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Photobiomodulation: Cellular, molecular, and clinical aspects

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ABSTRACT

Photobiomodulation (PBM) is a noninvasive photonic-based therapy, capable of dealing with immune-inflammatory, neurological, and musculoskeletal disorders, as well as healing oral and chronic skin wounds. During PBM light is applied at a specific wavelength, either in the visible or near-infrared (NIR) ranges. Photophysical and photochemical processes might stimulate or inhibit various biological processes, depending on the target tissue, the wavelength of light, irradiance, fluence, repetition rate (pulse frequency), spot size, optical data of the tissue to be irradiated and treatment regimen. There are several randomized clinical studies demonstrating the PBM benefits as main or adjuvant therapies. Of importance to this review, there is a large piece of evidence in the management of skin or venous ulcers, and diabetic foot. In this review, the PBMs efficacy as adjuvant therapy to deal with chronic human ulcers were discussed concerning the photophysical parameters and clinical aspects. Beside, we overview the state-of-the-art regarding the cellular and molecular modulatory mechanisms photo-activated by red and NIR light.

1. Introduction

Photobiomodulation (PBM) is "*a more accurate and specific term for the therapeutic application of low-level light compared*" to Low-level light therapy (LLLT) as defined in Tsai & Hamblin [1]. According to the Medical Subject Headings (MeSH) Identifier "D028022", Established Data 2002–01–01, LLLT corresponds to "*treatment using irradiation with light of low power intensity so that the effects are a response to the light and not due to heat. A variety of light sources, especially low-power lasers are used*". Therefore, PBM is a noninvasive light based-therapy capable of controlling musculoskeletal pain as well as fibromyalgia [2]. Beside PBM has been highlighted as a novel therapeutic modality for the treatment of several neurological diseases, immune-inflammatory, and other morbidities, including skin ulcers and oral mucositis [3]. PBM was found to be safe and effective to mitigate oral mucositis associated with chemotherapy in cancer patients [4,5]. In this review, we focused on chronic ulcers and how PBM might deal with them.

Chronic ulcers affect millions of people around the world, putting a strain on public and private healthcare systems [6,7]. The wound-healing process relies on highly integrated cell and biochemical signaling pathways that regulate hemostasis, inflammation, proliferation, and tissue remodeling [8–11]. And multiple factors can compromise wound healing. Among the systemic factors that are known to impact wound healing include genetic background, aging, and comorbidities such as diabetes [10].

Aside from them, local factors can also directly influence wound pathophysiology itself and so contribute to delayed healing, such as infection. Current studies have shown how bacterial diversity and instability increments alter the microbiota composition and how they might impact wound healing [12]. For example, proteolytic enzymes from the genera *Staphylococcus* may affect the extracellular matrix during wound contraction, leading to delayed healing [13]. Among the recent promising therapeutic strategies that emerge to eradicate biofilm and improve wound healing are based on light-induced cellular and molecular mechanisms [14].

As photonic-based therapy, PBM relies on electromagnetic radiation (light at red and near-infrared wavelengths) to stimulate or inhibit various biological processes, depending on the target tissue, the

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wavelength of light, irradiance, fluence, repetition rate (pulse frequency), spot size, optical data of the tissue to be irradiated and treatment regimen. The electromagnetic spectrum comprehends a range of frequencies (spectrum) and their respective wavelength (nm) and photon quantum energy (eV). The visible light extends from the 400 to 700 nm range, which corresponds to a respective photon energy of 3.1 eV - 1.77 eV. Beyond visible light, there is a near-infrared (NIR) portion of the electromagnetic spectrum, which is commonly used in PBM to relieve several neurological conditions [15]. Despite there is no universally accepted definition of the range of NIR radiation, typically it considers the range of 700 nm to 1.0 mm, which corresponds to 1.77 eV - 1.24 eV [16].

PBM usually employs light sources ranging from the visible spectrum such as blue (405 to 470 nm), red (600 to 700 nm), or NIR [15,17–20]. Concerning light spectra, PBM shows effective wound healing related to regeneration and anti-inflammatory signaling specifically in the red and NIR spectrum (wavelength between 630 and 850 nm) [21].

As a photonic-induced process, several aspects must be considered before choosing PBM as a therapeutic strategy, including the disorder type, target cell, light wavelength spectrum, irradiance (power density, intensity, or creep rate), and fluence (radiant exposure, dose, or energy density). To underline possible parameters or clinical baseline features we critically evaluated the selected clinical studies regarding PBM efficacy to alleviate skin ulcers. In this regard, we pointed out the main differences and pitfalls that may compromise clinical outcomes.

2. Methodology

To provide antecedents and examine evidence on the state-of-the-art regarding the photophysical, biological, and clinical aspects of PBM (red and near-infrared spectrum), we conducted an integrative/narrative review. We selected clinical studies focused on wound healing to uncover probable pitfalls that may affect PBM efficacy or safety. Beside, to overview the photobiology aspects and PBM effects on microbiota we contemplate original published articles, as well as reviews. All paper selection was carried out in library databases (Web of Science, PubMed, Google Scholar, and Scopus) contemplating published articles from 2010 to 2021. The descriptors applied in the search were selected from the controlled vocabularies Health Sciences Descriptors (DeCS) and Medical Subject Heading Terms (MeSH). The following descriptors and Boolean operators were used: "low-level light therapy OR photobiomodulation" AND "ulcer OR wound" AND "diabetic foot OR pressure OR venous" AND "clinical trial OR clinical study". In addition, we selected cited articles from reviewed publications. Studies published in potential predatory journals according to Beall's list [22], without a full text or that did not contemplate the proposed theme were excluded.

3. Photophysical, photochemical, and biological principles of PBM

The therapeutic PBM effects rely on photoinduced biological interactions targeting intracellular components, which depend on photophysical light aspects including reflection, scattering (non-productive), transmission, and absorption (productive). The light-tissue interactions may be influenced not only by the wavelength but likewise by the biological characteristics of the target cell, and corresponding endogenous chromophores and photoreceptors. In fibroblasts and neurons, for example, PBM may photoactivate distinct processes according to the light wavelength used [15,23].

As recently reviewed, PBM might activate different processes in the brain according to the light-spectra range: (1) the NIR region where there is a balance of calcium levels inside cells, whose channels are sensitive to light stimuli, and causes the vibration of water inside the cell; (2) the light in the red and NIR region shows a primary photoreceptor the enzyme cytochrome *c* oxidase (CcOx) - a terminal complex of the mitochondrial respiratory chain, responsible for oxygen consumption (about 90%), and so it is responsible for almost all energy produced in mammalian cells. Thus, the CcOx light activation may boost metabolism due to increased ATP synthesis; (3) the green light (around 500 nm) may photoactivate opsins, enhancing the calcium permeability, as well as modulating magnesium and sodium channels or activating other cell signaling pathways; and (4) the 400 nm visible spectrum range (i.e., blue light) improves cellular respiration by targeting cryptochromes and flavoproteins as well may promote the expression of vascularization factors [15]. Concerning tissue regeneration and anti-inflammatory response, the red and NIR spectrum (630 to 850 nm) were found to be more effective [20]. Whereas blue light inhibits proliferation red or infra-red light stimulates cell growth, which was associated with increased ATP levels and mitochondrial membrane potential (MMP), and only generated modest amounts of reactive oxygen stress (ROS) [24]. Thereby, in our revision regarding the PBM effects on wound healing, we focused on these light wavelength spectra (Fig. 1).

As reviewed, PBM may stimulate CcOx activity and improves tissue regeneration that relates to increased collagen production, increased microvascularization, anti-inflammatory response, and analgesia [18]. As a terminal enzyme IV complex of the respiratory chain, CcOx catalyzes the cytochrome *c* (cyt *c*) oxidation and contributes to a proton electrochemical gradient across the inner mitochondrial membrane of eukaryotes used by ATP synthase to produce ATP, the universal energy form of the cell [25].

CcOx is a multi-subunit enzyme complex that compromises four redox active metal centers - two heme iron (a and a_3) and two copper (Cu_A and Cu_B). It couples the transfer of electrons from cyt *c* - a 12 kDa small globular protein with a *c*-type heme, which consists of a porphyrin (tetrapyrrolic macrocycle) ligated to iron. The electron transfer properties of cyt *c* arise from the ability of its iron center to change the oxidation state, and so to bind $^3O_2$ and NO. From heme *a*, electrons from cyt *c* are transferred intramolecularly to the active site of CcCOx (i.e., heme a_3/Cu_B), where $^3O_2$ and NO bind. Contrasting NO, for $^3O_2$ binding a complete reduction of this binuclear site is necessary [26].

Despite not being experimentally confirmed, the most popular theory to explain exactly why CcOx photon absorption could boost cellular mitochondrial metabolism is based on inhibitory nitric oxide (NO) photodissociation related to probable photoactivation of cyt heme-containing porphyrin. By binding CcOx in a competitive high-affinity inhibition site (Fe^{+2} heme a_3) and a noncompetitive, lower-affinity site (Cu^{+2} Cu_B), NO may reversibly inhibit mitochondrial respiration. Being not covalently connected to the binuclear center (a_3/Cu_B) NO can reversibly and specifically inhibit CcOx in competition with $^3O_2$. For example, at low $^3O_2$ tensions (or the consumption rate is increased) NO interacts predominantly with the fully reduced CcOx - i.e., (Fe^{+2}/Cu^{+}) center, in competition with $^3O_2$ [27].

As proposed by Hamblin, a relatively low-energy photon (e.g., in the NIR region) could displace NO, allowing boosted respiratory metabolism and consequently increasing energy generation [19]. Despite suggesting "*appropriate caution about data interpretation*" Mason et al. showed the CcOx NIR spectrum [28]. And based on these findings some insights arise regarding CcOx being a photoreceptor, probably due to its heme center. Based on NIR spectral signatures (650–980 nm) the CcOx spectrum (700 to 980 nm) is related to the cooper center Cu_A, which has a maximum at 835 nm in the beef heart enzyme. Concerning red light, they found a 655 nm spectral signature for the oxidized heme a_3/Cu_B binuclear center [28]. Once photoactivated CcOx can increase enzymatic activity, consequently increasing oxygen consumption and enhancing ATP production. In addition, the NO increment generated in response to CcOx photoactivation (i.e., ATP production and mild ROS) might modulate several transducing signals [29,30], as shown in Fig. 1. Beyond the cellular phenomena associated with CcOx stimulation, PBM might target other chromophores including those present in cell membranes, such as flavins, as well as water as an alternative chromophore [19].

When PBM stimulates CcOx activity there is a resulting increase in

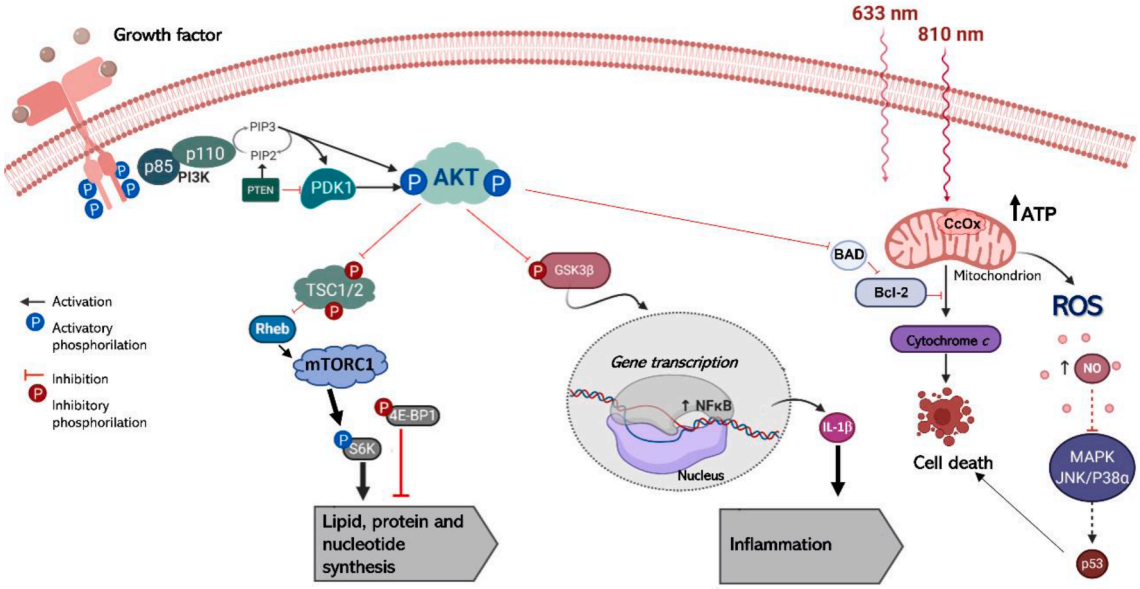

Fig. 1. Molecular mechanisms of photobiomodulation related to tissue regeneration.
Legend: PBM may activate PI3K kinase that phosphorylates phosphoryl phosphatidyl-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PI3P), which in turn recruits PDK1 kinase. Once phosphorylated PDK1 activates AKT resulting in a transducing cascade signal that inhibits the TSC1/2 complex, glycogen synthase kinase 3 beta (GSK3β), and the pro-apoptotic protein BAD. Once activated AKT relieves the inhibitory TSC1/2 complex effect on Rheb and stimulates the mTORC1 activity, leading to boost synthesis of nucleotide, protein, and lipid towards tissue regeneration. Aside from mTOR, photoactivated-AKT might evoke anti-inflammatory responses and anti-apoptotic signals via suppression of GSK3β and BAD, respectively. PBM also leads to NO (nitric oxide) increment responsible for modulating pro-survival routine by lessening MAPK/JNK/p38α. the light in the red and NIR region shows a primary photoreceptor the enzyme cytochrome *c* oxidase (CcOx), with consequent increases in ATP synthesis and mild ROS generation. Created with BioRender.com.

mitochondrial membrane potential above normal basal levels, which generates ROS as NO, see Fig. 1. Although there is a brief increase in NO generation, this is quite modest, but capable of raising the level of ATP and eventually might regulate the action of p53 via suppression of the MAPK/JUNK/p38α cascade [31] (Fig. 1).

Aside from the direct PBM effects on mitochondrial metabolism, red light (635 nm) irradiation was found to significantly upregulate the gene expression of key proteins related to cellular proliferation, such as *AKT1, PIK3CA,* and *CCND1* following in mesenchymal stem cells [32]. *AKT1* encodes AKT Serine/Threonine Kinase 1 which once active may increase cell proliferation and suppresses apoptosis, while its activity blockage leads to cycle arrest and apoptosis [33,34]. *PIK3CA* encodes the 110 kDa catalytic alpha PI3K subunit which uses ATP to phosphorylate PtdIns, PtdIns4P, and PtdIns(4,5)P2 [33,34]. Thereby, PBM modulates PI3K/AKT/mTOR/eIF4E pathway. Zang et al. corroborated this premise showing an increase in phosphorylation of $AKT^{serT473}$ after red-irradiation (1.2 J/cm^2, 632.8 nm, 12.74 mW/cm^2). Confirming a PI3K/AKT signaling axis, PI3K class I inhibitor (wortmannin) suppressed the photoinduced *in vitro* proliferation of fibroblast cells [35]. Interestingly, to counterbalance the PI3K/AKT signaling related to S/G1 progression and apoptosis suppression, photoinduced cells with red light significantly downregulated the expression of the proteins PTPN6 (Protein Tyrosine Phosphatase Non-receptor Type 6) and STK17B (Serine/Threonine Kinase 17b). These proteins may have negative regulation of cell proliferation [36] or positive regulation of the fibroblast apoptotic process [37], respectively.

The PBM-mediated counterbalanced effects regarding the activation of PI3K/AKT signaling may explain the safety and efficacy that are usually observed throughout clinical studies attempting to manage wound healing of diabetic-associated ulcers [38–41]. PI3K/AKT is important in the maintenance of cellular homeostasis, regeneration, and cicatrization [21].

The downstream transducing signal intrinsically related to PI3K/AKT is the mTORC1 complex (Fig. 1), whose reduced function prevents cell growth, epithelial-mesenchymal transition (EMT), and wound healing. Aside from them, its activation is essential for cell migration [42–44]. mTORC1 consists of mTOR protein, RAPTOR, mLST8, PRAS40, and DEPTOR proteins [45]. By sensing several extracellular and intracellular signals mTORC1 promotes anabolic processes that might sustain cellular metabolism, protein synthesis, cell growth, and survival [46]. Together these benefits therapeutically target mTORC1 as a strategy to treat aging-related disorders [47], as well as promote wound healing and cutaneous scarring [42,44].

As we can see in Fig. 1, the activation from the cell survival mechanism and PI3K/AKT/mTOR metabolic activation may lead to a cascade of events related to protein synthesis, lipids, and nucleic acids. PBM by activating mTOR via PI3K/AKT promotes phosphorylation of ribosomal protein S6 (prS6) [48] on residues$^{ser235/236,\ ser240/244}$ [49]. Active PBM prS6 is probably a result of mTOR activation of $pS6K^{(Ser235/236)}$ with consequent cell proliferation [50,51]. Hence, one of the main mechanisms of wound and ulcer healing is the activation of the PI3K/AKT/mTOR pathway that promotes collagen synthesis and angiogenesis in the reparative process of ulcers, especially in the regeneration of diabetic foot ulcerations [21].

Also via PI3K/AKT axis, PBM inhibits GSK3β [52,53]. By using red light (632.8 nm, 12.74 mW/cm^2, 2 J/cm^2) occurs inhibition of GSK3β via AKT activation which promotes the cytosolic accumulation of β-catenin, with consequent translocation to the nucleus where it acts as a transcriptional cofactor with TCF/LEF to promote cell survival [52]. Moreover, the photoinduced effects on GSK3β inactivation might impact the apoptotic process [52,53]. By decreasing the interaction between GSK3β and the pro-apoptotic protein BAX, PBM prevents the translocation of BAX to mitochondria, with consequent suppression of intrinsic apoptosis (Fig. 1). In parallel, the effect of PBM on the inhibition of GSK3β by AKT also implies translation and stabilization of cyclin protein D1, with consequent cell proliferation [49]. AKT photoactivation with consequent Inhibition of GSK3β may benefit the healing process mainly in cases of diabetic foot with atypical tissue regeneration related to higher expression of GSK3β, NFκB, p53, and p16INK4a [54].

Based on the PI3K/AKT/GSK3β pathway, we speculated that PBM

could still regulate the signaling of the transcription factor NFκB (Fig. 1). As reviewed GSK3β might positively modulate NFκB expression [55]. NFκB allows inflammation not only by regulating cell proliferation, apoptosis (Bcl-2, survivin), differentiation of keratinocytes, and morphogenesis but also by directly enhancing the production of pro-inflammatory cytokines [56].

NFκB suppression is associated with phosphorylation and direct inactivation of GSK3β by AKT (Fig. 1), generating a counterpoint in laser NFκB photoactivation at 810 nm [19]. Paradoxically, there is an anti-inflammatory response attributed to PBM with consequent reduction of TNF-α e interleukins - IL-1β, IL-8 e IL-12 [57,58]. Hence, PBM seems to mediate a significant reduction in NFκB activation in cells stimulated with a Toll-like receptor agonist 9 (TLR9) [57].

Beside PI3K/AKT, PBM may modulate the expression of other genes encoding proteins engaged in extracellular matrix remodeling and cell adhesion, including *DDR2, PTPN6*, and *STK17B* cells [32]. PBM significantly upregulated *DDR2 (*Discoidin Domain Receptor Tyrosine Kinase 2) that encodes a collagen-induced tyrosine kinase receptor, which plays a pivotal role in the communication of cells with their microenvironment, capable of inducing activation of signal transduction pathways involved in angiogenesis, cell adhesion, proliferation, and extracellular matrix remodeling, and in turn, accelerates regenerative processes [30,33,34].

The wound healing process might be surrogated or delayed when one of the four phases (hemostasis, inflammation, proliferation, and tissue remodeling) is out of sequence or even missing, and this often results in cutaneous ulceration. At the molecular level, such surrogation of the wound healing process may result either in functional inhibition or deficiency of growth factors. Beside, we might observe a persistent inflammatory phase without improving the resolution phase [59,60]. And PBM positively impacts the healing process by modulating the expressions of pro-inflammatory or anti-inflammatory proteins, as well as cell growth factors. For instance, the TNF-α increment results in the alteration of the macrophages and monocytes phenotypes [19]. Accordingly, such modulation has beneficial clinical implications for the treatment of pressure ulcer (PU) [61,62].

After PBM (660 nm, 2 J/cm^2) the PU wound area in diabetic patients due to a significant alteration in the gene expression profile of inflammatory-related proteins (downregulated *TNF-α* and upregulated *TGF-β1* or *VEGF*), which contributes to the reduction of the lesion area and improvement of the aspect of lumbosacral ulcer [61].

Following PBM (658 nm, 4 J/cm^2) occurs a significant reduction in the wound level of TNF-α and serum level of the interleukins IL-2 and IL-6, as well as increased wound level of VEGF and TGF-β1 which may underline improved outcomes of the ulcers in sacral and pelvic regions due to the inflammatory phase regulation [62]. PBM at wavelengths of 808 and 940 nm did not significantly change the expression of these repair-related proteins, which explains the low effectiveness of NIR light in the treatment of PU [62,63].

However, the decrease in local Levels of TNF-α was not observed at least for chronic venous ulcers after red light (625 nm, 4 J/cm^2) [64]. Another recent study corroborates this low alteration of TNF-α in oral ulcerated areas [65]. On the other hand, a reduction of pro-inflammatory cytokines after PBM was reported in patients with expected healing outcomes at the end of clinical follow-up (e.g., IL-1, IL-2Ra, IL-8, IL-16, MIG, M-CSF, TNF-α, and TRAIL), while anti-inflammatory cytokines increased (e.g., IL-4, IL-5, IL-10, and G-CSF) [65].

4. Clinical application of PBM

PBM clinically emerged 50 years ago [66]. Currently, it has been considered a noninvasive photonic promisor intervention with low cost and multifunctional applications since it is capable of eliciting beneficial effects on neurological, skin ulcers, musculoskeletal, joint inflammatory processes, and oral mucositis, as well as immunopathology like rheumatoid arthritis [12,20,66,67]. However, low efficacy might occur due to a non-standardized approach or to a lack of comprehension of PBM's photophysical or photochemical aspects. For example, light sources with different emission wavelength ranges are being used, which might induce contrasting phototherapeutic efficacy in several pathophysiologic conditions, including alopecia, regeneration of chronic wounds, and neurodegenerative diseases, as reviewed [66].

PBM positively modulates target cells and their microenvironment, which results in beneficial therapeutic outcomes such as pain relief, wound healing, photorejuvenation, and tissue or neural regeneration (TSAI; [19]). However, those outcomes depend on the fluence distribution within a tissue, which changes the illumination geometry and wavelength [68]. And both the parameter light wavelength and irradiance have been highlighted as the most important and determining factor in the laser-tissue interaction.

As mentioned above, when absorbed by the tissue the laser light causes biochemical energy-related effects. And as greater the light wavelength the superior the depth achievement, which in turn implies less absorption by the thickness tissues. On the other hand, shorter wavelengths have a more superficial targeting since they are more absorbed by the epidermis or dermis. Light tissue targeting relies on the range of chromophores present throughout the skin, which has scattering and absorption coefficients, and in turn, is highly wavelength dependent. Thereby, the penetration depth of 1% of the intensity is reached at 1.0 mm with blue light (400 nm), meanwhile is about 3.0 mm and 5.4 mm with a wavelength of 550 and 750 nm, respectively [68]. Thus, to optimize therapeutic techniques, light-tissue interactions must be thoroughly understood. Otherwise, heterogeneous protocols will not ensure clinical effectiveness to alleviate wound healing.

By using red light (e.g., 658 nm), the PBM efficacy may be accomplished since the photons might be more absorbed in the most superficial part of the PU with grades 2 or 3 according to European Pressure Ulcer Advisory Panel (EPUAP) and the National Pressure Ulcer Advisory Panel (NPUAP) [62,69]. On the other hand, NIR light due to its less absorption by more superficial tissues compromises the PBM effectiveness concerning superficial PU [62,69,70].

According to a systematic review, the PBM clinical benefits concerning PU are insufficient to ensure effectiveness, and studies with higher methodological quality and minor risk of bias should be performed [63]. Though, these clinical endeavors should use parameters similar to those which have found significant results of 658 nm at 4 J/cm^2 fluences [61,62,69].

Meanwhile, PBM still requires further clinical studies to prove its effectiveness to treat PU, it has emerged as a promisor therapeutic avenue to treat diabetic foot ulcers (DFU) and chronic venous ulcers (CVU). Here, we review important aspects to be considered for the effective management of ulcerations using PBM, especially in its analgesic and regeneration outcome concerning DFU and CVU, which enables desirable wound healing.

4.1. Parameters

Unlike high-power or surgical lasers, low-intensity light devices used in PBM do not promote thermal effect on tissues [20,30,71]. In addition to lasers, LEDs have been successfully employed [72]. Even though, both source of photons seems to be lesser important as a variable that might influence the PBM efficacy. Both light wavelength and the photons' number (fluence) have a significant influence on the PBM efficacy to promote *in vitro* cell proliferation [24].

Red light (600 - 700 nm) has a higher photon quantum energy (2.07 to 1.77 eV) than NIR (808 nm = 1.53 eV) and can more easily promote electrochemical changes in tissues. On the other hand, NIR light increases mostly a molecular vibrational state, which may lead to a transient thermal effect (at least 2 °C in tissue with thickness from 3.0 to 5.0 mm) and increased metabolic activity. And if the photons' number increases beyond a particular level the cellular benefits disappear, and

when it is even further increased, inhibition and cellular damage may turn out [20,24].

Literature suggests that such photon increment beyond hormesis might result in loss of MMP, production of excessive ROS, and release of excessive free NO, which together trigger a cell death mechanism (Fig. 1). According to the Medical Subject Headings (MeSH) Identifier " D059165", Established Data 2012–01–01, hormesis is defined as "*biphasic dose responses of cells or organisms (including microorganisms) to an exogenous or intrinsic factor in which the factor induces stimulatory or beneficial effects at low doses and inhibitory or adverse effects at high doses*".

Though, the range of irradiance, fluence, and treatment regimen at which these transitions affect hormesis is not widely endorsed. Ranging irradiance of red light (670 nm) from 8.0 to 40 mW/cm^2 to reach a 2.5 J/cm^2 fluence can result in the same desired wound closure rate on the 7th day in a murine PU model after twice-daily treatment. On the other hand, the irradiance ranges from 8.0 to 40 mW/cm^2 to reach 5.0 J/cm^2 leading to distinct outcomes after one-day treatment [73].

Beyond fluence, irradiance, and light wavelength, some parameters affect it: total energy; repetition rate (pulse frequency); spot size (cm^2); the number of treatments and optical data of the tissue to be irradiated - considering light absorption and scattering; tissue absorption characteristics and its type of cellular population and its physiological state [20].

The spot size is considered one of the factors that is directly related to PBM's optical doses since it is associated with the light reaching the tissue surface and the actual target tissue. Spot size or the treatment area plays a key role in the penetration depth effect and light dispersion in tissue, and in turn, has important clinical implications. As defined by Ash et al. *"with increasing spot size, there is a reduction in the amount of lateral scattering; this results in greater penetration for larger spot sizes. As a result, lower energy densities can be applied when using larger spot sizes to achieve the same penetration depth for treatment. Variation in spot size is also important depending on the condition being treated, if the treatment region is over a large area then a larger spot size is used and for isolated small lesions in blood vessels for instance, a smaller spot size would be recommended resulting in increased intensity at the target"* [68].

Moreover, the evaluation of the modus *operandi* of light in tissues needs to be considered. In PBM, the use of continuous mode has been considered, by some authors, as the gold standard in the applications of light in tissue regeneration, both neuronal and wound healing [74]. Even though, pulsed mode promotes greater penetration of light into the target tissue. Nevertheless, some studies show there is no difference in the healing process regardless of the modus *operandi* - continuous or pulsed mode [74].

It seems that using the same parameters of irradiation might occur negative as well as positive outcomes considering independent studies. Hambling and colleagues suggested that such differences among the studies might be due to the mitochondria amount in the target tissue [20]. Depending on the mitochondria amount, if higher or lower, may occur effective or ineffective PBM response. Corroborating this premise, an *in vitro* study showed that both blue (400/450 nm) and NIR-light (810 nm) can promote increased cell metabolic activity that was intrinsically associated with less ATP production and mitochondrial respiration in myoblasts compared with myotubes - a cell type with higher mitochondrial content. Consequently, myotubes promptly produce a higher level of ROS after PBM, which was found greater increased after blue light compared with NIR [75]. However, it is worth considering the type of experiment - *in vitro* or *in vivo*. Indeed, Hambling group concluded that "*ineffective studies in vivo are more likely to be due to under-dosing regardless of the number of mitochondria*" [20].

The wound area closure, according to *in vitro* experiments, differs not only due to photophysical parameters but also depending on the target skin cell type, skin color, and tissue thickness [23,76–78].

Whereas the red wavelength (655 nm) was more successful on keratinocytes to decrease wound area, the 808 nm of wavelength was significantly effective on fibroblasts to induce wound healing totally and enhance cell viability [78]. It seems that fibroblasts are more responsive to 808 nm light than keratinocytes. Corroborating this premise, Engel and colleagues showed that at least in the case of oral *in vitro* cells, unlike keratinocytes fibroblasts may deal with ROS NIR-induction since they have higher levels of catalase activity, which properly impacts cell survival under photooxidative stress. Unlike oral fibroblasts, oral keratinocytes are less prompted to survive when NIR-irradiation occurs at higher irradiance (e.g. 50 mW/cm^2) since they have lower basal levels of catalase activity, and in turn failed to relieve laser-induced ROS [76].

Beyond considering different cell types that constitute human skin, it is worth taking into consideration the *in-situ* localization within the dermis, at least for fibroblasts as revealed by Mignon et al. [23]. They demonstrated that papillary (superficial dermis less than 500 μm) and reticular (deep dermis) fibroblasts isolated from human adult facial skin showed differences in their transcriptomes and metabolic activity after irradiation with both blue (450 nm) and NIR (850 nm) lights. Unlike blue light, NIR light not modulated the up-regulation of genes linked to ROS (e.g. *SOD2*). In reticular fibroblasts, NIR-light up-regulated the expression of the *MCM5* gene that can increase metabolic activity through increment of cell proliferation. Moreover, papillary fibroblasts showed up-regulation of genes associated with metabolism, xenobiotics metabolism, proteostasis, and protein production, which were down-regulated in reticular fibroblasts after NIR-irradiation. Beside they shared down-regulated cell adhesion pathways. And only in papillary fibroblasts both the cell signaling and hormone biosynthesis were found down-regulated [23].

Despite the findings demonstrating how PBM might trigger differences in the transcriptome of human dermal fibroblasts (papillary x reticular), it is worth considering that these experimental outcomes may not be directly extrapolated to the *in vivo* condition. Both inter-cellular communications and extra-cellular environment interactions should play an important role in the cellular response to irradiation. For example, papillary fibroblasts cultured *in vitro* lack the cell interaction with epidermal keratinocytes that they otherwise would experience *in vivo* [23].

Depending on the color skin (light and dark) and skin thickness (3.0 or 5.0 mm) light reflectance and temperature differ regarding visible and NIR light. As expected, as the thickness of tissue increased the predicted transmittance of light decreased [77], mainly in the absence of melanin a pigment found to be capable of absorbing visible light [79].

Considering the reflectance, a significant portion of incident red or NIR light may be lost in light color skin (e.g. 12%), which slightly differs concerning the thickness of the tissue. Even though, it was significantly less for dark skin, particularly for red light [77]. As dark skin contains epidermic keratinocytes enriched with the melanin pigment, there was also less light transmittance of almost 60% and 30% after irradiation of 3.0 mm-thickness tissue with red and NIR light, respectively [77].

Noteworthy that reflectance not changed as fluence increased (2 to 12 J), though, it was slightly less in thick tissue (i.e., 5.0 mm) [77]. Nevertheless, the fluences ranging significantly impacted the temperature effects of visible and NIR-light in a greater magnitude. The increase of temperature for both red and NIR irradiation was lesser than those found in longer wavelengths. Overall, 808 nm light increased the temperature rather than 635 nm, though, the differences were less prominent in the case of 5.0 mm-thick dark skin. And these effects are probably due to the higher transmittance and reflectance of 808 nm light [77]. Taken together, whereas the increment of temperature, transmittance, and reflectance of light are parameters that should be considered for NIR-based PBM, the status of pigmentation should be taken into account for PBM using visible light (635 nm).

Consequently, the light wavelength application implies specific conditions like the target tissue thickness and the presence and type of endogenous chromophores. Aside from melanin, other intracellular molecules may have distinct dispersion and absorption coefficients, and in turn, are highly dependent on wavelength. As reviewed in Souza-Barros et al. "*melanin and subcutaneous lipids are two of the main light*

absorbers in superficial tissue as the blood volume is small. Maximal melanin absorption occurs at wavelengths shorter than 510 nm but significant absorption still occurs for red light, 600–700 nm. The effect of melanin, which also includes light scattering, becomes increasingly smaller in the NIR range beyond 800 nm. Conversely, maximal lipid absorption occurs in the NIR range around 760 and 930 nm" [77].

As light wavelength increases, there is less absorption by the more superficial tissues, and in turn, photo-modulatory effects would virtually occur in a higher depth range. On the other hand, shorter wavelengths have a more superficial target since they are more absorbed by the epidermis or dermis. The light between 300 and 750 nm might reach a depth penetration range of 0.37 to 5 mm, depending on the maximum light intensity, if 13.5% or 1%, respectively [68].

Thus, based on this computational estimated behavior of light regarding skin thickness, the penetration of blue (450 nm) and green (500 - 550 nm) light would be restricted to the papillary epidermis-dermis (up to 1.5 mm) and dermis-reticular (up to 3 mm), respectively [68]. Red light (600 - 650 nm) would be limited in deep dermis-reticular and hypodermis (between 4.0 and 4.5 mm). On the other hand, the NIR light (700 to 750 nm) would involve beyond the hypodermis, in deeper tissues of thicker skin as occurs in acral regions [80,81]. On the other hand, considering body areas with 2.4 mm skin thickness such as the abdomen [82], we need to consider an even greater penetrance of pulse light. Despite this knowledge that may be considered during the design of PBM clinical protocols, are still necessary further *in vivo* studies concerning the interaction and light penetration within the tissue matrix of distinct body areas (thin vs. thick skin).

Therefore, if the light spectra would not promptly be chosen concerning skin thickness PBM efficacy might be compromised. And literature data underlie such consideration. Regardless if PU has a larger ulcerated area above 30 cm^2 [69] or less [70], 940 nm would not benefit healing. Studies are reporting lower PBM efficacy at 940 nm in the elderly (81.3 $\pm$ 9.6 years) with grade III *decubitus* PU with smaller ulceration areas (i.e., up to 4.0 cm^2), but probably thinner skin due to aging [70]. The light at 940 nm did not remarkably reduce the ulcerated area (2.46 $\pm$ 2.64 cm^2 to 1.94 $\pm$ 4.44 cm^2) in comparison to the conventional therapy, which reduced the ulceration by 41% (3.38 $\pm$ 3.86 cm^2 to 2.00 $\pm$ 3.84 cm^2) [70]. Therefore, we concluded that in cases of stage III PU,according to EPUAP/NPUAP the ideal therapeutic light range would be 600–660 nm though.

As reviewed in the next section, most randomized clinical studies employ red light in continuous mode, but without a consensus regarding the following parameters: light wavelength range, the energy source (LED or diode-laser), irradiance, fluence, time, and treatment sessions.

4.2. Clinical studies

For the establishment of an elective protocol for PBM as adjuvant therapy for ulceration healing, only controlled-randomized clinical trials containing all parameters, baseline demographics, and clinical characteristics were considered. Overall 7 studies were compared based on their protocol, demonstrating the PBM outcomes as adjuvant therapy for DFU [38–40], CVU [64,83,84], and PU [69]. The primary outcome of the vast majority of studies is the complete healing of ulcers of different etiologies, followed by pain relief (Table 1). Tough, the evaluation of gene expression of factors such as IL-2, IL-6, TGF-1, TNF-α, VEGF, and PDGF should be explored for a better understanding and detailing of the PBM's molecular underlying mechanisms.

In general, as shown in Table 1, the PBM protocols differ among the parameters used - the fluence ranged from 3.0 to 10.0 J/cm^2 at 17.5 to 65 mW, and light wavelengths - 625 nm [64], 635 nm [84], ~660 nm [38,40], 685 nm [39], 810 nm [83], and 658 nm, 808 nm or 940 nm with [69]. Therefore, there is a lack of consensus on standardized treatment parameters, such as wavelengths, fluence, irradiance, and *operand* mode.

Regardless of the laser type, potency (W), sessions number, and ulcerated area, red light (625 - 685 nm) with 3 to 10 J/cm^2 fluence resulted in an increased benefit to patients with PU and DFU resulting in a higher healing index of about 50% compared to the control group [39,40,69]. These studies showed higher clinical benefits compared to that using LED (625 nm at 4 J/cm^2 or laser (635 nm at 2.95 J/cm^2), which did not show superior efficacy compared to the control group concerning CVU healing [64,84]. Regardless of the parameters employed PBM was not significantly superior, compared to the control, to alleviate CVU, as observed for PU and DCU (Table 1). Of note Light at 658 nm for PU, unlike NIR light, resulted in outcome superiority at 113%. And in the case of DFU with an ulcerated area less than 10 cm^2 occurs healing around 70% after red light [38,39]. Therefore, for better outcomes, some aspects need to be considered before PBM, including the extension and ulceration's localization.

Despite photobiomodulation leads to clinical benefits beyond cicatrization like pain relief [38,84], we observed a lack of the harmonization of current protocols aimed at the management of cutaneous ulcers. In this sense, we suggested the conduction of well-designed and blind controlled-randomized clinical trials, using red light 660 nm (1.88 eV) under 4 J/cm^2 for treatment of PU or DFU, with disease baselines description of eligible patients sharing the same age range and similarity regarding area and depth of ulceration.

Another important point to be considered before electing PBM for ulcer treatment is the acknowledgement of the microbiological profile (microbiota) present in ulcerated tissues. Tissue colonization by microbial agents or microbial communities is certainly a determinant factor for wound healing. Both due to the presence of strains with different degrees of virulence and the biofilm formation on the surface of wounds or ulcers, which hinder the effectiveness of skin regenerative underlying mechanisms [85].

5. Conclusions and prospects

PBM can affect cellular metabolism, homeostasis, and stress defense. However, the data found in the literature are preliminary and do not allow for harmonizing reliable protocols. Therefore, robust investigations using randomized clinical studies should be carried out to evaluate the PBM efficacy as adjuvant therapy, and the implications on tissue regeneration of skin ulcers.

After an integrative review of the parameters used, with ulcer healing as the main outcome, we observed that regardless of the treatment protocol, the efficacy after PBM was not significantly higher concerning the control to mitigate CVU, as observed for PU and DFU. However, some studies showed a lack of complete reporting of their protocols (e.g. potency, spot size, irradiance, or time of irradiation) which makes it difficult for clinicians and future researchers to replicate them. Complete reporting should also include the distance of the light device from the tissue surface, spot size (cm^2), skin color, and use of the current terms "photobiomodulation" instead of "LLLT"; "spot size" rather than "dot size", "irradiation area" or "spot area"; "irradiance" rather than "intensity", "power density"; "potency" rather than "power"; and "fluence" rather than "radiant exposure", "energy density", "dose of energy" or "dose" are recommended for future use.

Regardless of laser type, power, and the number of sessions, the use of laser at 658 nm (1.88 eV) under fluence 4 J/cm^2 resulted in a superior healing index than conventional treatment for PU and DFU. Therefore, there is a clinical benefit after PBM, but new randomized-controlled clinical investigational studies are needed for its harmonization and clinical dissemination.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 1
PBM as adjuvant therapy for ulceration healing: clinical aspects, irradiation parameters, and outcomes of the randomized-controlled investigational studies.

Ulcer	Baseline disease	Ulcer area (cm^2)	Age (years)	N	nm (eV)	Irradiation system	Spot size (cm^2)	Irradiance (mW/cm^2)	Potency (W)	Time (seconds)	Fluence (J/cm^2)	Sessions number	Follow-up weeks	Control	Wound healing	Pain relief	Refs
DFU	DM II WC[a] I < 200 mg/dL[c]	14.8 ± 5.6	54.1 ± 12.9	30	660 ± 20 1.88 eV 1.82 eV	Diode laser	–	50	–	60	~3	15	2	Daily cleaning and dressing guidance	9.3 ± 4.1 g ($p<0.001$) 37.3 ± 2.28%[h] 148.7%*	–	[40]
DFU	DM II WC[a] I-II NSS [b] > 7	10.7 ± 25.7	60.2 ± 9	13	685 1.81 eV	BTL laser device	1.0	50	0.05	200	10	27	4	Placebo[f]	73.7 ± 10.2%[h] ($p = 0.03$) 55.9%*	–	[39]
DFU	DM WC[a] II-III 140 - 350 mg/dL[c]	1.83 ± 1.08	53.11 ± 8.85	9	660 1.88 eV	Laser pulse Ibramed	0.06	49	0.03	12	6	16	4	Daily cleaning and dressing guidance	0.32 ± 0.26 [g] ($p = 0.031$) 76.45 ± 18.30%[h] 49.1%*	Yes 2.22 ± 2.72 before 0.77 ± 1.71 after 69%**	[38]
CVU	CEAP[e] C6 ABI[d] < 0.9	10.9 ± 9.98	~60	14	625 1.98 eV	LED	1.0	–	0.025	160	4	30	30	Unna boot	No significant difference	–	[64]
CVU	ABI[d] > 0.8 Doppler ultrasound demonstrating venous reflux	10.13 ± 6.23	67.9 ± 14.78	13	635 1.95 eV	Erchonia ML-Scanner (MLS) laser	–	2.46	0.0175	1.200	2.95	24	12	Placebo[f]	2.28 ± 2.78 [g] ($p<0.001$) 77.10 ± 25.70%[h] 11.4%*	Yes 44.69 ± 23.93 before 1.15 ± 4.16 after 75.7%**	[84]
CVU	CEAP[e] C6 Superficial and deep reflux	17.92 ± 12.0	61.02 ± 8.2	21	810 1.53 eV	Gallium-aluminum-arsenide diode laser	–	–	0.065	–	4	42	48	Conservatively treated with drug therapy	No significant difference	–	[83]
PU	IIA, IIB and III (EPUAP/NPUAP)	32.87 ± 31.3	68.2 ± 10.0	18	658 1.88 eV	Gallium-aluminum-arsenide diode laser	0.1	–	0.05	–	4	20	12	Placebo[f]	8.42 ± 14.23 [g] ($p<0.001$) 74.4%[h] 112.6%*	–	[69]
		34.88 ± 36.1	69.0 ± 12.0	17	808 1.53 eV		0.1	–	0.05	–	4	20	12	Placebo[f]	21.07 ± 26.02 [g] ($p = 0.005$) 39.6%[h] 13.1% *	–	
		30.23 ± 29.2	67.4 ± 11.2	18	940 1.32 eV		0.1	–	0.05	–	4	20	12	Placebo[f]	19.23 ± 23.88[g] ($p = 0.005$) 36.4%[h] 4%*	–	

Criteria eligibility[a]: Ulcer stage according to the Wagner Classification (WC).
Criteria eligibility[b]: Neuropathy symptoms score (NSS).
Criteria eligibility[c]: Fasting blood glucose values.
Criteria eligibility[d]: Ankle-brachial index (ABI).
Criteria eligibility[e]: Clinical manifestations. etiologic factors. anatomic distribution of the disease. pathophysiological findings (CEAP) scale.
Placebo[f]: Sham PBM as an adjuvant to the standard treatment.
Outcome[g]: Total ulcer area (cm^2) after treatment.
Outcome[h]: Wound Healing Index (WHI): [(initial area - final area)/initial area] x 100.
Outcome: PBMs WHI compared to the control group.*
*Outcome**: Ulcer Pain Visual Analogue Scale (VAS) rating reduction in comparison to the control group.*

Data availability

No data was used for the research described in the article.

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Supplementary materials

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