression and anxiety, reduction of pain, and increased cortical oxygenation.

These benefits have been demonstrated in CNS damage and trauma, and also could be effective in MS. In one animal model caused by administration of cuprizone (classic demyelinating model) which is accompanied by oligodendrocyte apoptosis, metabolic failure, endoplasmic reticulum stress, and generation of reactive oxygen species, PBM therapy produced improvement in motor performance, lessened the demyelination, increased the number of oligodendrocyte precursor cells, and modulated the activation of microglia and astrocytes [42].

PBM is capable of inducing photobiological processes inside the cells. Therefore, the mechanism of PBM at the cellular level has been attributed to the absorption of NIR radiation by specific cellular chromophores. Chromophores are photoacceptor molecules that can absorb photons leading to a photochemical reaction occurring. It is generally believed that mitochondria located within the nervous system are the primary sites for photochemical reactions occurring in cells, and cytochrome c oxidase (CCO), the terminal enzyme of the mitochondrial respiratory chain, is likely to be most important molecule in this regard. It is proposed that PBM can increase mitochondrial membrane potential, accelerate electron transport, and raise ATP synthesis, increased oxygen consumption, membrane flexibility, and synthesis of NADH and NADPH oxidase. During these changes, there is a brief burst of reactive oxygen species (ROS) generated in the cells [43, 44]. PBM can cause the release of calcium from mitochondria and pronounced changes in calcium metabolism [45].

Data obtained from the measurement of R/NIR laser irradiation using wavelengths of 600-890 nm has shown that PBMT can pass through the skin and the muscle to reach the damaged site of various nerves. Interestingly, penetration through the skin was higher than muscle, and penetration increased as the wavelengths increased from 650 to 890 nm was only minor at 500nm. The depth of penetration can be up to 3 cm. For 670 nm laser, approximately 6.6% and for 830 nm,

around 11.3% of the photons penetrated to the injury site [46, 47]. However, an important point is that the absorption of cytochrome c oxidase (CCO) is much higher at 670 nm than 830 nm, which may explain why 670 nm light was more effective in pain relief, despite the fact that the number of photons reaching the injury site was lower at 670nm. Some researchers believe that the beneficial effects of R/NIR laser therapy for treatment of peripheral nerve damage depend on some laser-specific factors such as homogeneity, narrow beam profile or coherence, and presence of speckle pattern [46].

There are some controversies about laser effects on the oxidative state of the cells. For instance, on one hand, it was reported that light can produce a shift in the overall cellular redox potential toward greater oxidation, higher ROS generation, and cell redox activity. These factors are typical of oxidative stress, which is considered to contribute to the pathogenesis of neurodegenerative diseases [48–50]. On the other hand, studies have shown that PBM can increase antioxidants such as glutathione, superoxide dismutase, and catalase that can work together to remove H_2O_2, one of the members of the ROS class, and convert it to water [35]. This apparent paradox can be explained when it is realized that the increase in ROS caused by PBM is fairly modest and brief in nature, and can therefore serve to induce the antioxidant defense system. Inducible antioxidants can enhance the recovery of neurons by restoring mitochondrial activity and normalizing energy production. PBM can also produce local vasodilation through increasing the release of nitric oxide [35, 44]. While it can be hypothesized that light causes NO dissociation from CCO, this has not as yet been proved, but increased CCO activity has been shown to be beneficial for mitochondria [50–52].

Another exciting point is that 633 nm PMB upregulated expression of calcitonin gene-related peptide (CGRP) [53, 54]. CGRP protein expression is increased in pathological conditions, such as inflammation, stress, injury, tension, and acute and chronic pain [54–56]. Increased CGRP is accompanied by an increase in the neuronal expression of brain-derived neurotrophic factor (BDNF) and the phosphorylated form of the cyclic adenosine monophosphate-responsive element-binding protein (CREB) [57–59]. PBM has been reported to promote BDNF expression through CREB and ERK phosphorylation and its effects on intracellular calcium (Ca^{2+}) [60].

It was reported that red PBM could change the energy metabolism in mitochondria and cause an increase in ATP synthesis [35, 61, 62], whereas another report suggested that PBM can cause a reduction in the activation of the purinergic receptor (P2X3) by ATP [35]. P2X family receptors are located in the dorsal root ganglion (DRG), and play a crucial role in facilitating pain transmission at both peripheral and spinal sites [63, 64]. Of course, P2X receptors mediate wide-ranging physiological functions such as affecting energy supplies to cells and tissue. They also govern Na and Ca passage into the cells and tissues [64, 65]. Thus, it seems PBM can act toward normalizing many cellular functions.

Beside the abovementioned mechanism concerning P2X3, PBM could play a role in the recovery of damaged peripheral nerves due to increased expression of growth-associated protein-43 (GAP-43) which governs the growth and development of large neurons. It was shown that GAP-43 was upregulated in the early stages of rat sciatic nerve regeneration triggered by PBMT, and the myelin sheath thickness was restored after injury [66, 67].

Also, PBM has been shown to accelerate the speed of nerve regeneration, and improve neuronal survival in the axotomized neurons [53, 68]. PBM irradiation could slow down the process of anterograde degeneration, which may lead to a subsequent delay in invasion by macrophages. The nerve regeneration process can be impaired in nerve segments if macrophage invasion is prevented.

PBM irradiation onto the sciatic nerve increased the action potential and this effect persisted for up to 8 months after stopping the irradiation [69]. PBM could also accelerate and potentiate the peripheral nerve regeneration process in rabbits within 14 days of irradiation [70]. In a comparison between PBM using red laser (680 nm), red LED (650 nm), and blue LED (450 nm), the laser was most effective to increase sciatic nerve regeneration after injury [70].

Mechanism of PBM in the brain and spinal cord injury

The light used in PBM has the ability to pass through the scalp and the skull bone, so it could be effective for brain injury treatment due to its anti-inflammatory and regenerative properties [71–74]. PBM may also be used for spinal cord injuries [36, 75, 76]. PBM can penetrate through several tissues, including the spine, scalp, and skull, and reach into the brain and spinal cord. Its effect in the reduction of inflammatory markers and improvement in the cavity size after spinal cord injury has been reported [36, 77, 78].

PBM therapy applied to the head can also be thought of as a kind of technique for non-invasive brain stimulation [71, 74]. Specific infrared wavelengths are able to penetrate deeply into the brain [74] [53]. It has been estimated that PBM could reach a depth of 20–30 mm from the cortical surface in rabbits (810 nm laser), monkeys (670 nm laser; personal observation), and humans (810 nm laser) while a similar distance of ~30 mm has been reported to reach the spinal cord in rats (810 nm laser) [71, 79–82].

Nevertheless, there is some controversy about the best wavelength for penetration into the brain. An experiment study showed that 660 nm and 810 nm penetrated deeper compared to 980 and 1064 nm. In this regard, 660 nm was shown to be the best, and was a little better than 810 nm [83].

Other studies suggested that wavelengths in the 600–700 nm range should be chosen for treating superficial tissues, while wavelengths between 780 and 950 nm would be better for treating deeper-seated tissues due to the longer optical penetration depth through tissue [82, 83]. Our meta-analysis study covering effectiveness showed that wavelengths shorter than 670 nm could have a better effect on the functional recovery of experimental animals after spinal cord injury [84].

In some studies, PBM therapy has been used as a combination approach that could lead to improvements in standard therapy, for example in helping the tissue to absorb proteins or to incorporate stem cells especially after spinal cord injury [36, 75].

Clinical trials of PBM therapy in humans have shown beneficial effects in acute ischemic stroke [81, 85, 86]. Provided treatment was initiated within 24 h after the stroke onset [86]. After a stroke, PBM may be neuroprotective by upregulating TGF-β1 and suppressing NOS. It can also cause a significant increase in superoxide dismutase (SOD) and a decrease in systolic aspartate transferase in whole brain tissue [87, 88].

The anti-anxiety effects and the anti-depressive effects of transcranial PBM therapy have been reported. In some studies, an average power density of 1.6 mW/cm^2 can be transmitted through the skin, and skull to reach the cortical surface [89, 90]. The best response was found with a laser dose of 8 J/cm^2 in a chronic restraint stress mouse model, which was probably due to increasing 5-HT and decreasing NO levels in the prefrontal cortex and the hippocampal areas [90, 91]. Moreover, transcranial PBM therapy can be carried out via an intranasal approach, and might have a larger anti-depressant effect [92–94]. Some clinical studies have confirmed that intravenous and transcutaneous PBMT can both have anti-depressant benefits in the acute and chronic treatment of depression [95–98]. PBM therapy can provide functional improvements in cognitive impairment [99–101], motor deficits [102], and, particularly, improvement of crossed cerebellar diaschisis (reduction of activity in the cerebellum after a contralateral acute stroke) [103]. PBM also improved sustained attention, extinction memory, and working memory in patients with different brain conditions [104].

Many preclinical animal studies have been done to explore the effect of the laser after spinal cord injury, and the results showed that laser beams shone on the site of the lesion led to functional recovery of movement and repair of the spinal cord lesion. PBM therapy reduced the expression of GSK3-β, an enzyme that inhibits the regrowth of axons, and causes neuropathic pain after a spinal cord injury [36, 75].

PBM might be effective to combat autoimmune neuroinflammatory diseases, such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) [105–109]. The mechanisms of action of PBM against MS, using 660 nm, 904 nm, and 670 nm lasers, have been evaluated. Preventative treatment with PBM during the induction phase of experimental autoimmune encephalitis (MS model) led to upregulation of anti-inflammatory cytokines (IL-4, IL-10) and inhibited the progression of the disease by the reduction of TNFα, IFN- , IL-17, IL-1b l, and NO levels [105, 110–112]. Moreover, PBM inhibited clinical signs, and reduced neuroinflammation and oxidative damage [109, 112–114]. A study was conducted on effect of PBM therapy (810 nm laser) on a mouse model of familial ALS. However, the results showed that PBM did not extend survival or improve measures of motor performance [109].

According to some reports, PBM can ameliorate Alzheimer's symptoms at the behavioral and molecular level such as an increase in BDNF expression [105, 106].

Conclusion

PBM therapy can inhibit apoptosis and inflammation, stimulate angiogenesis, and increase neurogenesis and synaptogenesis, and therefore has great potential for therapeutic applications in the nervous system. No reports of adverse events have been directly attributed to PBMT or laser therapy. PBM therapy could be helpful for patients with traumatic brain disorders, spinal cord injuries, or peripheral nerve injuries. Huntington's and Batten can be mentioned as diseases that have not been investigated by PBM therapy while laser therapy in these diseases may help to reduce the damage to the brain.

Acknowledgements The authors would like to thank Radiation Biology Research Center (RBRC), Physiology Research Center of Iran University of Medical Sciences (IUMS), and International Consortium for Personalized Pain Medicine (ICPPM).

Funding This study was supported by a scientific project grant (NO: 97-3-75-13319) financed by Iran University of Medical Science (IUMS). MRH was supported by US NIH Grants R01AI050875 and R21AI121700.

Declarations

Ethical approval This study was approved by ethical code IR.IUMS.REC.1397.1087.

Conflict of interest MRH was supported by US NIH Grants R01AI050875 and R21AI121700. MRH declares the following potential conflicts of interest. Scientific Advisory Boards: Transdermal Cap Inc., Cleveland, OH; BeWell Global Inc., Wan Chai, Hong Kong; Hologenix Inc. Santa Monica, CA; LumiThera Inc., Poulsbo, WA; Vielight, Toronto, Canada; Bright Photomedicine, Sao Paulo, Brazil; Quantum Dynamics LLC, Cambridge, MA; Global Photon Inc., Bee Cave, TX; Medical Coherence, Boston MA; NeuroThera, Newark DE; JOOVV Inc., Minneapolis-St. Paul MN; AIRx Medical, Pleasanton CA; FIR Industries, Inc. Ramsey, NJ; UVLRx Therapeutics, Oldsmar, FL; Ultralux UV Inc., Lansing MI; Illumiheal & Petthera, Shoreline, WA; MB Lasertherapy, Houston, TX; ARRC LED, San Clemente, CA;

Varuna Biomedical Corp. Incline Village, NV; Niraxx Light Therapeutics, Inc., Boston, MA. Consulting: Lexington Int., Boca Raton, FL; USHIO Corp., Japan; Merck KGaA, Darmstadt, Germany; Philips Electronics Nederland B.V. Eindhoven, Netherlands; Johnson & Johnson Inc., Philadelphia, PA; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany. Stockholdings: Global Photon Inc., Bee Cave, TX; Mitonix, Newark, DE.

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Burns & Trauma, 2021, 9, tkab008
https://doi.org/10.1093/burnst/tkab008
Research Article

Research Article

Photodynamic therapy accelerates skin wound healing through promoting re-epithelialization

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Received 17 December 2020; Revised 1 February 2021; Editorial decision 4 March 2021

Abstract

Background: Epidermal stem cells (EpSCs) that reside in cutaneous hair follicles and the basal layer of the epidermis are indispensable for wound healing and skin homeostasis. Little is known about the effects of photochemical activation on EpSC differentiation, proliferation and migration during wound healing. The present study aimed to determine the effects of photodynamic therapy (PDT) on wound healing *in vivo* and *in vitro*.

Methods: We created mouse full-thickness skin resection models and applied 5-aminolevulinic acid (ALA) for PDT to the wound beds. Wound healing was analysed by gross evaluation and haematoxylin–eosin staining *in vivo*. In cultured EpSCs, protein expression was measured using flow cytometry and immunohistochemistry. Cell migration was examined using a scratch model; apoptosis and differentiation were measured using flow cytometry.

Results: PDT accelerated wound closure by enhancing EpSC differentiation, proliferation and migration, thereby promoting re-epithelialization and angiogenesis. PDT inhibited inflammatory infiltration and expression of proinflammatory cytokines, whereas the secretion of growth factors was greater than in other groups. The proportion of transient amplifying cells was significantly greater *in vivo* and *in vitro* in the PDT groups. EpSC migration was markedly enhanced after ALA-induced PDT.

Conclusions: Topical ALA-induced PDT stimulates wound healing by enhancing re-epithelialization, promoting angiogenesis as well as modulating skin homeostasis. This work provides a preliminary theoretical foundation for the clinical administration of topical ALA-induced PDT in skin wound healing.

Key words: Photodynamic therapy, Wound healing, 5-aminolevulinic acid, Re-epithelialization, Epidermal stem cells, Transient amplifying cells

Highlights

- The rate and quality of wound healing is an active area of research.
- Photodynamic therapy might improve wound healing.
- Photodynamic therapy enhanced epidermal stem cell differentiation and migration.
- Epidermal stem cells are the basis for re-epithelialization and this is promoted by photodynamic therapy.

Background

Wound healing is a well-organized physiological process requiring a complex interplay of resident mesenchymal and epithelial cells to perform the 4 stages of the process: haemostasis, inflammation, re-epithelialization and remodelling [1]. These stages overlap spatiotemporally and interact in complex ways to ultimately complete the healing process. Wound healing is an active area of clinical research, especially concerning factors that improve the healing rate or quality. It is critical to identify efficient, fast acting and economical treatments without side effects to enhance wound healing.

Epidermal stem cells (EpSCs), located in the hair follicle and interfollicular epidermis, participate in maintaining the homeostatic functions of the skin [2]. EpSCs and their daughter cells maintain the normal cutaneous structure by first migrating from their quiescent niches and then undergoing proliferation and differentiation [3]. In the case of skin damage, activated EpSCs are recruited to the epidermis and migrate towards the centre of the wound linearly, resulting in re-epithelialization of the wound and regeneration of the intact epidermis [4].

Photodynamic therapy (PDT) has been used to treat cancers, infectious diseases and inflammatory conditions, including acne vulgaris, rosacea, genital warts and others [5]. PDT uses a combination of a nontoxic dye, known as a photosensitizer, and specific wavelengths of harmless light, thereby photochemically generating reactive oxygen species (ROS) that affect cell signalling and selectively produce cell damage or death in target tissues and microorganisms [6]. The main products of photochemical activation are ROS, which mediate the regulation of intracellular signal transduction *in vivo*. Although the exact details of the regulatory process have not yet been elucidated, it is clear that ROS target signal transduction systems from the cell surface to the nucleus [7].

PDT dose is a critical factor in determining ROS concentration during photochemical reactions [8]. As opposed to the cellular toxicities caused by high levels of ROS, low-dose PDT influences proliferation and differentiation without significantly increased cell death [9], consequently promoting the differentiation of pluripotent stem cells, such as mesenchymal stem cells, osteoblast precursor cells [8], neural stem cells [10] and others. The regulatory effects of exogenous ROS on stem cells have been demonstrated *in vitro* [10]. Recently, a study demonstrated that *in situ* ROS production in murine skin activated hair follicle stem cell proliferation, stimulating hair growth in the quiescent phase and promoting burn healing [11]. Nevertheless, little is currently known about the precise effects of exogenous ROS produced by photodynamic activation on EpSCs during wound healing. We hypothesized that lower doses of PDT than those traditionally used to kill cancer cells might produce sufficient ROS to stimulate EpSCs. We tested and verified the idea *in vivo* and *in vitro* and found that low-dose PDT did enhance EpSC differentiation and migration that was helpful for the wound healing process, without significant cytotoxicity, by promoting re-epithelialization, angiogenesis and inflammatory regulation.

Methods

Isolation and culture of EpSCs

Mouse EpSCs were acquired from the skin of newborn (day 0–2) mice as previously described [12]. In brief, the separated mouse skin was incubated in 0.5% neutral protease (Roche, Switzerland) at 4°C overnight. The epidermis was carefully separated from the dermis and digested with trypsin (Gibco, USA). Then, the single isolated cells were suspended in keratinocyte serum-free medium (Invitrogen, USA) complemented with $CaCl_2$ (0.05 mM), cholera toxin (10 M; Sigma, USA), streptomycin/penicillin (100 IU/ml) and mouse epidermal growth factor (10 ng/ml; Becton Dickinson Biosciences, USA) and cultured at 37°C in 5% CO_2. The Animal Ethics Committee of the Third Military Medical University approved all protocols involving animals. Identification of the primary cells was achieved using flow cytometry and staining with the following fluorochrome-labelled murine monoclonal antibodies: α6-integrin (CD49f) (Invitrogen, USA), transferrin receptor (CD71) (Becton Dickinson Biosciences, USA) and keratin 14 (K14), keratin 15 (K15) and keratin 10 (K10) (all from Santa Cruz Biotechnology, USA).

Preparation of the experimental animal model

We used C57BL/6(C57) mice (male, 18–20 g, specific pathogen-free) at 6 weeks of age. Mice were anaesthetized and shaved before induction of excisional wounds. After disinfecting with 75% alcohol, 2 excisional wounds were

created by cutting down to the musculus panniculus carnosus with a sterile round skin biopsy punch (6 mm diameter) on the dorsal surface, with the 2 wounds at least 15 mm apart. The wound area was measured by tracing the wound margin and was calculated based on the photographs taken with a digital camera after the treatment using Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, USA). The rate of wound healing was calculated as follows: healing rate (%) = $(WA_i - WA_n)/WA_i \times 100\%$, where WA_i represents the initial wound area and WA_n represents the residual wound area on the nth day post-surgery.

PDT *in vivo* and *in vitro*

We used freshly prepared 5-aminolevulinic acid (ALA) (Shanghai Fudan-Zhangjiang Bio-Pharmaceutical Co. Ltd, China). For the i*n vivo* experiment, application of PDT was performed 24 hours after induction of the excisional wounds [13]. We applied a 3% ALA solution to the wounds with a 1 cm margin occluded with a polyurethane dressing (3 M Healthcare, USA). To implement PDT, a red light (635 nm) was applied to the dorsal wound surface after 3 hours of incubation in darkness for a total dose of 6 J/cm^2 using a light-emitting diode (LED) lamp (Omnilux Revive, Photo Therapeutics, Inc., UK). Animals in the irradiation group received only red light and those in the ALA group received only ALA; control group animals underwent the natural recovery process. None of the animals were exposed to light for at least 48 hours after the treatment.

For the *in vitro* experiment, cultured EpSCs were randomly divided into 4 groups, namely the control, irradiation, ALA and PDT groups. EpSCs were incubated with 0.1 mM ALA for 6 hours in a serum-free cell culture medium. In the PDT group, the cells were washed 3 times with phosphate-buffered saline and immediately irradiated with an LED light (635 nm) at a dose of 4 J/cm^2 in a serum-free medium. After irradiation, EpSCs were cultured in fresh medium for further assays.

EpSC apoptosis assay *in vitro*

A total of 5×10^5 EpSCs were collected by centrifuging for 10 minutes at 4°C after dissociation with 0.25% trypsin. Following the manufacturer's protocol, cells were stained with Annexin V-FITC and propidium iodide (Invitrogen, USA) and detected using flow cytometry. The percentage of apoptotic cells was measured using FlowJo software (Tree Star Inc., USA).

Scratch wound migration assay *in vitro*

An *in vitro* wound healing model was used to study EpSC motility in 6-well plates, as previously reported [14]. Briefly, EpSCs were cultured to near confluence in 6-well plates and incubated in a complete medium supplemented with 0.1 mM ALA and 4 μg/mL mitomycin C (Sigma, USA) for 6 hours. The scratch wounds were created in the monolayer (0 hours) with a sterile 200 μL pipette tip. The cells were illuminated by red light and cultured with a fresh culture medium post-PDT. They were monitored for 48 hours using an inverted phase microscope. The control groups included light control, dark control and normal control. The scratch areas were quantified using IPP 6.0 software. The rate of migration was calculated as follows: rate of migration (%) = (initial area − residual area)/initial area × 100%.

ROS detection

ROS production in the wound during PDT was evaluated *in vivo* using ROS-sensitive 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, USA) [11]. After preparation of excisional wounds and PDT application as previously described (incubation of treated animals for 3 hours in the dark, followed by irradiation with 6 J/cm^2 of 635 nm light), we topically applied 50% ethanol (1 mg/ml) with DCFH-DA on wound areas 20 minutes after irradiation. ROS levels generated in the wound area were evaluated using an In Vivo Imaging System (IVIS) Spectrum (Xenogen, CA) 45 minutes after irradiation. The filter settings were 465 nm for excitation and 560 nm for emission. The ROS-related green emission was also evaluated under a fluorescence microscope using frozen sections.

Histological examination

On day 5 post-surgery, mice were sacrificed and the harvested wound tissues were carefully removed. Haematoxylin–eosin (HE) staining and histological analyses were performed as previously reported [14]. The length of the neo-epidermis and the granulation thickness were measured using IPP 6.0 software. Immunohistochemistry (IHC) staining was undertaken using the following antibodies: anti-insulin-like growth factor-1 (IGF-1) (1:200), anti-interleukin (IL)-1 (1:50), anti-IL-23 (1:400), anti-tumour necrosis factor-α (TNF-α) (1:300), anti-platelet endothelial cell adhesion molecule (CD31) (1:100), anti-vascular endothelial growth factor A (VEGFA) (1:100) and anti-proliferating cell nuclear antigen (PCNA) (1:200) (all from Abcam, UK).

Statistical analysis

All data are displayed as mean ± standard deviation. Statistical differences were calculated using one-way analysis of variance for comparisons among multiple groups simultaneously, followed by the least significant difference test. Two-way analysis of variance was used to compare multiple time points using SPSS 20 software (IBM, USA). The significance level was set at $p < 0.05$.

Results

PDT induces ROS production around skin wound regions

We measured the accumulation of protoporphyrin IX (PpIX) in the wound regions after ALA solution application. We found that topical application of ALA on the skin wounds resulted in significant absorption of PpIX around the wounds, as demonstrated by reddish-pink fluorescence.

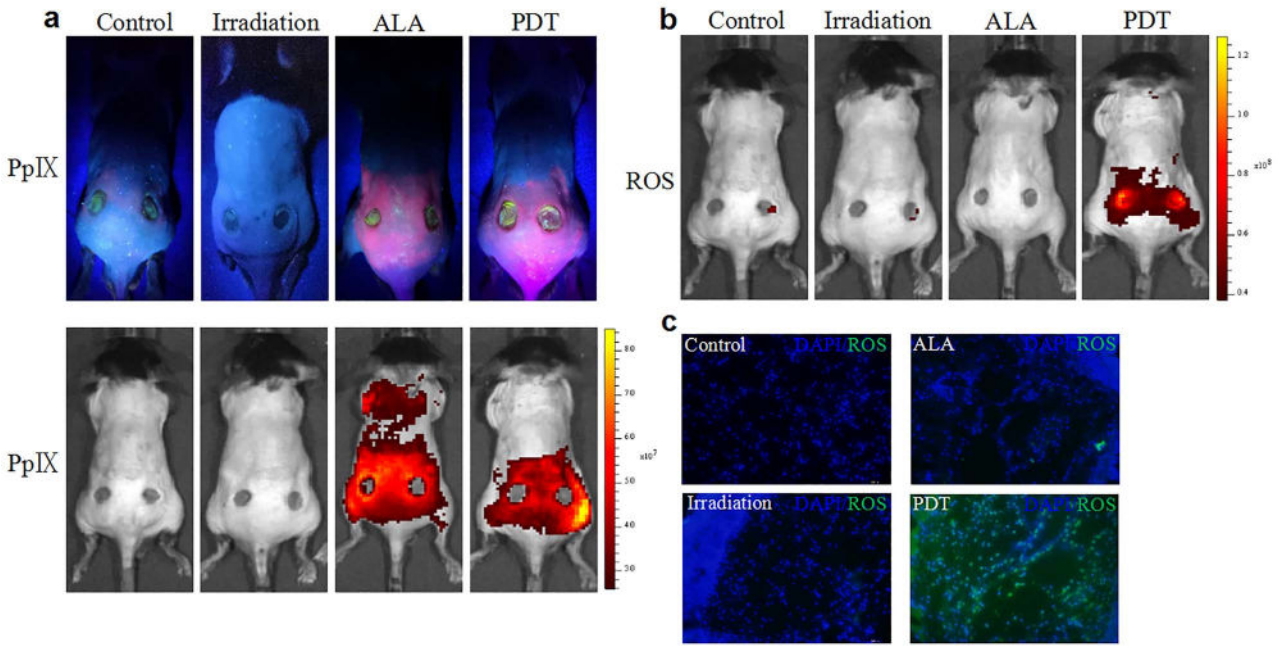

Figure 1. 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) switches on *in situ* reactive oxygen species (ROS) production in skin wound regions. **(a)** Accumulation of protoporphyrin IX (PpIX) induced by topical treatment with ALA in skin wound regions as compared to control groups. **(b, c)** PpIX-dependent ROS production monitored by 2′,7′-dichlorodihydrofluorescein diacetate. *DAPI* 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride

The fluorescent signal emitted by PpIX was markedly enhanced when measured using the IVIS living imaging system (Figure 1a). Subsequent irradiation of treated skin with red light (635 nm) promoted transient production of ROS in the tissue of wounds and margin areas (Figure 1b, c).

ALA-induced PDT promoted skin wound closure and wound re-epithelialization

We found that the wounds appeared to be clean and new epidermis could be observed at the edge of the wounds on days 3 and 5 in the PDT group compared with the other 3 groups (Figure 2a). The wound healing rate in the experimental groups was significantly higher than in the other 3 groups on days 3 and 5 (Figure 2b, c). These macroscopic findings were highly consistent with histological observations. On day 5 post-surgery the wounds of the control mice were covered with fibrin-rich clots, whereas the clot had been replaced by cellular and vascularized granulation tissue in PDT animals (Figure 2d).

Wound re-epithelialization was evaluated by measuring the length of the new epidermis in the wounds using HE staining. The average length of the neo-epithelium in the PDT group was notably longer than that of the other groups. The average lengths of the neo-epithelium on day 5 in the control, irradiation, ALA and PDT groups were 296.63 μm, 326.91 μm, 486.04 μm and 563.34 μm, respectively (PDT *vs* control, $p < 0.01$; PDT *vs* irradiation, $p < 0.01$; and PDT *vs* ALA, $p < 0.05$) (Figure 2d, e). Likewise, the longitudinal diameters between the tips of the epithelial tongues were dramatically shorter in the PDT group than in the other groups on day 5 after wounding (PDT, 972.87 μm *vs* control, 2584.62 μm, $p < 0.01$; PDT, 972.87 μm *vs* irradiation, 2871.71 μm, $p < 0.01$; and PDT, 972.87 μm *vs* ALA, 1692.68 μm, $p < 0.05$) (Figure 2f).

The effect of ALA-induced PDT on EpSC differentiation, migration and apoptosis

Re-epithelialization is closely regulated by the proliferation and differentiation of epidermal cells. We reasoned that the positive effect of low-dose ALA-induced PDT on cutaneous cells, particularly epidermal cells, would produce a physiological response. To investigate the proliferation of keratinocytes *in vivo*, we measured expression levels of PCNA, a biomarker for cell proliferation, in the neo-epidermis around the wound using IHC staining. We found that PDT appeared to cause a significantly greater number of PCNA positive cells at the wound margin than the other groups at day 5 (Figure 3a, b). Due to the critical role of IGF-1 in the proliferation of epidermal cells, we measured the production of IGF-1 in the neo-epidermis. IGF-1 production was markedly higher in the epidermis around the wounds of the PDT group than in the other 3 groups based on the IHC analysis on day 5 (Figure 3c, d).

The impact of ALA-induced PDT on the differentiation of epidermal cells at the wound edge was further evaluated using flow cytometry. The proportion of transient amplifying cells (TA cells) characterized by the CD49f-bright/CD71-bright ($CD49f^{bri}CD71^{bri}$) phenotype was significantly higher in the PDT group than the other groups (PDT, 27.98% *vs* control, 13.32%, $p < 0.01$; PDT, 27.98% *vs* irradiation,

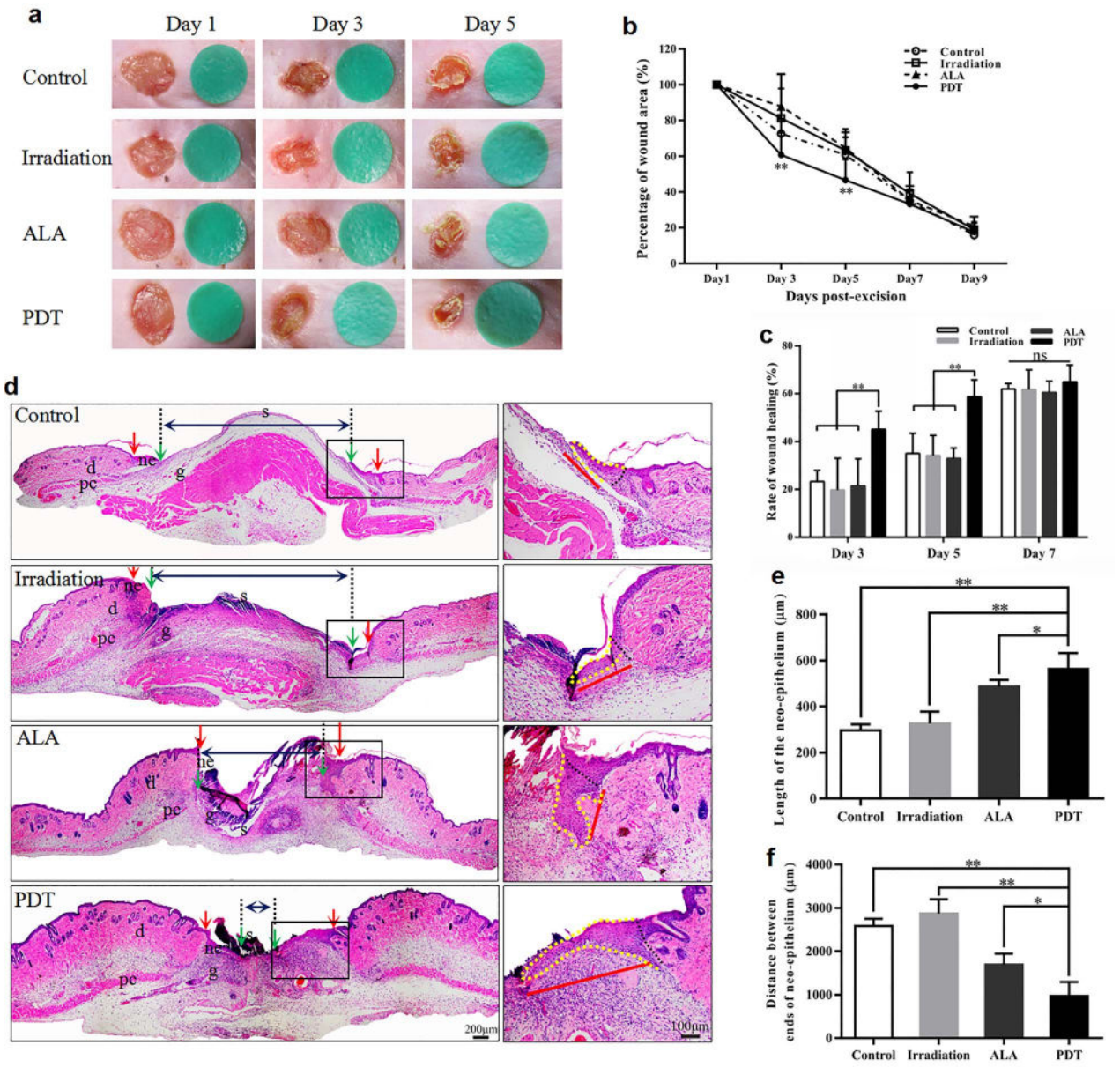

Figure 2. 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) promotes wound healing and wound re-epithelialization. **(a)** The macroscopic appearance of the wounds at different time points. **(b, c)** Wound healing at different time points (n = 5 per group). **(d)** The newly formed epithelium in the wound tissue at day 5 post-wounding in different groups after haematoxylin–eosin staining (blue double-ended arrows represent the distance between the epithelial tips; red arrows indicate wound edges; green arrows indicate tips of epithelial tongues; red lines indicate the neo-epidermis). Scale bars: 200 μm (left) and 100 μm (right). **(e)** Quantification of the neo-epidermal length (n = 5 per group). **(f)** Quantification of the distance between the epithelial tips (n = 5 per group). Statistical analysis: $^{**}p < 0.01$, $^{*}p < 0.05$. *pc* panniculus carnosus, *d* dermis, *ne* neo-epithelium, *g* granulation tissue, *s* scab

14.05%, $p < 0.01$; and PDT, 27.98% *vs* ALA, 17.58%, $p < 0.05$) (Figure 3e, f). The same trend was observed in post-mitotic differentiating cells (PMD cells) with the CD49f-dim ($CD49f^{dim}$) phenotype, with significantly higher proportions in the PDT group than in the light control and dark control groups. However, no obvious differences were found between each group for EpSCs with the $CD49f^{bri}CD71^{dim}$ phenotype. The expression of K10, K14 and K15, another set of biological markers used to define the differentiation state of keratinocytes, was measured using flow cytometry with intracellular cytokine staining. Figure 3g, h shows that the expression of K14 was dramatically higher in the PDT group than in the control groups ($p < 0.01$). The opposite result was observed for the expression of K10, with a significant decrease in the PDT group. Expression levels of K15 in the PDT mice were significantly higher than in the ALA group. However, no significant differences were observed compared to the blank control or light groups (Figure 3g, h).

Differentiation, proliferation and migration of EpSCs are the cellular bases for cutaneous re-epithelialization. We investigated the potential influence of low-dose ALA-induced PDT on EpSCs *in vitro*. PDT significantly increased the

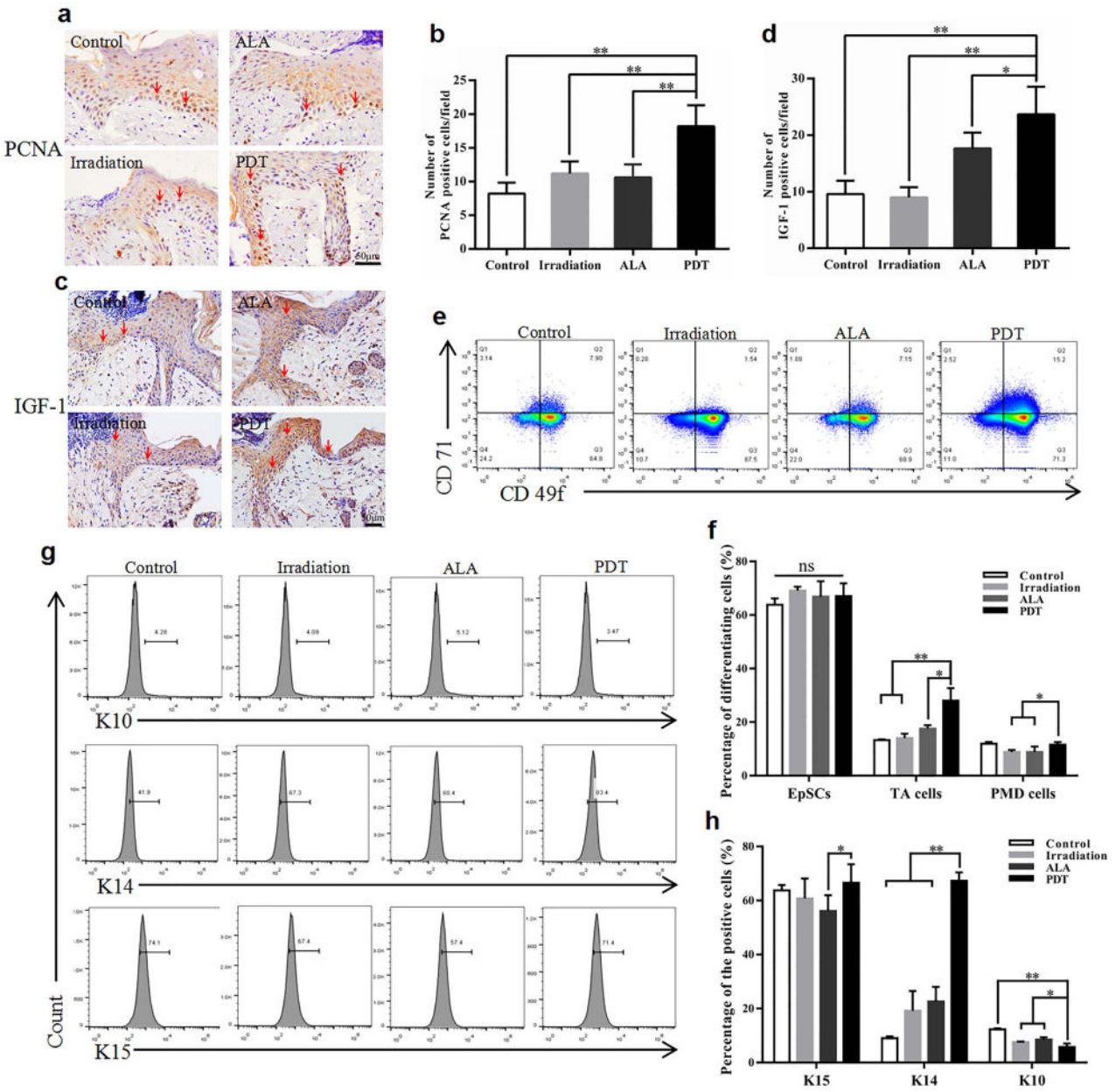

Figure 3. 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) promotes the proliferation and differentiation of epidermal cells. **(a, c)** Typical images of immunohistochemistry staining of proliferating cell nuclear antigen (PCNA) and insulin-like growth factor-1 (IGF-1) at day 5 post-surgery. The red arrows indicate PCNA positive and IGF-1 positive keratinocytes. Scale bars: 50 μm. Quantitative counts of **(b)** PCNA positive keratinocytes and **(d)** IGF-1 positive keratinocytes per field (500 × 500 pixels/field). The percentages of epidermal stem cells (EpSCs), transient amplifying cells (TA cells) and post-mitotic differentiating cells (PMD cells) in the epidermis at wound margins on day 3 post-wounding were detected with **(e)** staining for surface biological markers and **(g)** intracellular markers using flow cytometry. **(f, h)** Quantitation of the epidermal cells characterized by both sets of biological markers. Statistical analysis: $^{**}p < 0.01$, $^{*}p < 0.05$. *ns* no significance, *CD71* transferrin receptor, *CD49f* α6-integrin, *K10* keratin 10, *K14* keratin 14, *K15* keratin 15

proportion of TA cells compared with the other groups, which was in line with the *in vivo* result. Precisely the opposite occurred in EpSCs, with a significant decrease in the PDT group (Figure 4a, b). In contrast, compared with the control and light groups, the expression of K14 in EpSCs was significantly greater in the PDT group (Figure 4c, d). The impact of PDT on the migratory capacity of EpSCs was measured using the scratch assay (Figure 4e, f). The migration of the isolated EpSCs was not significantly higher in the ALA-induced PDT group than in the other groups (Figure 4e, f). There was no apparent effect of PDT on apoptosis among the 4 groups (Figure 4g, h).

Granulation tissue formation and angiogenesis in the dermis

Granulation tissue formation and angiogenesis are the bases of efficient wound repair. The effect of ALA-induced PDT was evaluated using HE staining and IHC analysis. ALA-induced PDT increased granulation tissue remodelling, as the average thickness in PDT mice was 661.03 μm on the fifth day,

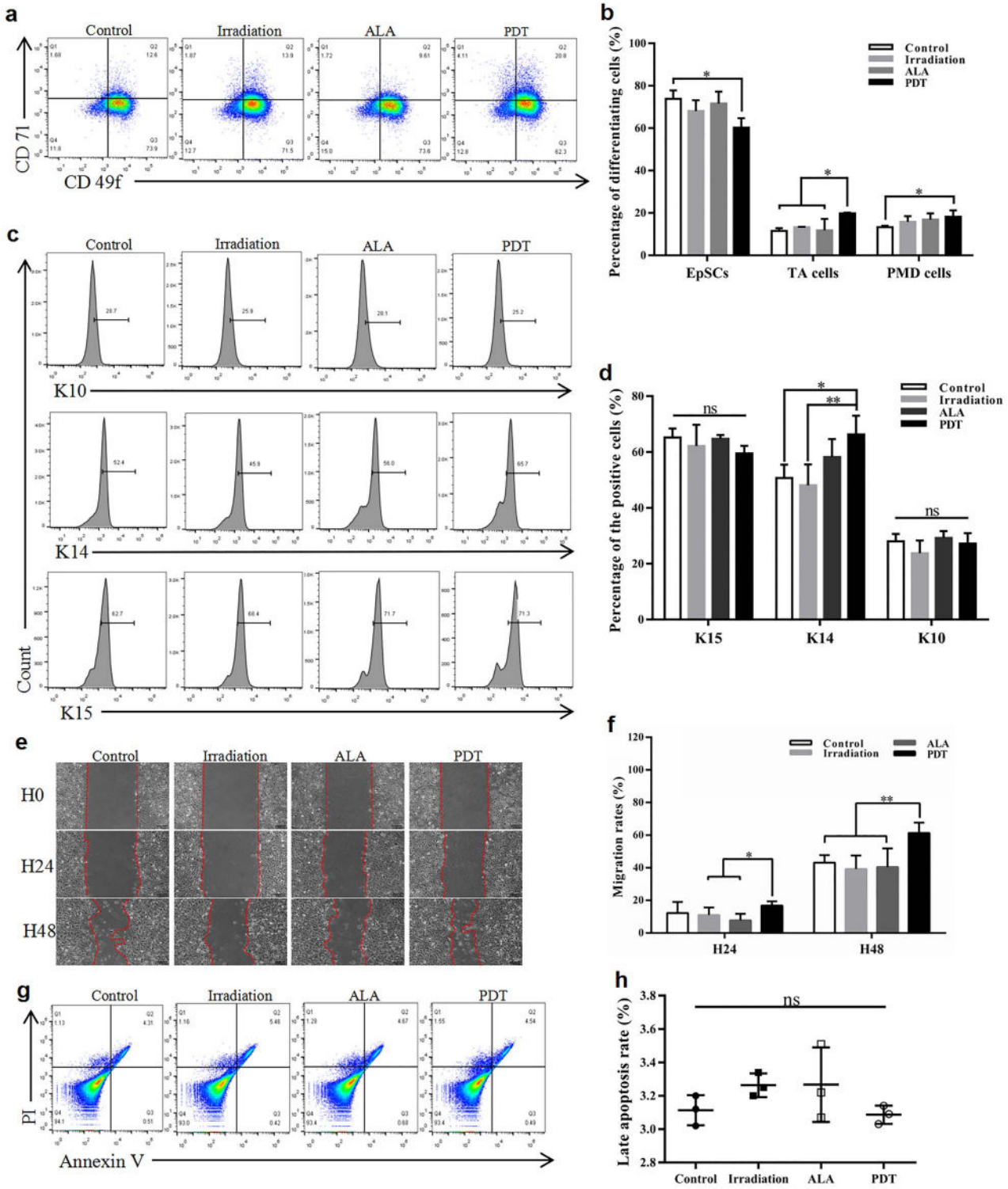

Figure 4. The effect of 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) on epidermal stem cell (EpSC) differentiation, migration and apoptosis *in vitro*. Cultured EpSCs were treated with different conditions according to the groups. The levels of EpSC surface markers **(a, b)** and intracellular markers **(c, d)** were analysed using flow cytometry 24 hours post-treatment. **(e)** Typical images of cell migration and recovery in scratched areas at 24 hours (H24) and 48 hours (H48). The red dashed line shows the leading edge of migrating cells. Scale bars: 100 μm. **(f)** Quantitation of the remaining area that has not yet been covered (n = 6 per group). **(g)** Apoptosis was evaluated using Annexin V/3,8-Diamino-5-[3-(diethylmethylammonio) propyl]-6-phenylphenanthridinium diiodide (PI) staining. **(h)** Quantitation of the late apoptosis rate. Statistical analysis: $^{**}p < 0.01$, $^{*}p < 0.05$. *CD71* transferrin receptor, *CD49f* α6-integrin, *TA cells* transient amplifying cells, *PMD cells* post-mitotic differentiating cells, *K10* Keratin 10, *K14* Keratin 14, *K15* Keratin 15, *H0* 0 hour, *ns* no significance

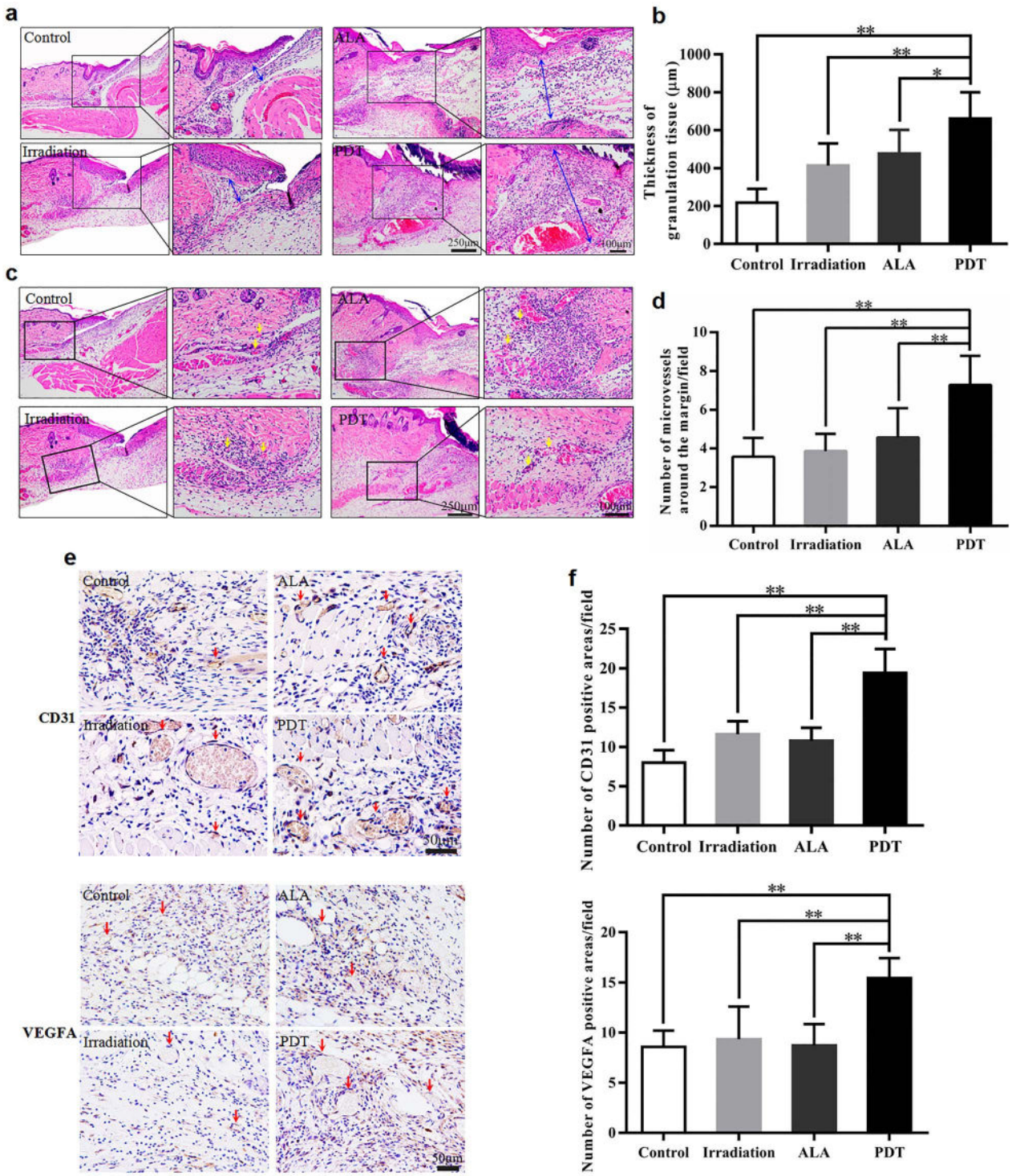

Figure 5. 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) promotes angiogenesis at the wound edge. **(a, c)** Representative haematoxylin–eosin stained images of the wound edge for each group on day 5. The blue double-ended arrows indicate granulation tissue and the yellow arrows indicate micro-vessels around the margin. Scale bars: 250 μm (left) and 100 μm (right). Quantitative determination of **(b)** thickness of granulation tissue and **(d)** number of micro-vessels around the wound margin in different groups. **(e, f)** Immunohistochemistry (IHC) analysis of wound sections on day 5 post-operation, **(e)** Typical images of IHC staining for platelet endothelial cell adhesion molecule-1 (CD31) and vascular endothelial growth factor A (VEGFA). The red arrows indicate the positive area. Scale bars: 50 μm. **(f)** Quantitative counts of CD31 positive and VEGFA positive areas per field (800 × 800 pixels/field). Statistical analysis: $^{**}p < 0.01$, $^{*}p < 0.05$

which was much thicker than that in the control, irradiation and ALA groups (217.57 μm, 413.12 μm and 476.62 μm, respectively; PDT *vs* control, $p < 0.01$; PDT *vs* irradiation, $p < 0.01$; and PDT *vs* ALA, $p < 0.05$) (Figure 5a, b).

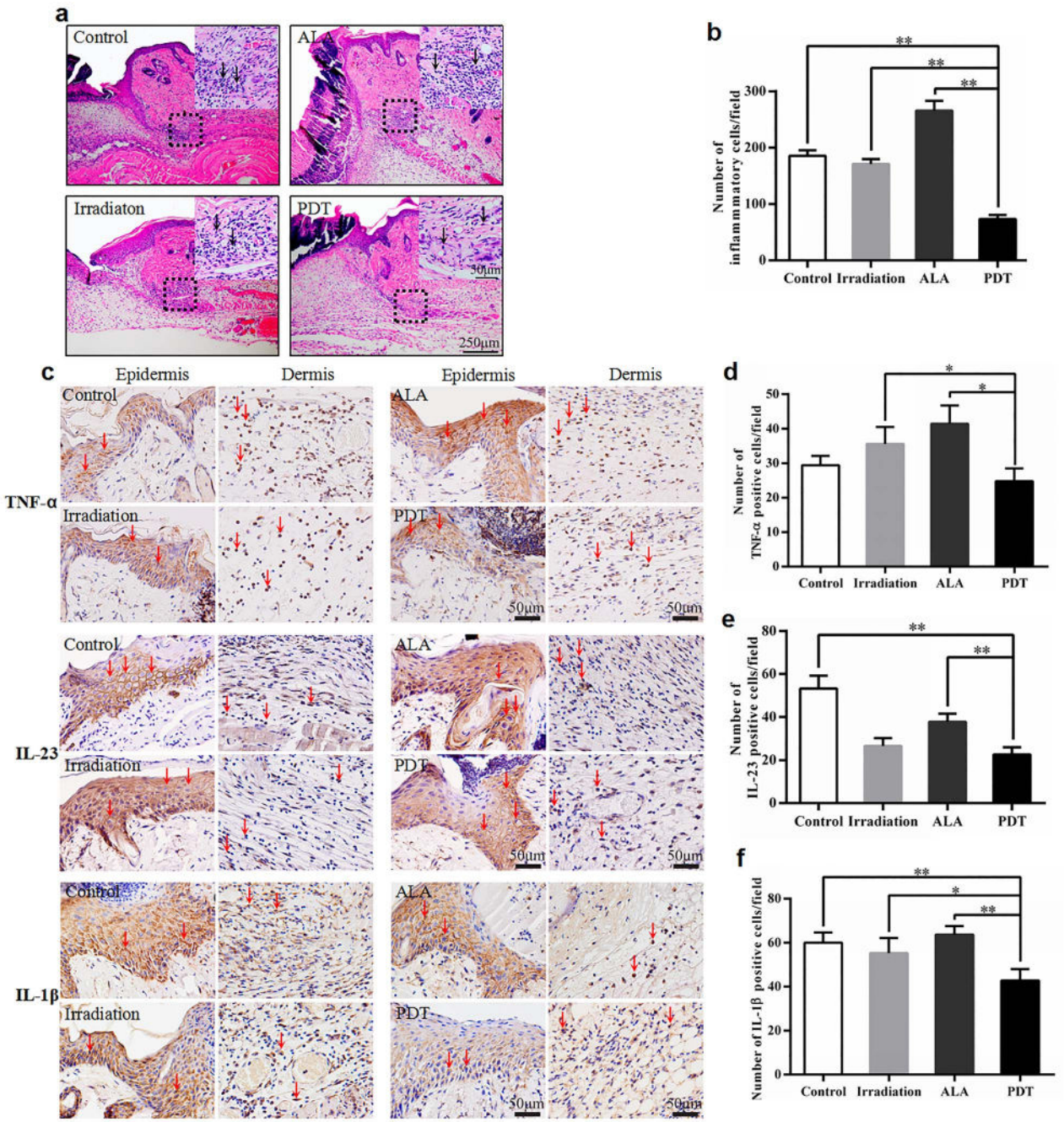

Figure 6. 5-aminolevulinic acid (ALA) photodynamic therapy (PDT) inhibits the inflammatory response. **(a)** Representative haematoxylin–eosin stained images of the injury site for each group. The black arrows indicate inflammatory cells. Scale bars: 250 μm (main images) and 50 μm (insets). **(b)** Quantitative determination of the number of inflammatory cells for each group (500 × 500 pixels/field). **(c–f)** Immunohistochemistry (IHC) analysis of skin wound sections at day 5 post-operation. **(c)** Representative images of IHC staining of tumour necrosis factor-α (TNF-α), interleukin-23 (IL-23) and interleukin-1β (IL-1β). The red arrows indicate the positive cells. Scale bars: 50 μm. Quantitative counts of **(d)** TNF-α positive cells, **(e)** IL-23 positive cells and **(f)** IL-1β positive cells per field (500 × 500 pixels/field). Statistical analysis: $^{**}p < 0.01$, $^{*}p < 0.05$

Furthermore, the average number of neo-capillaries at the wound edge was significantly greater post-ALA-induced PDT treatment when compared with other groups (PDT, 7.29 *vs* control, 3.57, $p < 0.01$; PDT, 7.29 *vs* irradiation, 3.85, $p < 0.01$; and PDT, 7.29 *vs* ALA, 4.57, $p < 0.01$) (Figure 5c, d).

To confirm these results, expression levels of CD31, a marker of angiogenesis, were measured using IHC. The growth of neo-vessels into the wounds was significantly greater in the PDT group, consistent with the observations on HE staining (Figure 5e, f). Because VEGFA is a critical growth factor for angiogenesis, we further examined its production.

Compared with the control, irradiation and ALA groups, we observed that expression levels of VEGFA were dramatically higher in the wound tissue of the PDT group at day 5 after PDT (Figure 5e, f).

The expression of proinflammatory cytokines and inflammatory infiltrate during wound healing

Inflammatory responses establish the first initial host defense against wounds and play critical roles in the entire healing process [15]. Moderate inflammation is advantageous to the normal healing process, while excessive inflammation is harmful. To evaluate the immunomodulatory effect of ALA-induced PDT on wound repair, we analysed the inflammatory cell infiltration and cytokine expression in mouse skin wound tissue. ALA-induced PDT dramatically reduced inflammatory cell infiltration at day 5, as the average value was less in PDT mice than in the other groups (PDT, 73.40 *vs* control, 185.40, $p < 0.01$; PDT, 73.40 *vs* irradiation, 170.80, $p < 0.01$; and PDT, 73.40 *vs* ALA, 265.60, $p < 0.01$) (Figure 6a, b). There were significantly lower levels of proinflammatory cytokines, including IL-23, IL-1β and TNF-α, in the wound tissue of PDT mice than in the control, irradiation and ALA groups (Figure 6c, d e, f).

Discussion

Low-dose ALA-induced PDT accelerated wound closure in an acute mouse skin excision model, as demonstrated by the induction of re-epithelialization, neo-vascularization, granulation and inflammatory infiltration. In addition, the length of the neo-epithelium and the distance between the epithelial tongue tips were improved by ALA-induced PDT.

Healing acute injuries demands a rapid and efficient re-epithelialization process to recover cutaneous integrity [16,17]. Cutaneous healing requires expansion of the neo-epithelium across the wound bed [18], a process sustained by EpSCs that arise from niches found in the interfollicular epidermis and hair follicles [19,20]. In response to injury, EpSCs play a pivotal role in the re-epithelialization process. They are transiently activated to proliferate and differentiate into migratory cells from their otherwise habitually quiescent niches [21]. The re-epithelialization process during the proliferation phase of wound repair requires the activation of quiescent EpSCs [22], their proliferation and migration over the site of injury and their differentiation into a stratified epidermis. Activated EpSCs show upregulation of PCNA (Figure 3a) and are divided into 3 subtypes—CD49f^{bri}CD71^{dim}, CD49f^{bri}CD71^{bri} and CD49f^{dim}—with the characteristics of EpSCs, TA cells and PMD cells, respectively [23–25]. Under homeostatic conditions, the repair and maintenance of stratified skin epithelium relies on epithelial progenitor cells, including both EpSCs and TA cells [23,26]. TA cells are the progeny cells of EpSCs by asymmetric division with a proliferative capacity and have been identified as a pool of rapidly proliferating cells [3,27]. We found that ALA-induced PDT appeared to increase the proportion of TA cells characterized by both sets of biological markers (Figure 3e, f, g, h).

Successful stratification and differentiation of the neo-epithelium are essential for regaining the protective skin barrier after wound closure [28]. As previously reported, K14-positive cells migrate into the wound bed, followed by a mixed population of supra-basal keratinocytes expressing various inducible keratins during acute wound healing [26,29]. However, no experiments have yet been performed to define the role of ALA-induced PDT on EpSCs *in vivo*. The protein expression levels of K10 were lower in PDT mice, suggesting that K10-positive cells remain at the wound margin and contribute essentially nothing to the formation of the neo-epithelium [29]. These results indicate that ALA-induced PDT might enhance re-epithelialization by improving EpSC proliferation, differentiation and migration. Li *et al.* reported that activated dendritic epidermal T cells around the wound edge secreted IGF-1 to contribute to the formation of neo-epithelium, thereby accelerating the healing process [30]. In the present study, IGF-1 expression was greater in the PDT group, suggesting that ALA-induced PDT stimulates IGF-1 expression to accelerate wound healing (Figure 3c, d). The *in vivo* results inspired us to perform *in vitro* experiments to investigate the effect of ALA-induced PDT on cultured EpSCs. We found that ALA-induced PDT enhanced EpSC migration (Figure 4e, f), the proportion of TA cells (Figure 4a, b) and the expression of K14 (Figure 4c, d), suggesting enhanced cell differentiation post-ALA-induced PDT treatment.

Angiogenesis is also an essential factor for accelerated wound healing. Garcia *et al.* described the positive effects of PDT on proliferation and angiogenesis in burns [31]. We found that wounds in PDT mice displayed thicker granulation tissue and more neo-capillaries (Figure 5a, b, c, d). Thus, enhanced angiogenesis and granulation tissue formation could also help explain the rapid closure of the wounds [32]. On day 5 post-treatment, low-dose PDT promotes the expression of VEGFA, a key growth factor for angiogenesis in wound tissue (Figure 5e, f) [33,34]. It also reduced the inflammatory infiltrates and there were relatively lower expression levels of proinflammatory cytokines, including IL-23, IL-1β and TNF-α (Figure 6). Thus, we speculate that ALA-induced PDT inhibits proinflammatory cytokines and inflammatory infiltrates at the middle stage of wound healing.

Conclusions

Low-dose ALA-induced PDT activates cell proliferation, angiogenesis and regulates skin homeostasis, promoting wound healing by activating *in situ* ROS production. Our study is the first to focus on the role of EpSCs in ALA-induced PDT-stimulated wound healing. Our findings suggest that topical low-dose ALA-induced PDT might be used clinically for skin wound healing.

Abbreviations

ALA: 5-aminolevulinic acid; CD31: platelet endothelial cell adhesion molecule-1; CD49f: α6-integrin; CD71: transferrin receptor; DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate; EpSCs: epidermal stem cells; HE: haematoxylin–eosin; IGF-1: insulin-like growth factor-1; IHC: immunohistochemistry; IL: interleukin; IPP: Image-Pro Plus; IVIS: In Vivo Imaging System; K10: keratin 10; K14: keratin 14; K15: keratin 15; PCNA: proliferating cell nuclear antigen; PDT: photodynamic therapy; PMD cells: post-mitotic differentiating cells; PpIX: protoporphyrin IX; ROS: reactive oxygen species; TA cells: transient amplifying cells; TNF-α: tumour necrosis factor-α; VEGFA: vascular endothelial growth factor A.

Availability of data and materials

All data generated and/or analysed during the current study are included in this published article.

Authors' contributions

ZY: conceptualization, formal analysis, investigation, original draft preparation, writing of the original draft. XH: conceptualization, formal analysis, methodology. LZ: investigation, methodology. YH: investigation, visualization. XZ: investigation, resources. JY: resources. ZJ: conceptualization, methodology. YL: conceptualization. HS: visualization, supervision. GL: project administration, methodology. MRH: writing, review and editing. WH: funding acquisition, writing of the original draft. RY: funding acquisition, study design, writing, review and editing. All authors read and approved the final manuscript.

Funding

This work was supported by National Natural Science Foundation of China (grant No. 81571902 and 31872742).

Ethics approval and consent to participate

All protocols involving animals were approved by the Animal Ethics Committee of Third Military Medical University and performed in accordance with international animal welfare standards.

Conflicts of interest

The authors declare that they have no competing interests.

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Indexed in: PubMed

Review

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Special Issue

Therapeutic Mechanisms of Photobiomodulation

Edited by

Dr. Ann Liebert, Prof. Dr. Liisa Laakso and Dr. Hosen Kiat

MDPI

https://doi.org/10.3390/biomedicines11020237

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Review

Photophysical Mechanisms of Photobiomodulation Therapy as Precision Medicine

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Abstract: Despite a significant focus on the photochemical and photoelectrical mechanisms underlying photobiomodulation (PBM), its complex functions are yet to be fully elucidated. To date, there has been limited attention to the photophysical aspects of PBM. One effect of photobiomodulation relates to the non-visual phototransduction pathway, which involves mechanotransduction and modulation to cytoskeletal structures, biophotonic signaling, and micro-oscillatory cellular interactions. Herein, we propose a number of mechanisms of PBM that do not depend on cytochrome c oxidase. These include the photophysical aspects of PBM and the interactions with biophotons and mechanotransductive processes. These hypotheses are contingent on the effect of light on ion channels and the cytoskeleton, the production of biophotons, and the properties of light and biological molecules. Specifically, the processes we review are supported by the resonant recognition model (RRM). This previous research demonstrated that protein micro-oscillations act as a signature of their function that can be activated by resonant wavelengths of light. We extend this work by exploring the local oscillatory interactions of proteins and light because they may affect global body circuits and could explain the observed effect of PBM on neuro-cortical electroencephalogram (EEG) oscillations. In particular, since dysrhythmic gamma oscillations are associated with neurodegenerative diseases and pain syndromes, including migraine with aura and fibromyalgia, we suggest that transcranial PBM should target diseases where patients are affected by impaired neural oscillations and aberrant brain wave patterns. This review also highlights examples of disorders potentially treatable with precise wavelengths of light by mimicking protein activity in other tissues, such as the liver, with, for example, Crigler-Najjar syndrome and conditions involving the dysregulation of the cytoskeleton. PBM as a novel therapeutic modality may thus behave as "precision medicine" for the treatment of various neurological diseases and other morbidities. The perspectives presented herein offer a new understanding of the photophysical effects of PBM, which is important when considering the relevance of PBM therapy (PBMt) in clinical applications, including the treatment of diseases and the optimization of health outcomes and performance.

Keywords: photobiomodulation; photophysical; oscillations; resonant recognition model; mechanotransduction; precision medicine

Citation: Liebert, A.; Capon, W.; Pang, V.; Vila, D.; Bicknell, B.; McLachlan, C.; Kiat, H. Photophysical Mechanisms of Photobiomodulation Therapy as Precision Medicine. *Biomedicines* **2023**, *11*, 237. https://doi.org/10.3390/biomedicines11020237

Academic Editor: Stefano Bacci

Received: 1 January 2023
Revised: 11 January 2023
Accepted: 12 January 2023
Published: 17 January 2023

1. Introduction

Photobiomodulation therapy (PBMt), formerly known as "low-level laser" or "light therapy", is the use of non-thermal light to enhance tissue repair and reduce pain [1–3]. While light therapy in its various forms has a long history dating back to ancient times and includes

the advocation of natural lighting by Florence Nightingale and the treatment of various skin conditions by Niels Finsen at the turn of the 20th century, modern PBMt had its beginnings in 1967 with Endre Mester's use of laser light to serendipitously heal skin conditions and regrow hair in a cancer model in mice [4]. The term low-level laser therapy (LLLT) has evolved into PBMt and includes light emitting diode (LED) devices. It is now used as a therapy for many conditions, including pain, tissue repair, inflammation, and neurological disorders. Over the decades PBMt has gained increasing acceptance; however, the full mechanisms of PBMt are yet to be entirely understood [3]. The action of photons on cytochrome c oxidase has been widely accepted as the primary component for the mechanisms underlying PBMt [3]. However, although cellular effects observed at red wavelengths were believed to primarily involve cytochrome c oxidase [5], new evidence has revealed that PBMt at 660 nm can enhance cell proliferation without cytochrome c oxidase modulation [6]. This revelation prompts the exploration of new perspectives on photobiomodulation (PBM) mechanisms and cellular interactions with light that may explain global effects within the body. In particular, the non-visual photophysical mechanisms of PBMt that are underpinned by bioelectromagnetic radiation and protein oscillations need to be revisited. This may unlock potentially novel intervention targets independent of cytochrome c oxidase that may enhance the potential of precision medicine using PBMt.

Mechanisms of PBM beyond the cytochrome c oxidase mechanism, including increased adenosine triphosphate (ATP) and the dissociation of nitric oxide (NO) from cytochrome c oxidase [7], have been proposed and investigated [3]. These include light-sensitive ion channels such as transient receptor potential channels that respond to low power laser irradiation [8], the increased direct synthesis of intracellular ATP [9], the modulation of mitochondrial and cell membrane-induced reactive oxygen species (ROS) that activate nuclear transcription factors [6,10–13], direct and indirect changes to oxidative stress [3], retrograde mitochondrial signaling [14], the modulation of electron transport chain enzymes and mitochondrial complexes (including the upregulation of complexes IV, negative regulation of complex III, and no regulation of complex II [15]), and other effects on gene expression [16].

This paper aims to investigate some direct and indirect systemic effects of PBMt that cannot be explained by mechanisms involving cytochrome c oxidase and how targeting these photophysical mechanisms may be important in the pursuit of photobiomodulation therapy as precision medicine. The seminal papers that guided the authors' thinking are presented in Table 1. Photophysical mechanisms can be somewhat arbitrarily divided into biophotonic effects, mechanotransduction, and photophysical effects involving the cytoskeleton and oscillations of other proteins. The perspectives presented here may offer a new understanding of the photophysical effects in PBMt, which is important when considering the relevance of PBMt in clinical applications for the treatment of diseases and optimization of health outcomes and performance. These mechanisms are especially important for neurogenic conditions that involve the disruption of cortical coherence and brain wave patterns (e.g., alpha, gamma, theta waves), including migraine headaches with cortical spreading depression [17], and central pain syndromes, including fibromyalgia [18], Parkinson's disease (PD) [19], and Alzheimer's disease (AD) [20]. Additionally, PBMt has therapeutic potentials for diseases involving ion channel disruption, known as channelopathies, which include cardiac disease with dysautonomia and various dysrhythmia [21]. Notably, some wavelengths used in PBMt have been shown to have no therapeutic effect [22]. Here, it is proposed that additional insights into PBMt mechanisms might be made from the consideration of the photophysical effects of light.

Table 1. Seminal studies informing on changes in the light therapy landscape.

Authors	Title	Contribution
Gurwitsch 1932 [23]	Mitogenetic Emission	Release of biophotons

Table 1. *Cont.*

Authors	Title	Contribution
Popp et al., 1984 [24]	Biophoton emission. New evidence for coherence and DNA as source	Release of biophotons
Kert & Rose 1989 [25]	Low level laser therapy	Clinical applications of PBM
Albrecht-Buehler 1992 [26]	Rudimentary form of cellular "vision"	Release of biophotons
Laakso et al., 1993 [27]	Quality of light—is laser necessary for effective photobiostimulation?	PBM coherence
Amano et al., 1995 [28]	Ultraweak biophoton emission imaging of transplanted bladder cancer	Biophotons for diagnosis
Cosic 2001 [29]	The Resonant Recognition Model of Bio-molecular Interactions: possibility of electromagnetic resonance	Resonant oscillation theory
Voeikov et al., 2003 [30]	Biophoton research in blood reveals its holistic properties	Release of biophotons
Amat et al., 2006 [31]	The electric field induced by light can explain cellular responses to electromagnetic energy: A hypothesis of mechanism	PBM coherence
Chow et al., 2007 [32]	830 nm laser irradiation induces varicosity formation, reduces mitochondrial membrane potential and blocks fast axonal flow in small and medium diameter rat dorsal root dorsal root ganglion: implications for the analgesic effects of 830 nm laser.	PBM modulation of cytoskeleton
Mathew et al., 2010 [33]	Signalling effect of NIR pulsed lasers on axonal growth	Biophoton signaling
Sun et al., 2010 [34]	Biophotons as neural communication signals demonstrated by in situ biophoton autography.	Communication with biophotons
Bokkon et al., 2010 [35]	Estimation of the number of biophotons involved in the visual perception of a single-object image: Biophoton intensity can be considerably higher inside cells than outside	Communication with biophotons from periphery to brain
Minke 2010 [36]	The history of the Drosophila TRP channel: the birth of a new channel superfamily	Photon activation of neuronal ion channels

Table 1. *Cont.*

Authors	Title	Contribution
Lavi et al., 2012 [11]	The Plasma Membrane is Involved in the Visible Light–Tissue Interaction	PBM membrane interactions
Hanczyc et al., 2013 [37]	Multiphoton absorption in amyloid protein fibres	Photons for diagnosis
Liebert et al., 2014 [38]	Protein conformational modulation by photons: A mechanism for laser treatment effects	Biophoton theory for PBM
Niggli 2014 [39]	Biophotons: ultraweak light impulses regulate life processes in aging	Biophotons for diagnosis
Tang & Dai 2014 [40]	Spatiotemporal imaging of glutamate-induced biophotonic activities and transmission in neural circuits	Communication with biophotons in the brain
Budagovsky et al., 2015 [41]	Cell response to quasi-monochromatic light with different coherence	Oscillation theory
Shi et al., 2016 [42]	Photon entanglement through brain tissue	Quantum entanglement theory
Cosic & Cosic 2016 [43]	The treatment of Crigler-Najjar syndrome by blue light as explained by resonant recognition model	Clinical application of resonance theory
Poznanski et al., 2017 [44]	Solitonic conduction of electrotonic signals in neuronal branchlets with polarized microstructure	Soliton and nerve theory
Cantero et al., 2018 [45]	Bundles of brain microtubules generate electrical oscillations	Photon modification of microtubules in neurons
Johnson & Winlow 2018 [46]	The Soliton and the Action Potential—Primary Elements Underlying Sentience	Soliton and nerve theory
Fekrazad 2018 [47]	Photons Harmony for Cell Communication	Biophotons and PBM
Santana-Blank & Rodríguez-Santana [48]	Photobiomodulation in Light Our Biological Clock's Inner Workings	PBM and circadian oscillations
Facchin et al., 2019 [49]	Physical energies to the rescue of damaged tissues	Biophotons and PBM
Zomorrodi et al., 2019 [50]	Pulsed near infrared transcranial and intranasal photobiomodulation significantly modulates neural oscillations: a pilot exploratory study	PBM and neural oscillations

Table 1. *Cont.*

Authors	Title	Contribution
Wang et al., 2019 [51]	Transcranial photobiomodulation with 1064-nm laser modulates brain electroencephalogram rhythms	PBM and neural oscillations
Lima et al., 2019 [6]	Photobiomodulation enhancement of cell proliferation at 660 nm does not require cytochrome c oxidase	PBM photophysical mechanisms
Pope et al., 2020 [52]	Wavelength-and irradiance-dependent changes in intracellular nitric oxide level	PBM photophysical mechanisms
Esmaeilpour et al., 2020 [53]	An Experimental Investigation of Ultraweak Photon Emission from Adult Murine Neural Stem Cells	Biophotons for diagnosis
Sordillo & Sordillo 2020 [54]	The mystery of chemotherapy brain: kynurenines, tubulin and biophoton release	Clinical application of biophotons
Mahbub et al., 2020 [55]	Non-invasive real-time imaging of reactive oxygen species (ROS) using auto-fluorescence multispectral imaging technique: A novel tool for redox biology	Autofluorescence
Zangari et al., 2021 [56]	Photons detected in the active nerve by photographic technique	Biophotons and nerve theory
Staelens et al., 2022 [57]	Near-Infrared Photobiomodulation of Living Cells, Tubulin, and Microtubules	Photon modification of microtubules in neurons
Korneev et al., 2022 [58]	Exploring Structural Flexibility and Stability of α-Synuclein by the Landau–Ginzburg–Wilson Approach	Solitons
Moro et al., 2022 [59]	The code of light: do neurons generate light to communicate and repair?	Biophoton communication
Moro et al., 2022 [60]	The effect of photobiomodulation on the brain during wakefulness and sleep	Biophoton and circadian rhythms

2. Biophotons and PBM

One form of electromagnetic (EM) radiation in the body is known as biophoton emission, which refers to photons of light emitted by a biological system [23,24,26,30,61]. Biophotons, often referred to as ultra-weak photon emissions (UPEs), can be detected and measured by several techniques [62,63]. These emissions can originate when ROS form [64]

in the cell and, in neurons, can influence potassium channel activity and be formed as a by-product of membrane depolarization [65]. Biophotons can also be released following endogenous ROS mechanisms in the mitochondrial and cell membranes, especially neurons of the central and peripheral nervous systems.

An interesting source of endogenous biophotons are those detected during neutrophil burst events [66,67], whereby the occurrence of oxidative stress appears to be quantifiable to UPEs detected as a biological readout [66]. This provides the basis of a potential non-invasive detection method that can be observed in real-time [55]. Indeed, circulating neutrophils in the bloodstream may have the capacity to be targeted for biophoton detection via the measurement of oxidative stress [30]. This is noteworthy since studies have shown that PBMt-stimulated oxidative bursts from neutrophils, as a measure of their production of ROS, resulted in increased functional profiles translating to elevated fungicidal capacity [68]. The manipulation of oxidative stress, particularly those deriving from neutrophil activity, is an interesting concept since it is understood that one of the key effects of PBMt in cellular systems is the modulation of ROS [3,10,69].

Recent studies have focused on the possible EM aspect of axonal conduction, including the optical propagation of photons through myelinic waveguides [70]. Axonal activity utilizes energy generation and exchange in the same way as other cellular and biological processes in the body. Early studies on neuronal function detected heat and infrared radiation transfers between the nerve during an action potential [71,72]. Further, the presence of infrared and visible light wavelengths has recently been shown in a variety of tissues and nerve cells [34,73,74]. In addition, the idea that photon emissions may be able to carry cellular information via the propagation of EM radiation has also been postulated [70]. If present, its implications for diagnostic and therapeutic use are significant.

An example of the potential importance of biophoton signaling is in the neuronal axon and dendrites. This could constitute a form of neural code and communication [59] and can be apparent in the central and peripheral nervous systems during periods of sleep and wakefulness [60]. This has been flagged as an important part of a systems biology approach [38]. New evidence has come to light in the explanation of this phenomena in the nodes of Ranvier [75].

Research studies have reported axonal responses in the presence of infrared and visible light irradiation in human neurons [76]. There has also been growing evidence for the endogenous generation of detectable cellular EM fields, with investigations on multiple biological effects that are attributed to a broad spectrum of wavelengths [77]. Additionally, it has been observed that emission intensity positively correlates with an increase or change in physiological activity, particularly under stress. For example, the presence of reactive oxygen species has been associated with the upregulation of UPEs [66,74,77,78].

In 2009, although it was well-known that biophotons existed in plants and bacteria, their existence and potential role as a cellular communication component in neurons was undetermined. However, there is now strong evidence for biophotons transmitting information within the body [79]. Like action potentials, biophotons may be involved in cell-to-cell communication to facilitate downstream cellular processes [70,80]. The potential effects of EM radiation within the infrared or visible spectrum on the expression of the endogenous biophotons have been postulated during neural excitability and signaling, culminating in a potential EM theory of neural communication [59,75]. Specifically, one breakthrough study showed that stimulating spinal motor or sensory nerve roots with light caused an increase in biophoton emissions at the end of the nerve root [34]. However, administering a neural conduction inhibitor blocked these effects. This result implies that biophotons are transmitted by axons in the same way as electrical signaling and are therefore likely to be a form of signaling [34].

Subsequently, our group had hypothesized that protein-to-protein interactions can occur when external photons are applied and proposed a mechanism of protein conformation to explain some of the effects of PBMt. A biophoton emission mechanism was hypothesized to be facilitated by these interactions, stimulated by the formation of ROS [38]. Following

this hypothesis, a potential photophysical pathway has started to emerge highlighting the EM properties of neuronal axons and the emission of photons [75], particularly in the light-sensitive structures found in the gaps within the axonal myelin sheath called the nodes of Ranvier [81–83] (Figure 1).

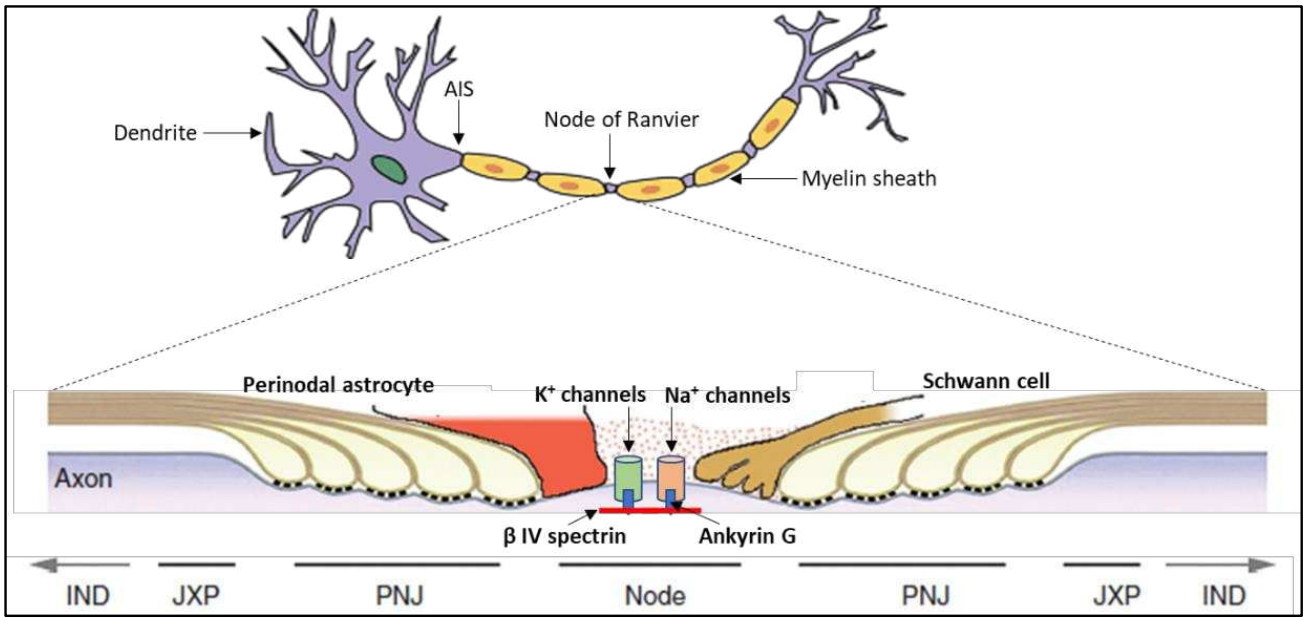

Figure 1. A detailed schematic of a Node of Ranvier.

The movement of propagated electrical signal between these nodes is significantly faster compared to signal transduction in unmyelinated axons, using a process called "saltatory conduction" [84]. Recently, studies investigating the EM properties pertaining to the physiology and function of the node of Ranvier have been conducted in both heart tissue and neurons [75,85], whereby EM-driven changes appear to affect a range of physiological pathways. For example, a recent study on EM radiation found that the accumulation of ion channels clustered in the nodes of Ranvier behaved like an array of nanoantennae emitting at wavelengths below 1600 nm [56,75]. This infrared emission is likely to propagate from node-to-node along the myelinated axon and may have an important role in nerve cell communication. It is possible that these EM emissions can trigger biological processes, such as triggering the release of neurotransmitters at the synaptic cleft, and may play an intimate role in neurotransmission [75].

The application of additional irradiation from an external source, such as through the delivery of PBMt, may be hypothesized to modulate the EM properties of these ion channels to elicit a biological response that has a direct influence on neurotransmission. Indeed, Chow et al. and others have shown that the application of low-level laser irradiation on neurons in-vitro, induced axonal varicosities in the same way as pharmacological anesthetics, resulting in the blockade of neurotransmission and therefore conferring an analgesic effect [32,86,87]. Interestingly, a recent study has suggested that neuronal spheroids may be involved in the pathology of Alzheimer's disease [88]. From the model proposed by Zangari et al. [75], it is possible that the application of PBMt may act directly on the nodes of Ranvier and therefore modulate the signaling properties of proteins clustered within them, such as ion channels. This mechanism may be present in other cell types with similar light-responsive ion channels and has possible clinical implications for diseases involving channelopathies [89].

There is increasing interest in the connection between PBM and biophoton release [67]. The hypothetical connection between the production of biophotons and PBMt is shown in Figure 2. Placing intervention, biological target, and consequential downstream metabolite together provides potentially new hypotheses regarding the control and regulation of ROS

in health, such as adaptive immunity and inflammation mechanisms from the production of nicotinamide adenine dinucleotide phosphate (NADPH)-derived ROS from phagocytes [90]. In this model, a lack of phagocytic ROS production may result in immunodeficiency and autoinflammation during an immune response; however, the overproduction of ROS can result in tissue damage and disease states due to cumulative oxidative stress [90]. The reports that biophotons influence aging [39] and metabolic disease states such as cardiovascular disease [91] and infectious disease [92] suggest that endogenous biophotons are not only present in physiological conditions but can also be used to quantifiably detect physiological events relevant to pathophysiology such as occurrence of oxidative stress. Therefore, they can also function as a potential target for the modulation of downstream effects. In summary, the neuroimmune modulatory effect of PBMt on production of biophotons through neutrophil burst may be an important aspect of the PBMt mechanism that has been so far under-researched due to previously unreliable technology to accurately measure biophotons.

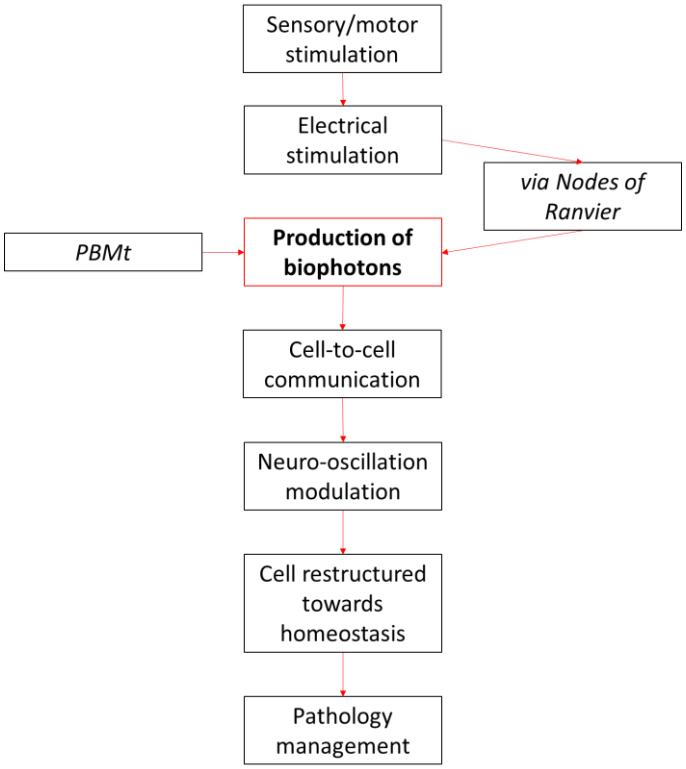

Figure 2. A conceptualization of how biophotons and photobiomodulation may contribute to cell-to-cell communication and the modulation of neural oscillations.

3. PBMt and Mechanotransduction

The therapeutic effects of PBMt for related conditions including acute trauma from concussions and other traumatic brain injuries, degenerative diseases such as dementia, and behavioral and psychiatric disorders such as post-traumatic stress disorder, can be explained using the concept of biophysical alterations to the cell. These may include alterations to the cytoskeleton, the Ca^{2+} stores of the cell membrane, and oscillations of cellular proteins (see Figure 3). It is possible that PBM-induced changes in neuronal patterns and oscillations [50,93,94] are due to direct modifications of the cytoskeletal [45,95] (see Figures 4 and 5). These may alter neurological electrical fields, associated brain wave pattern changes, subsequent symptomatic improvement, or possibly mitochondrial or Ca^{2+} oscillations [96]. Cantero et al. [45] have postulated that microtubules within neurons are able to generate electrical oscillations, modulate ion channels, and generate cytoskeleton regulated electrical activity (Figure 5). This may have implications for higher brain functions such as memory and consciousness. For example, improvements have been observed in

PD patients treated with transcranial PBMt, with enhancements in mobility, balance, fine motor skills, and cognition [97]. Here, it is hypothesized that microtubule depolymerization and the accumulation of dopamine and ROS in the dopaminergic neuronal cytosol causes disruptions to the microtubule and cytoskeletal dynamic, which results in mitochondrial dysfunction and an increased risk of PD [98]. The application of transcranial PBMt to PD patients may augment dopaminergic neuronal microtubule bundles and enhance cytoskeletal function, thereby reducing microtubule depolymerization, ROS, and dopamine cytosol accumulation. This may reduce mitochondrial dysfunction.

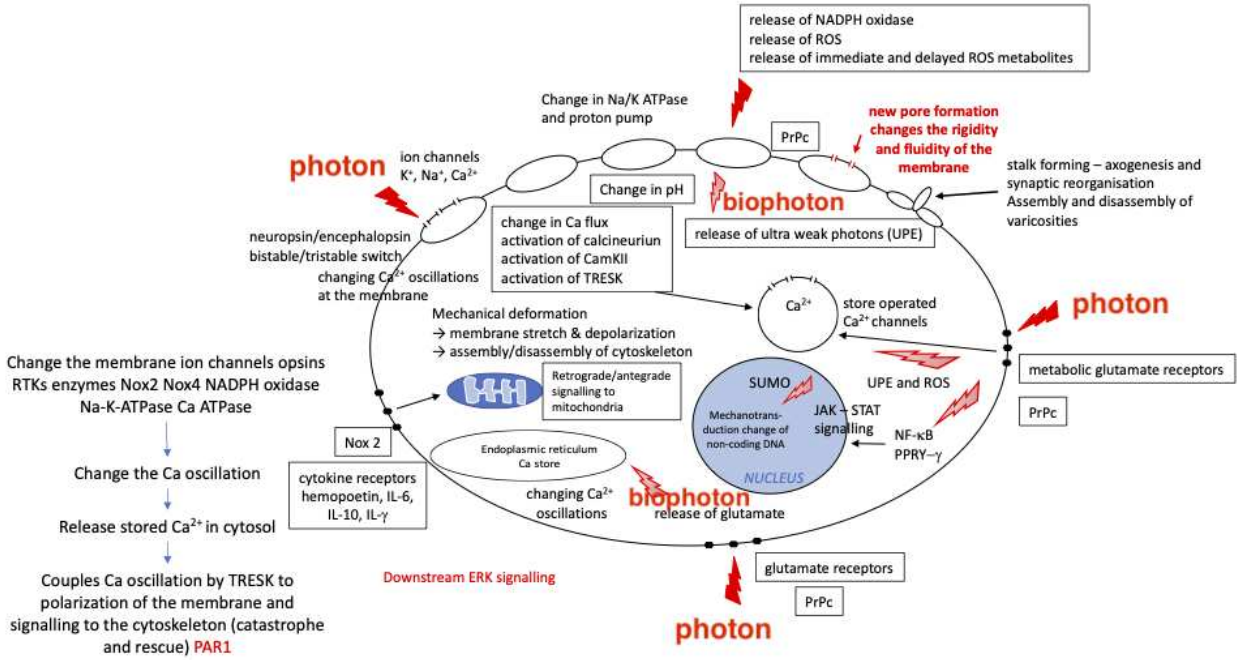

Figure 3. Mechanotransduction influences at a cellular level.

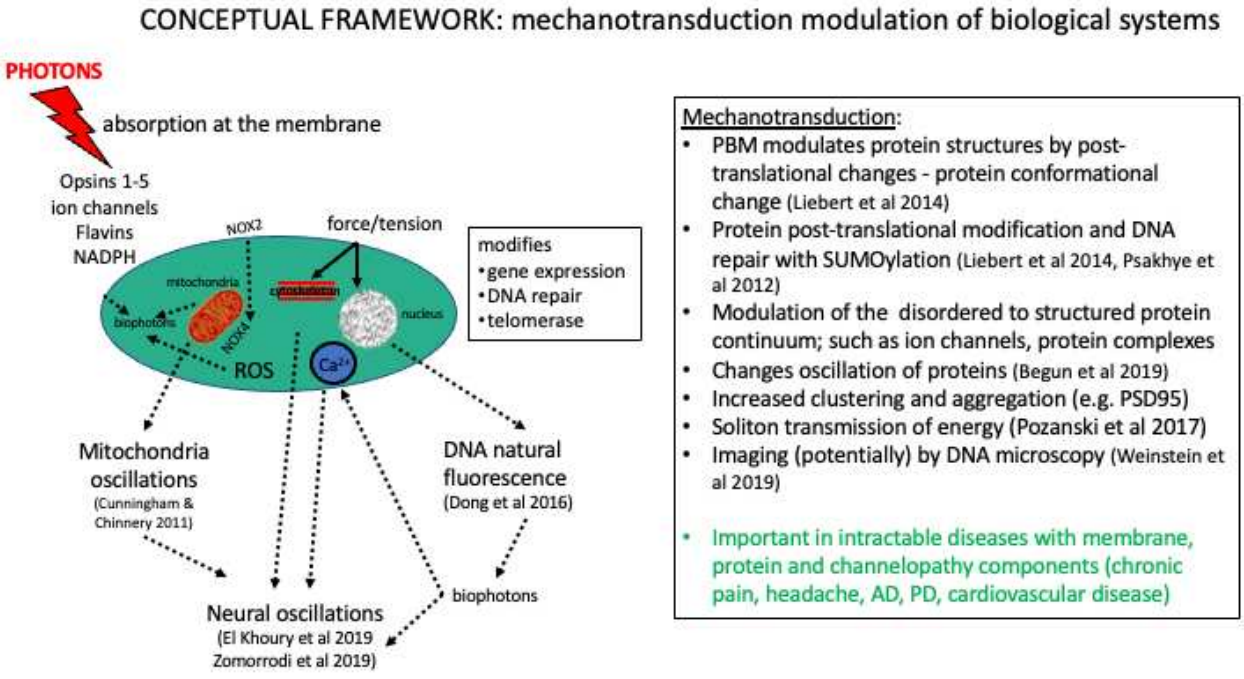

Figure 4. A conceptual framework for mechanotransduction effects of photobiomodulation [38,44,50,99–103].

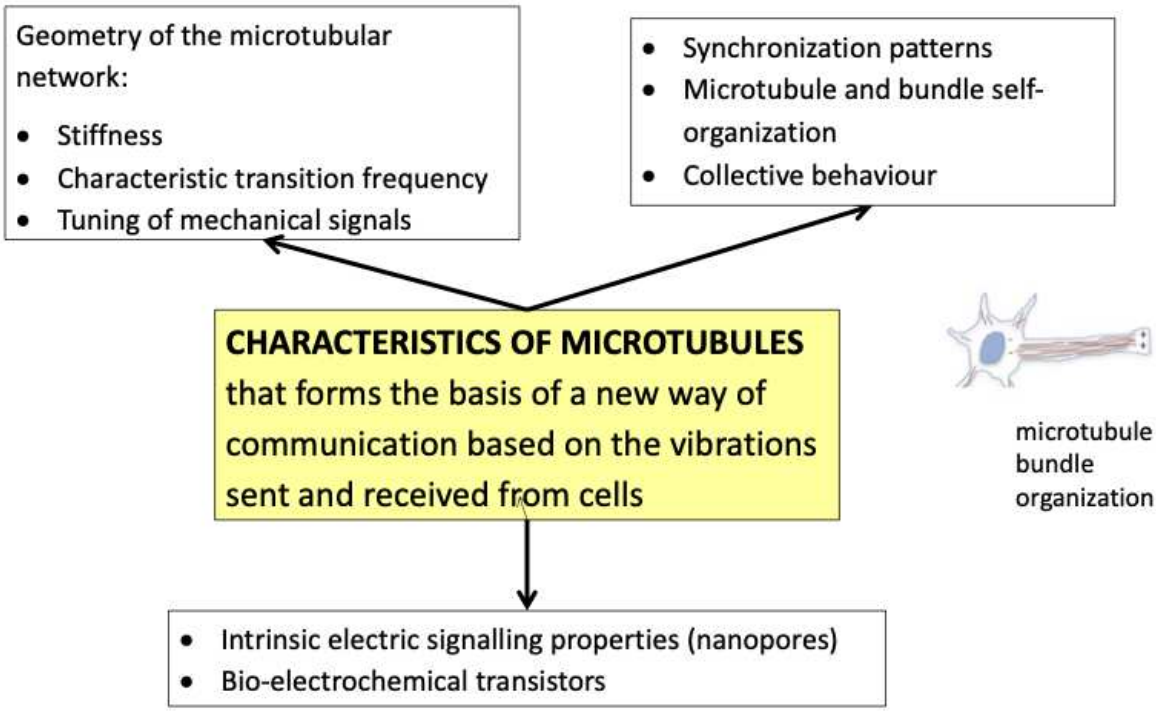

Figure 5. Proposed mechanism for cytoskeleton affecting global brain wave oscillation (adapted from Facchin et al., 2019 [49]).

3.1. PBMt Modulation of the Cytoskeleton

The cytoskeleton of the cell is made up of microfilaments, intermediate filaments, and microtubules. In addition to their biological functions, they have the capability to interact with electric signals, mechanical action, and EM fields to enable interplay between these properties. Mechanotransduction in cells (transduction of mechanical stimuli into biological signals) can be initiated when the microtubular network is exposed to EM fields in the THz frequency [95]. This means that light, as used in PBMt, is also able to modulate mechanotransduction in this system. Indeed, this mechanism may be involved in the reported effects of PBMt in cellular processes, including ATP synthesis [104], stem cell production [104,105], activation of ion channels (e.g., transient receptor potential (TRP) channels [106,107] and transient receptor potential cation channel subfamily V (TRPV) [108]), and reversal of neurological pathologies [109,110] such as in AD [111] and PD [112].

Expanding on this hypothesis, PBM has been demonstrated to induce observable changes to neuronal structures and mechanotransductive properties in the cytoskeleton, which seems to be a direct consequence of cytoskeletal modulation. Laser, but not LED, irradiation to the mouse dorsal root ganglion (DRG) neurons and cultured neonatal rat DRG neurons, using 830 nm and 20 mW continuous wave laser at doses of either 6 J over 5-min or 15 J for 15-min, resulted in "beading" or varicosity formation along the length of the neurons in the peripheral nervous system [32]. Additionally, live imaging using confocal microscopy showed the cessation of mitochondria movement along the cytoskeleton and accumulating at these varicosities. The formation of these varicosities reflects a disruption to the microtubular and cytoskeletal structures. This coincides with the decline in mitochondrial membrane potential (MMP) energy states, which are a reflection of ATP depletion within the mitochondria. Importantly, these varicosities are reversed 24 h after the cessation of PBM stimuli [32]. Similar changes have now been observed in the central nervous system, with improvements in synaptic plasticity and alterations in the cytoskeleton, including dendrites, with PBM [95,113,114].

There are several possible explanations for the physical and functional changes in neuronal cytoskeletal structure that relate to the non-phototransduction mechanisms of PBM. The polymerization and depolymerization of microtubules are energy-intensive process, occurring at about 10-min intervals. The reduction of available ATP halts polymerization, which leads to the disruption of cytoskeletal integrity and therefore the transport of signaling molecules and functions requiring ATP. This change in protein conformation can also be seen following absorption of light energy [115]. For example, the direct application of PBM at 810 nm to microtubules in-vitro causes incremental structural disassembly, resulting in a reduction in the rate and total amount of tubulin polymerization [38]. These effects appear to be dependent on the overall concentration of tubulin since as the tubulin content increases PBM irradiation also increases tubulin polymperisation rates and total polymer mass [57]. It is worth noting that laser irradiation also produces ROS including NO and singlet oxygen, which can lead to the disruption of the cytoskeleton, as demonstrated by the formation of varicosities in DRG neurons in response to hydrogen peroxide (H_2O_2) [116]. This possibly provides both a direct and an indirect mechanism, resulting in neuronal cytoskeletal modification.

3.2. PBMt Modulation of Ion Channels

It is also known that PBMt can directly target and modulate light-sensitive ion channels and signaling proteins to directly regulate microtubule function. Notable examples include firstly the weakly inward rectifying K channel (TWIK)-related spinal cord potassium channels (TRESK), which are important in photophobia, including in migraine with aura [86], and secondly the chromophore neuropsin. Both have important roles in neuroplasticity and memory and also regulate microtubule-associated protein 2 (MAP2) [117]. More recently, it has been determined that α-synuclein, which can modulate actin and microtubule activity in the cytoskeleton is important in neuronal function [118,119], with roles in axonal transport and the formation of the short microtubule subtypes [120]. Indeed, PBM has been shown to suppress the over-expression of α-synuclein in animal models of PD [121], which may occur as result of regulation of microtubule function by targeting relevant light-sensitive ion channels resulting in the modulation of neuroplasticity. A recent study by Buendia et al. [95] reported evidence of improvements in synaptic plasticity in a mouse model of AD, whereby application of transcranial PBMt resulted in the significant elevation of long-term potentiation in the Schaffer collateral fibers involved in signaling pathways between the CA3 and CA1 pyramidal neuron regions [122], potentially pointing to changes to a-amino-3-hydroxy-5-methyl-4-isoxanzolepopionic acid (AMPA) receptors and small-conductance Ca^{2+}-activated K^+ channel (SK2) channel plasticity, which in turn has been shown to participate in synaptic changes when an activity-dependent decrease contributes to changes in long-term potentiation [95].

3.3. PBMt Modulation of the Microbiome

There is also evidence to show that microbial-derived metabolites can induce actin cytoskeletal rearrangements with beneficial outcomes. Common microbial metabolites in the form of short-chain fatty acids such as butyrate and propionate caused observable alterations to filamentous actin directionality, with increased tight junction expression and protection from lipopolysaccharide-induced tight-junction mis-localization [123]. Blood-brain-barrier integrity was also improved with modulation of mitochondrial network dynamics [123]. Based on what is understood about the interaction of PBM with the gut microbiome [124], it is plausible that PBM-induced changes in microbiome populations would have a consequential impact on the circulating concentrations of microbial-derived metabolites and therefore possible actin cytoskeletal rearrangement.

3.4. PBMt and Glymphatic Clearance

The concept of cells undergoing a phase transition and the shifting of resonance (see Figure 4 and Section 4 below) may be applied to cellular proteins, such as α-synuclein,

which may have significant implications in the progression of PD pathophysiology [125]. α-synuclein has, as part of its function, an antimicrobial peptide characteristic and is important for the homeostatic metabolic function of the immune system [126]. The prionization of α-synuclein is evident in skin samples of PD patients [127], and this mechanism results in a missense of monomers of α-synuclein to aggregate as oligomers, resulting in a loss of cellular function [128]. The α-synuclein protein is present in the cerebrospinal fluid (CSF) [129] and in red blood cells [130] with physiological functions within the lymphatics and is crucial in effective lymphatic drainage and control of tissues within the CSF. The latter has been shown in mice with A53T PD, demonstrating perivascular aggregation of α-synuclein and consequentially impaired polarization of aquaporin (AQP)4 [131]. Here, cervical glymphatic ligation causes severe dysfunction in mice, with an accumulation of misfolded α-synuclein, glial activation, inflammation, dopaminergic loss, and motor deficits. Based on these observations, it is highly probable that impairment of the brain's glymphatic system can result in increased incidence of neurovascular, neuroinflammatory and neurodegenerative diseases, including the increased production of CSF by the choroid plexus and extra-choroidal sources, arterial wall pulsatility, the entry of CSF into the brain parenchyma via AQP4 channels, the accumulation of CSF within the perivenous space, and the disrupted function of the meningeal and cervical lymphatic vessels [132].

It is hypothesized that laser mechanisms underlying the positive effects of PBMt treatment for PD models [133] may have photophysical aspects related to the glymphatic system [132]. PBMt may be able to influence AQP function, including AQP4, by decreasing expression of the protein [134,135]. Since AQP4 supports CSF distribution, which is under circadian control, it is an important target in the reduction of circadian differences in drainage in the lymph nodes [60,136]. By extension, it is further hypothesized that PBMt may target the expression of AQP4. This is important because reduced AQP4 diminishes the difference in day and night levels of glymphatic influx and drainage to the lymph nodes, which suggests temporal considerations for the application of PBM in this regard.

Evidence of PBM effects on lymphatic drainage function in the brain reveal numerous recent animal studies that support the novel application of PBM to the cranial and extracranial lymphatics to control diseases where CSF outflow abnormalities are present [137]. For example, application of PBM was shown to improve amyloid-β clearance from the brain with a reduction in the density of smaller plaques [138]. This was postulated to be due to direct PBM-triggered control on lymphatic pumping and contractility, with observations that PBM application at low fluencies caused a relaxation of the mesenteric lymphatics and a reduction in systolic and diastolic contraction amplitude resulting in vessel vasodilation. This increase in lymphatic endothelium permeability allows for increased transport of larger molecules via the lymphatic vessels [139]. Interestingly, the same study reported that PBM application at higher fluencies resulted in complete blockage of vessel contractility [139], providing the possibility of fine control of lymphatic drainage as required by modulation of PBM parameters during application.

Another study reported that PBM stimulated clearing function with the accumulation of experimental tracers in the deep cervical lymph nodes from the cisterna magna. In addition, PBM-induced dilation of the mesenteric lymphatic vessels were also observed and associated with a reduction in the resistance to lymph flow [140]. Furthermore, it was also shown that the PBM irradiation of immune cells, such as macrophages, resulted in the upregulation of migration from the lymphatic vessels to the surrounding tissues, alongside increased lymphatic permeability. It is thought that this is likely to occur due to a reduction in transendothelial electrical resistance integrity and the overall expression of junction proteins such as vascular endothelial (VE)-cadherin [140].

PBM-treated AD mouse models displayed increased amyloid-β protein level accumulation in the deep cervical lymph nodes, potentially indicating an increase in the efficiency for PBM-induced stimulation of amyloid-β clearance from the brain [141]. It is possible that this increase in clearance may be a result of improvements in blood oxygen saturation, which may lead to improved mitochondrial ATP production that can stimulate lymphatic

contractility to promote increased drainage and clearing activities within the meningeal lymphatic system [132]. Further experiments by the same group reported PBM associated enhancements in neurobehavioral status in these animals as a result of altered blood-brain-barrier permeability and possibly transendothelial integrity [140]. Another study that investigated the lymphatic clearance of other cell types, such as red blood cells, found that transcranial PBM application following intraventricular hemorrhage improved cell evacuation from the ventricles and enhanced symptomatic outcomes [142]. In this study, red blood cells were found to be transported from the ventricles to the deep cervical lymph nodes more quickly than non-irradiated animals, and the rate of red blood cell elimination was found to be higher following PBM intervention [142]. Furthermore, these animals also appeared to show faster recovery from intraventricular hemorrhage following PBM intervention, with a significant reduction in mortality and reduced stress compared to non-treated animals [142].

It is worth noting that changes to permeability and endothelial function within the clearance and drainage systems of the lymphatics may, in part, be due to increases in circulating NO. Indeed, PBM has been shown to increase blood flow in both humans and animals [143,144], based on the proposition that PBM causes the disassociation of NO from cytochrome c oxidase [3]. In previous work, PBM irradiation has been shown to increase neuronal NO from activation of endothelial NO synthase resulting in vessel vasodilation [144].

4. PBMt and Photophysical Mechanisms

4.1. Cell-to-Cell Oscillation

Interestingly, some proteins and networks in cells are comparable to vibrating systems that interact with each other and with other EM fields and hence transduce EM energy into mechanical force and other biological processes [49], which is another form of signaling in the body. In other words, light acts on the energy envelope and these vibrations are transmitted to the cellular processes resulting in an enhancement of the energy that contributes to enhanced physiological function. This in itself has been described as a vibrating system [49].

Using DNA microscopy [103], a change in cellular phase transition can be visualized, which may reflect a change in protein structure that will allow for different functions and perturbations. Phase transitions are not related to chemical functions but to forces and energy parameters within the structure of the cell, including some other thermodynamic properties. The aromatic ring structure of a protein, as indicated by specific paired and un-paired electron distributions, dictates its resonance frequency.

Research into the neural mechanisms of PBMt commonly explores changes in cellular activity [145]. In this context, the electrical energy produced by the action potentials of neurons is of particular importance [146]. However, non-sinusoidal "global" neural oscillations, typically measured by EEG [147], are another form of electrical activity in the brain which are often overlooked. These brain waves are formed by the synchronized activity of individual neurons within a network, creating symphonic wave-type electrical oscillations at a lower frequency than the action potential of a single neuron [148]. Macromolecules resonate at a higher frequency compared to the slower oscillations of brain waves. Currently, there is evidence that PBMt changes the cortical connectome in the same way as ambient light [149–151], which is important because the oscillatory networks; theta, beta, alpha, gamma, are widely acknowledged as being important in health and disease [152,153]. In particular, gamma oscillations (25–140 Hz) have been well reviewed [154].

In one study, PBMt at different wavelengths was able to affect the neural oscillations, particularly in the gamma range, by increasing gamma oscillations and improving coherence during task-related activity [93]. Interestingly, oscillations were unchanged at rest after PBMt exposure; however, subsequent studies have shown that modulation of oscillations is also possible during rest [50]. In addition, transcranial PBMt with near-infrared (NIR) light at a wavelength of 810 nm and frequency of 40 Hz changed the default mode network

and increased the power of the higher oscillatory frequencies of alpha, beta, gamma [50]. It is likely that this modulation of the default mode network is influenced by neurochemical concentrations of glutamate and gamma aminobutyric acid (GABA), as a high concentration of glutamate in the posterior cingulate cortex and pre-cuneus area is reportedly linked to reduced neural deactivation [155]. Interestingly, cortical spreading depression, which is characterized by increased glutamate and extracellular K^+ concentration, is one cause of migraine with aura [156], another pathology characterized by abnormal cortical coherence [157]. Additionally, migraine without aura is associated with disrupted default mode network connectivity [17].

The application of different wavelengths, by specific order of application or simultaneous application, can significantly change the oscillation activity in neurons [133]. These changes may be due to the cross-interaction of melanocortinergic and dopaminergic systems resulting in neural modulation [158], which in turn depend on membrane action potential wave-forms in pyramidal neurons of the prefrontal cortex [159,160]. These modulations may have direct implications for the way that PBMt should be used to modulate neural oscillations for the treatment of pathologies that manifest in impaired neural oscillatory networks. This includes an understanding of the types of neural oscillation variation.

4.2. Wavelength Specificity and Protein Interactions—Photophysical Resonance

The commonality between laser-produced light and proteins is in their mutual oscillatory properties. Indeed, two entities vibrating at equal natural frequencies can interact with and activate one another. This is part of the so-called resonance phenomenon. Specifically, this model portrays protein-to-protein interactions as occurring via resonant EM energy transfer [29]. This experimentally-validated hypothesis [161] has significant implications for PBMt because the EM resonance is within the range of infra-red and visible light [29]. The spectral and space analysis of a protein can determine the distribution of free energy electrons, which have specific frequencies for specific functional groups [162,163].

These principles present the prospect, in precision medicine, of laser intervention to target specific proteins. The inherent resonant properties of proteins are shared with the coherent light produced by lasers. Since the EM resonance energy transfer is integral to protein-to-protein activation, it is expected that the laser wavelengths used for PBMt can interact with the same proteins resonating at equal frequencies. Indeed, this hypothesis accounts for the importance of wavelength in achieving beneficial results in therapy. In particular, signaling proteins have autofluorescence characteristics in the infrared range [161], which may explain why infrared and near-infrared wavelengths of PBMt are most effective for treating neurological performance [164], generalized anxiety disorder [165], and PD [166].

4.3. Fluorescent/Auto-Fluorescent Proteins

Beyond the photophysical resonance of endogenous molecules and cells, certain fluorescent/auto-fluorescent molecules will also have an influence, if not interplay, with existing oscillations, or those modulated by PBMt. In this context, cellular auto-fluorescence, as defined by Surre et al. [167] denotes the production of intrinsic natural fluorescence deriving from fluorescent cell structures and metabolites, with common examples including flavins, NAD, aromatic amino acids, lipofuscins, advanced glycation end products, and collagen [167–169].

Indeed, it has been shown that DNA molecules can naturally fluoresce once they briefly exit their "dark state"; an extended time period wherein they do not absorb nor emit light, and the fluorescence process can be initiated by applying a certain wavelength of light [100]. It is possible that non-auto-fluorescent proteins, when subjected to the correct excitation wavelength, will also absorb light and initiate downstream processes. Moreover, the implications of fluorescent proteins being specific directional light absorbers and emitters suggests that interventions that utilize light to elicit a physiological response, or in the detection of biological activity, may also be dictated or modulated by the directional

properties of proteins. Indeed, targeting cellular autofluorescence for measurements of metabolic activity and other diagnostic investigations has been previously shown and the numbers of tissue and cellular types found to be able to produce autofluorescence is increasing, with studies reporting naturally occurring detectable fluorescence from white blood cells [170], fibroblasts [171], the liver [172–175], and the kidney [55].

Finally, the connection between endogenous resonance and fluorescence is revealed by linking two separate studies that investigated novel methods to discriminate cancerous cells. The first used biophotonic analysis to demonstrate that stressed, cancerous cells emitted shorter wavelengths of light compared to non-cancerous cells, which emitted photons within the near-infrared range [161]. This demonstrates that the EM nature of a cell is altered according to its health. Equally important is the identification of a resonance fingerprint of cancer DNA, at approximately 1.6 THz, which is a specific signal possibly due to aberrant methylation [176]. Together, these studies highlight the potential for an interactive relationship between endogenous resonance and biophoton emission. Resonant polarity and direction are also currently being investigated to determine the importance of the angle of light emission [177], which further demonstrates the difference between results using laser PBMt with coherent light, compared to LEDs.

5. Current Clinical Applications

The clinical applications of the photophysical pathways go hand-in-hand with the phototransductive mechanisms underlying the effects of PBMt in health and disease. Any pathology that involves photosensitive proteins that are responsive to light-induced oscillation modulations, that can induce global oscillatory interactions, could be considered to be amenable to PBMt as a therapeutic option.

5.1. Resonance

A clinical example of light therapy and its interaction with resonance theory at the protein scale is the treatment of Crigler-Najjar syndrome. This rare syndrome is characterized by a lack of 5′-diphosphglucuronosyltransferase 1-A1 (UDP) activity, which plays a role in glucuronidation of unconjugated bilirubin in the liver [178] (see Figure 6). To date, the most effective treatment of this syndrome in babies and young people is administering blue light phototherapy to the patient [43].

The effectiveness of blue light phototherapy for Crigler-Najjar syndrome may be due to the EM modulation of cellular proteins. Experimentally, the function of human protein UDP in bilirubin metabolism has been analyzed using the resonant recognition model (RRM), which showed characteristic frequencies associated with the blue light spectrum [43]. This presents a biophysical relationship between the UDP protein characteristic frequency and the wavelength of blue light. It is therefore proposed that blue light may serve as an "imitator" of the resonant activity typically present in UDP [43].

There may be other diseases besides Crigler-Najjar that can be managed in a similar way, including illnesses characterized by abnormal neural oscillatory activity (especially with reference to changes in the microtubular and cytoskeletal network) and those with pathologies in the central and peripheral nervous systems, such as PD, AD, chronic pain and inflammation, autism, and migraines [18,157,179–186].

5.2. Neutrophils

Practical applications of modulation of neutrophil processes are important in the treatment of inflammatory lung disease and other diseases of inflammation [187]. Mechanotransduction plays a key role in neutrophil activation and deactivation [188]. An understanding of the photophysical aspects of mechanotransduction activation in neutrophils will be crucial in precision medicine for conditions with motor dysfunctions, such as neurodegenerative conditions and cardiac diseases.

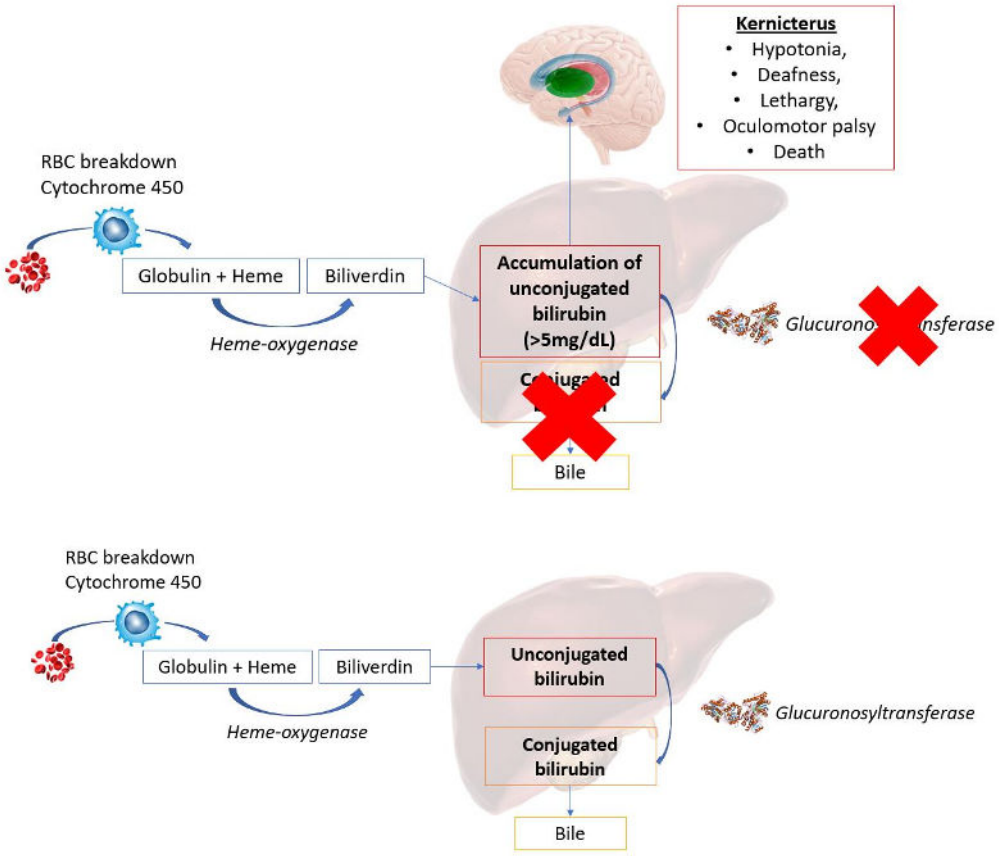

Figure 6. A schematic comparing functional and dysfunctional glucuronidation of unconjugated bilirubin in the liver. Dysfunctional glucuronidation is characteristic of Crigler-Najjar syndrome.

PBMt has a considerable effect on neutrophil function particularly in increasing neutrophil phagocytosis efficiency and in modulating the concentration of neutrophils produced during an immune response, particularly in the lungs [68,187]. This may be important in the observed decrease of neutrophils with aging [189], the impairment of neutrophils in cardiac diseases [190,191], PD [192–195] and increasingly in viral load sequelae, including the current COVID-19 pandemic [196]. A recent review supported PBMt as an adjunctive treatment of lung inflammation and for rehabilitating other affected organs by modulating neutrophil influx and inhibiting the macrophage inflammatory protein-2 and thereby reducing pulmonary edema [197].

It can be postulated that dysfunctional non-visual phototransduction processes, including photonic production, may also reflect disease processes involving neutrophils, including in the heart, since neutrophil membranes are a major source of photonic production [198,199]. This is particularly important in diseases concerning DNA aberrant methylation, such as chronic pain [200,201] and cancer [202–204]. Additionally, there are several diseases that involve abnormal neutrophil activation [205–207], for example, an abnormal lung response to air-borne toxins such as those found in air pollution, causing the dysfunctional activation of neutrophils [208,209], which may be modulated by PBMt. There is evidence that PBMt can modulate neutrophil activation, both by decreasing excessive neutrophil response and making the neutrophil burst more effective [197,210]. There is also increasing evidence that physiological processes have a biophotonic emission signature [53] that is different from the signature of pathophysiological processes [161,211,212].

5.3. Channelopathies

Mechanotransduction plays an essential role in myocardial mechano- and electrophysiologic function. It involves an assembly of protein complexes to mediate the sensing

and transmission of mechanical or electrical loads. These proteins, largely within the sarcomere, intercalated disc, and sarcolemma of myocardial cytoskeleton, trigger cascading intra- and inter-cellular processes, possibly effecting anatomic and physiologic or pathophysiologic alternations [213].

Genetic mutations in intracellular processes of mechanotransduction are the responsible agents in some of the channelopathies and cardiomyopathies, such as arrhythmias, including sudden death and heart failure [214,215]. Importantly, the disruption of mechanotransduction pathways has also been shown to play a significant role in initiation, and the progression, of other cardiovascular diseases including atherogenesis, hypertension, and atrial fibrillation [216,217].

PBMt may offer novel therapeutic applications in relation to mechanotransduction anomalies of the heart. PBMt has been recently shown in experimental models to resolve atrial fibrillation [218] and cardiac pacing utilizing an optogenetics approach [219]. Additionally, a recent study revealed that an optogenetic approach can also be used to activate dopaminergic neurons of the substantia nigra pars compacta in an experimental animal model [220]. This was achieved through upregulating levodopa (L-DOPA) production by recovery of tyrosine hydroxylase, which has implications for PBMt.

5.4. Analgesia and Anaesthetic Effects

The analgesic properties of PBMt, beyond the resolution of cortical coherence and brain wave pattern disruptions, are supported by a wealth of data that provide insight into the possible delivery of pre-emptive PBMt in the prevention and development of persistent pain [221], including neuropathic pain [87] chemotherapies [222], neck pain [223], low back pain [224], and pain following nerve or spinal cord injury [225]. Hypothetically, reversible cytoskeletal disruption may modulate pain by disruption of cytoskeletal and microtubular structures to physically interrupt ATP delivery and block neuronal depolarization to limit afferent signaling to the dorsal horn and through the disruption of fast axonal flow and limiting the transport of pro-inflammatory cytokines, as is evident via the appearance of dendritic varicosities.

A recent review evaluated the potential role of transcranial PBM as an adjuvant to enhance the effects of pharmacological anesthetics, coining the term "Optianesthesia" to describe this effect [226]. In this context, transcranial PBM at wavelengths of 808 nm or 810 nm had inhibitory effects on the cortex and hippocampus of healthy rats [227], showing possible therapeutic effects with reported attenuation of pharmacologically-induced seizures [228–230]. These observations were also reported when transcranial PBM was combined with valproic acid in the same model [231]. Furthermore, application of transcranial PBM has been shown to elicit anticonvulsant effects, showing evidence of abnormal electrical discharge inhibition. Based on these reports, it could be speculated that PBM delivered transcranially may be a promising adjuvant or add-on therapy in combination with general anesthesia to treat pediatric refractory status epilepticus and super refractory status epilepticus, which may in turn reduce some of the side effects experienced following administration of anesthetics [226]. To achieve this, it is possible that PBM may be altering consciousness reversibly via modification to quantum processes in microtubules that underly consciousness, in a similar way that general anesthetics can bind and affect microtubules to influence consciousness [232–234], including acting on quantum ion channels in neuronal microtubules specifically in brain regions known to be targeted by general anesthetics [57,232,235,236]. It is also suggested that transcranial PBM may aid in the distribution of pharmacological general anesthetics. There is evidence to suggest that PBM application has an arousal-dependent effect, that when applied during wakefulness is able to stimulate neuronal functions, such as increased mitochondrial activity and gene expression, as well as influence alpha, beta, and gamma waves and enhance neuronal protection and survival against distress and neurodegenerative diseases [60]. When PBM is applied during sleep, it is possible that there may be increased clearance of cerebral spinal fluid, which may be due to an increase in the permeability of AQP4 in astrocytes [60]. These

mechanisms may be relevant when the maintenance phases induced by using intravenous and inhaled anesthetics are considered, whereby the PBM-induced increase in cerebral spinal fluid flow in tandem with general anesthetic administration may have a synergistic effect in their speed of delivery and eventual distribution in the body.

5.5. Wounds and Aging

Increasing numbers of studies have reported evidence of downstream epigenetic changes following PBMt application, including changes to histone acetylation and DNA methylation that have consequential effects on functional cell maturation [237]. For example, PBMt for epithelial wound healing has shown accelerated epithelial migration and chromatic relaxation, along with increased levels of histone acetylation and the expression of cyclic AMP response element-binding protein (CBP) p300 and the mammalian target of rapamycin (mTOR) [238]. PBM was also shown to reduce levels of the transcription repression-associated protein methyl-CpG-binding domain proteins (MBD2), along with decreased numbers of epithelial stem cells and spheres [238], making it plausible that PBMt can induce epigenetic changes to epithelial cells to accelerate healing. Similarly, transcranial PBM was reported to increase signaling proteins related to both cell proliferation and cell survival [239]. Similar changes were also reported underlying oral ulcer repair using PBMt, which accelerated repair of oral ulcers and increased both histone 3 acetylation and NF-kB positive cells [240]. Interestingly, prolonged PBM application resulted in a reduction of histone 3 acetylation and NF-kB cells, suggesting that PBMt can stimulate keratinocyte migration during the initial phases of epithelial wound healing, followed by keratinocyte differentiation during the final stages [240]. Signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), p70 ribosomal protein S6 kinase (p70S6K) and protein kinase B (PJB) were all shown to be modulated following PBM application in a rat model. PBM was shown to activate STAT3, ERK and JNK signaling proteins in the cerebral cortex, while the increased expression of p70S6K and STAT3 and the activation of Akt were observed in the hippocampus. PBM was also shown to improve intracellular signaling pathways linked to cell survival, memory and glucose metabolism in a aging rat brain model [239].

6. PBMt and Neuro Oscillatory Networks: Clinical Implications

The ability to modulate impairments in neural oscillatory networks is especially relevant to PD [183,186], where neural gamma oscillations are impaired in individuals with the disease. Gamma oscillations are vital in cortico-basal ganglia loops which govern motor control [19], and thus their dysfunction gives rise to typical Parkinsonism symptoms such as resting tremors. Now that it has been shown that gamma oscillations can be modulated with transcranial PBMt [50], clinicians may be able to better manage the symptoms of PD. The early results of one ongoing study on PD patients using LED-lined cranial "buckets" have suggested an overall improvement in symptoms in more than half of the participants [241], although high-quality trials with larger participant numbers are needed.

Migraine is partly characterized by abnormal cortical coherence [179,182] and is also defined as a channelopathy [242], which is an impairment of ion channels and their receptors [243], and in turn shares a common etiology with epilepsy [244]. It has been postulated that genetic channelopathies have implications in chronic pain conditions, as well as acquired channelopathies such as from trauma and whiplash [245,246]. Migraine is one neurological condition where targeting neural oscillatory irregularities may be a plausible form of treatment. Migraineurs present with an abnormally increased amplitude of low-frequency oscillations (LFOs) in the thalamocortical networks [157]. The increased amplitude of these LFOs, which are characterized as delta oscillations, positively correlates with the increased frequency of headaches. Therefore, the defective thalamocortical brain-wave activity of migraineurs may predispose migraineurs to repeated episodes [157] and could therefore be a therapeutic target for the suppression of subsequent migraines. Transcranial PBMt has been shown to reduce the power of delta oscillations [50], and the

rhythmic cortical feedback to the thalamus influences thalamic oscillatory behaviors [157]. Thus, light that penetrates through the scalp and reaches the cortex may modulate low frequency oscillations in sub-cortical structures. Since it is now known that structures such as the suprachiasmatic nuclei in the hypothalamus show tissue-level rhythms during fetal development, before clock gene expression introduction [247], it is plausible to postulate that external triggers, such as PBM irradiation, could elicit a response from environmental factors affecting innate oscillation.

Gamma oscillations are also impaired in individuals with widespread centralized pain in fibromyalgia [18], schizophrenia, cognitive disorders, and other neurodegenerative diseases, including AD [180,181,185]. Autism has also been shown to involve impairment of cortical coherence [184] and would also be a potentially novel target for treatment with PBMt [248]. Interestingly, AD is of particular relevance due to its characteristic pathology of fibrillated amyloid-β. Indeed, amyloids typically have optical absorption properties, yet this feature is absent in the fibrillated state [37]. Other studies have demonstrated effects of PBMt on sleep enhancement [249], which is often reported as a positive side-effect of transcranial PBMt [165,250,251]. The fact that the oligomer length of amyloid-β bi-directionally modifies sleep [252], combined with the optical absorption properties of amyloid-β [37], presents the possibility that amyloid-β may be directly affecting sleep in patients and is a target for patients receiving PBMt. Further, neural oscillations are associated with quality of sleep and are vital to achieving deep stages of sleep (rapid eye-movement (REM) sleep) [253], suggesting that the observed effects may be explained by a neural oscillation modulation mechanism.

There is clear evidence that transcranial PBMt modulates cortical oscillatory behavior, however to date, there is a debate as to the mechanism involved. It is proposed that propagating biophotonic and electrical signaling may become synchronized so as to alter the slow waves in the body, including the brain. When groups of neurons synchronously "fire" action potentials, they can form larger, slower waves in the brain [148]. It is therefore possible that the high frequency oscillations of DNA may interact on a "local" scale with proximal DNA from other cells, to combine and produce global, slower forms of oscillation in the brain and body. Another possible mechanism by which PBMt affects global circuits is through interactions with other novel oscillations, such as calcium, mitochondrial, or astrocyte oscillations [254–256].

7. Future Implications of Photophysical PBMt Mechanisms Applied to Clinical Therapy

The implications of this photophysical hypothesis become significant when the development of treatment and management of diseases that are characterized by abnormal neural oscillations, such as migraine, PD, autism, and AD are considered. Since neural oscillations can be modulated by transcranial PBMt [50,93,257,258], this intervention may be an effective therapeutic application for these pathologies. The evidence of a modulatory mechanism in healthy participants is promising [93,94,259], although further studies are needed to evaluate the effect of PBMt treatment on symptomatology.

The RRM can be used in designing the formation of novel antimicrobial peptides in the treatment of skin cancers [260] and pathologies involving dysfunctions in neurogenesis [261,262]. Promoters or inhibitors designed using the RRM can modulate the quantity of biphoton emissions to either facilitate or block release. One study has shown that malignant cells emit blue wavelengths of light compared to healthy cells, which emit mainly biophotons in the infrared bandwidth [161]. Additionally, a recent study reported biophoton analysis to be useful in the discrimination of precancerous cells [161]. This non-invasive method utilizes wavelength-exclusion filters on cell cultures to determine the wavelength and amount of biophoton emission. By extension, this may have significant implications for the early detection of disease since the wavelength of the emitted biophotons appears to be indicative of cellular health. In summary, stressed cells may be subject to phase changes that influence the wavelength of light that they produce.

8. PBMt and Precision Medicine

The photophysical mechanisms described here combine known biological and biophysical phenomena to explain novel cellular signaling and their associated downstream effects [263]. The hypotheses presented here are multidisciplinary and comprises mechanisms beyond cytochrome c oxidase activation and offer a new perspective on the photophysical effects of PBMt and its relevance in the optimization of health outcomes and performance.

The potential signaling pathways employed during cell-to-cell communication utilizing biophotons [47] presents the possibility that externally applied light via PBMt may modulate these pathways and instigate cellular processes such as protein conformational changes in PBMt treated cells [38]. The RRM concept [162] underpins the local oscillatory protein signatures and forms the basis of our proposed perspective on the similarities between micro-oscillations observed following either PBM light-induced interactions with proteins or activation by ATP. The wavelength at which proteins oscillate influenced by endogenous cell biophotons, would inform on precision medicine. In the case of laser PBM, the light is coherent and oscillates at a specific frequency depending on the wavelength and thus can interact (according to RRM) with proteins resonating at equivalent frequencies. This is illustrated clinically through the blue light treatment of Crigler-Najjar syndrome, with the wavelength of light sharing a comparable resonant frequency with the UDP protein.

These local resonant interactions may also explain recent evidence for neural oscillation modulation by transcranial PBMt [50], which may be important in treating pathologies associated with the impairment of neural oscillations such as pain in fibromyalgia [18], schizophrenia, cognitive disorders, autism [184], migraine, and neurodegenerative diseases, such as PD and AD [180,181,185]. Beyond electrical signaling, there exists neurotrophic signaling and fast axonal flow, protein-oscillation communication, and electric oscillations of microtubules [264–266]. This latter mechanism may involve microtubule dynamics in the regulation of excitability in neurons, and involve the presence of ion channels, including potassium channels such as TRESK [45]. Clinically, this is important in migraine with aura and related diseases that involve this mutation, a well as other dysautonomia-related diseases, including PD and cardiac disease.

Future experimental and clinical studies on PBMt should determine the precise wavelengths efficacious for specific disease processes. Biophotonic analysis of the brain should be investigated during transcranial PBMt so as to determine if there is an increase in biophoton release and which wavelengths are being emitted.

9. Conclusions

PBM affects a wide range of biologic and pathologic processes. Our paper adds new pieces of information to the existing literature on PBM's complex mechanisms of action as modulators of cellular function and metabolic pathways as well as introducing host/microbiota interactions in health and disease. Knowledge of photophysical mechanisms would be beneficial in guiding the future design of experimental and clinical studies of PBMt. This could include synergistic transcranial and systemic applications of PBMt. The knowledge of photophysical mechanisms, especially oscillatory and resonance modulation mechanisms, could therefore be utilized to identify treatment outcomes with different precision applications of PBMt and foster future analytical studies of varying biophotonic activity at the tissue and molecular level. By considering the whole body as a system with interacting oscillating components, light allows an enhancement of the energy that makes the physiological processes tend towards better function. This may have implications for the vibrating body system as a whole and the loss of this vibration in aging. The potential mechanisms of PBM beyond cytochrome c oxidase presented here may well overlap in their modes of action, producing synergistic or complementary outcomes.

The perspectives presented here might also offer a new insight and drive future research into the global photophysical effects in PBMt, which may be important when

considering the relevance of PBMt in clinical applications, including the treatment of diseases, especially inflammatory diseases of various neurological and metabolic disorders with a focus on optimization of health outcomes and performance.

PBMt as a non-invasive, low-risk modality can deliver precision medicine for various diseases. The therapeutic regimen of light therapy and mode of delivery can then be individualized to target specific disease processes utilizing the most effective mechanistic pathways.

Author Contributions: A.L.—original ideas, background research, photophysical hypothesis, diagrams, substantial writing contributions pertaining to all areas of the paper, editing. W.C.—background research, substantial writing contributions pertaining to all concepts of the paper, referencing, editing. V.P.—background research, substantial writing contributions pertaining to all concepts of the paper, referencing, editing. D.V.—substantial writing contributions pertaining to biophysical and quantum physical concepts, editing. B.B.—original ideas, editing. C.M.—substantial writing contributions pertaining to all concepts of the paper. H.K.—substantial writing contributions pertaining to mechanotransduction and cardiac mechanisms. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: No new data was created.

Acknowledgments: We would like to thank Roger Adams for continued encouragement and inspiration, Roberta Chow for her mentorship in PBM, Jonathan Stone and John Mitrofanis for academic inspiration and the late Anthony Ancell for starting A.L., B.B. and H.K. on this scientific journey.

Conflicts of Interest: The authors state that no conflict of interest exists.

Abbreviations

AD	Alzheimer's disease
AQP	Aquaporin
ATP	Adenosine triphosphate
PBMt	Photobiomodulation therapy
CASP-9	Caspase-9
CSF	Cerebrospinal fluid
DRG	Dorsal root gangion
EM	Elctromagnetic
GABA	gamma aminobutyric acid
H_2O_2	Hydrogen peroxide
JAK-STAT	Janus kinases-signal transducer and activator of transcription proteins
KHz	Kilohertz
L-DOPA	Levodopa
LED	Light-emitting diode
LFOs	Low-frequency oscillations
MAP2	Microtubule-associated protein 2
MMP	Mitochondrial membrane potential
NIR	Near-infrared
NO	nitric oxide
PD	Parkinson's disease
REM	Rapid eye-movement
ROS	Reactive oxygen species
RRM	Resonant recognition model
SPD	Spectral power density
THz	Terahertz
TRESK	TWIK-related spinal cord potassium channel
TRP	Transient receptor potential
TWIK	the weakly inward rectifying K channel
UDP	5′-diphosphglucuronosyltransferase 1-A1
UPE	Ultra-weak photon emissions
VE	vascular endothelial

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