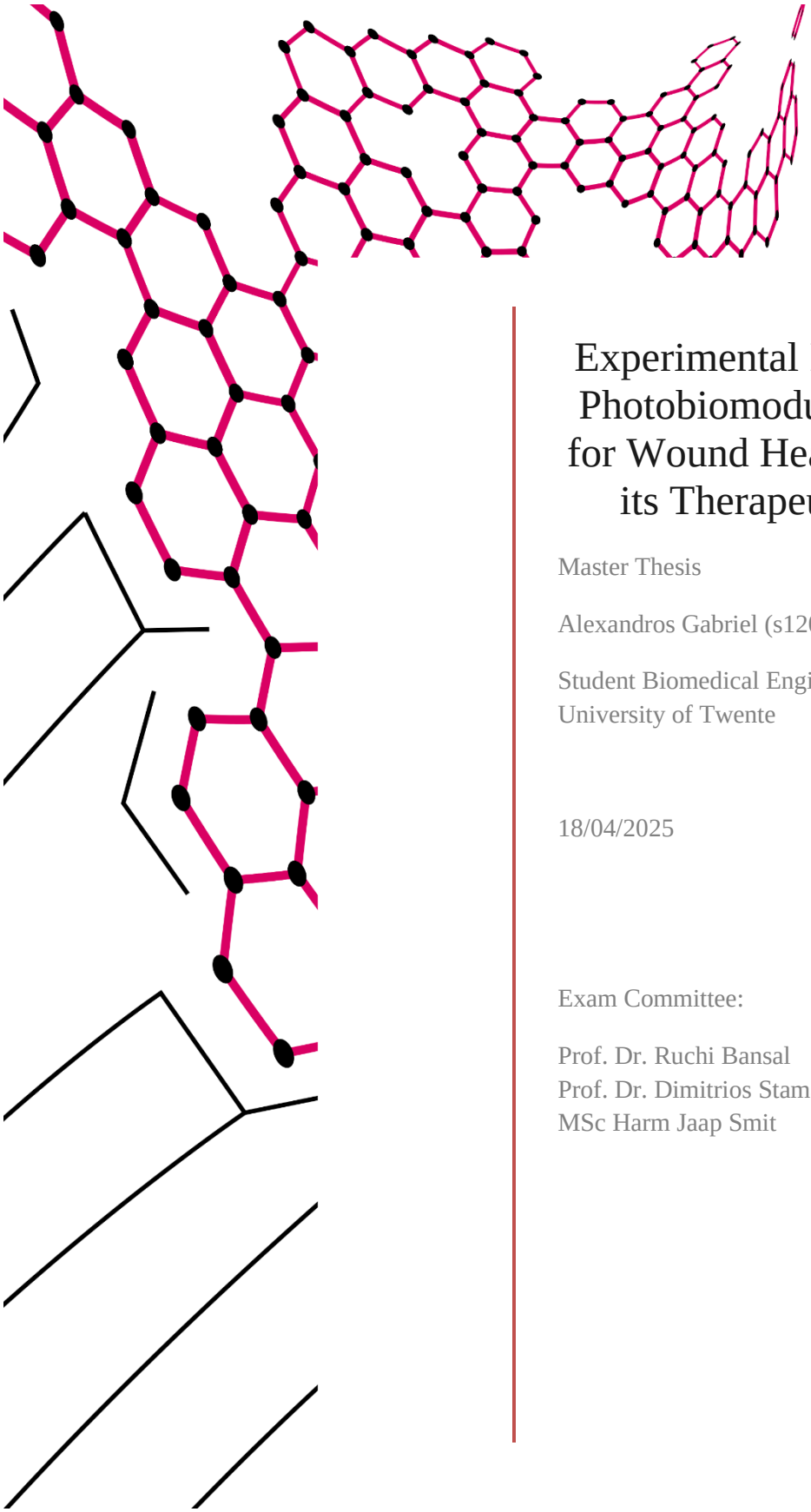

Experimental Investigation of Photobiomodulation Therapy for Wound Healing: Assessing its Therapeutic Potential

Master Thesis

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Abstract

Photobiomodulation therapy (PBMT) has gained some traction in recent years as a non-invasive treatment method for wound healing, however there is no widespread adoption in clinical settings. This shortcoming is mainly due to the incomplete understanding of the related molecular mechanisms as well as the therapeutic potential of PBMT. This study aims to validate the beneficial effects of PBMT on wound healing and gain a further understanding of the cellular processes involved. Using cell line of human dermal fibroblasts (HDFs), THP-1 monocytes, and primary human skin cells, we were able to systematically study the effects of PBMT on cell viability, contractility and migration. Furthermore, collagen and α-smooth muscle actin (α-SMA) expression, as well as gene and protein expressions of factors related to extracellular matrix (ECM) remodeling and inflammation were investigated.

Our results show that PBMT reduces transforming growth factor beta (TGF-β) induced contraction, fibroblast migration, as well as collagen and α-SMA expression. Moreover, we show that PBMT plays a role in fibroblast activation by regulating the expression of COL1A1, ACTA1, MMP2, MMP9, TIMP1, CTGF, COL3A1, factors related to ECM remodeling as well as regulating THP-1 monocyte gene expression factors iNOS, CCL2, TNF-α, IL-1β, CD163, IL-10 related to inflammation. While the results in this study were mostly not statistically significant, they do indicate that PBMT has a potential to both modulate tissue repair as well as the immune response. Results of the experiments using primary human skin cells were statistically not significant due to a limited sample size, however they do suggest that PBMT is effective for wound healing by reversing TGF-β induced gene expression profiles.

These findings provide a basis for further research and offer a look into the potential mechanisms that PBMT has on therapeutic wound healing.

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1. Introduction

Photobiomodulation therapy (PBMT), previously known as low level laser (or light) therapy (LLLT) was discovered in 1967 by Endre Mester at the Semmelweis Medical University in Hungary [1]. In simple terms, it is the utilization of non-ionizing radiation in the infrared and visible spectrum to trigger a photochemical response within cellular structures [2]. Wavelengths in the red 600-700 and infrared 770-1200 nm have shown positive results and have been used traditionally since discovery [3]. Recently blue and green wavelengths have been utilized [4, 5] however they lack in penetration depth as absorption and scattering by molecules and tissue become significantly greater with decreasing wavelength, while on the other hand at longer wavelengths water plays a significant role in absorption. The penetration depth is maximized around 810 nm [6]. In early research lasers were used however since then it is known that non-coherent light emitting diodes (LEDs) work equally well, and it could be argued that generally LEDs are a more attractive option since a higher surface area is irradiated for a more effective treatment, and costs are lower [2].

Although the benefits of PBMT have been known for some time, the clinical uses approved by regulatory agencies such as the FDA are limited. The current therapies focus on pain relief, inflammation, sports injuries, androgenetic alopecia, and oral treatment such as leukoplakia [7-9]. However, more recently evidence has emerged that supports PBMT for other ailments such as mood and sleep disorders [10]. The reluctance of widespread adoption of PBMT has largely been due to inconclusive understanding of the molecular and cellular pathways and mechanisms. However, the future of PBMT is at a precipice as in recent years much has been discovered about the mechanisms of action [11]. This research seeks to further validate the beneficial effects of PBMT for wound healing and gain a more thorough understanding of the molecular mechanisms involved.

In this thesis, the viability of PMBT on cell lines, and primary cells from human skin tissue will be evaluated. Firstly, we aim to determine if PBMT can modulate the contractility and migration of human dermal fibroblasts (HDFs). Thereafter we investigate the effects of PBMT on collagen and α-smooth muscle actin (α-SMA) expression which are critical in extracellular matrix (ECM) remodeling [12]. To gain a better understanding of the mechanisms involved we will study the gene expression of both HDFs as well as THP-1 monocytes, which can give us insights into the immune system response following PBMT. After validating the efficacy on immortalized cells, we will evaluate the potential of PBMT for wound healing on human skin cell disks obtained from human skin biopsies.

2. Theory

Wounds can have major consequences including pain, loss of body parts, and ruin a peoples quality of life [13]. Healing wounds is a complicated biological process involving a series of overlapping stages. These stages include hemostasis, inflammation, proliferation and remodeling [14]. The cellular and molecular events in each stage are characterized by different functions. Initially, hemostasis occurs at the wound site to prevent bleeding, which is achieved by platelet activation and the formation of a fibrin clot [15]. At the same time, the platelet formation releases growth factors as well as immune mediators, which activate the immune system and transition the wound into the inflammation phase [16]. In this phase, monocyte and neutrophil immune cells infiltrate the wound site to clear debris and eliminate pathogenic organisms [17]. While initial neutrophils activity is beneficial, they have been identified to persist in chronic wounds which can lead to delayed wound healing [15], and the inflammatory response can have a detrimental effect to the healing of the wound [18]. Factors derived from neutrophil activity such as matrix metalloproteinases (MMPs) have been identified with degrading healthy ECM and they have been detected in chronic wounds [19-21].

Macrophages are phagocytic in nature, clear necrotic tissue, and are involved in recruitment of leukocytes. They can be classically activated into so called pro-inflammatory M1 state by factors released from necrotic tissue (DAMPs) as well as factors released from natural killer cells (IFN-γ) [22]. Macrophages can further phagocytose apoptotic neutrophils, which triggers the macrophages to polarize into the anti-inflammatory M2 state [23]. These macrophages promote tissue repair by releasing anti-inflammatory factors, as well as growth factors to stimulate fibroblast proliferation and ECM synthesis such as VEGF and transforming growth factor-β (TGFβ) [24]. Chronic wounds are fixed in the inflammatory phase whereby the transition from M1 to M2 is impaired [25].

During the resolvent of the inflammation, the proliferation stage starts by the creation of new blood vessels, critical to supplying nutrients and oxygen for the creation of new (granular) tissue and sustain cell metabolism [26]. Remodeling involves the conversion of the temporary “scaffolding” granular tissue into scar tissue, or Collagen III into the stronger Collagen I. The kinetics underlying wound breaking strength, a measure of wound healing [27], in the latter three phases are shown in **Figure 1**. Collagen III and Fibronectin, which the ECM is initially comprised of during the inflammation stage is gradually replaced by Collagen I resulting in an increase in wound breaking strength [28].

Not all wounds are the same. Wounds can be classified into 5 different levels, based on complexity [29]. Level 0 are ‘normal’ healing wounds, level 1 are wounds that heal over time despite impairments due to patient factors, level 2 are wounds that that arise from local disease and therefore are a symptom of underlying pathology. Level 3 is similar to level 2 but is caused by systemic disease such as ulcers from diabetes, and finally level 4 is a consequence of molecular or cellular issues. The expectation of increased wound healing is dependent on the complexity of the wound [30]. Even palliative wound care can have substantial additive value to patients with chronic wounds [31, 32].

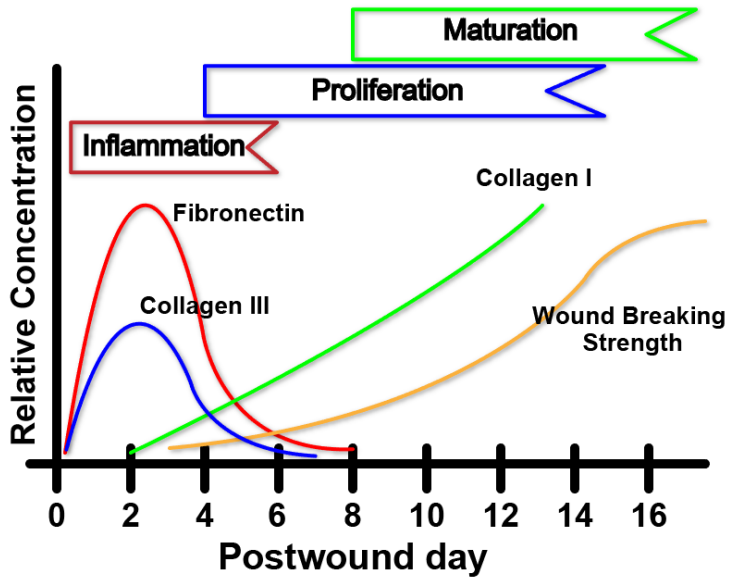

Figure 1. The factors deposited on the wound involving extracellular matrix components over time in the inflammation, proliferation, and maturation phase. Initially the ECM is composed of Fibronectin and Collagen III and gets replaced with Collagen I over time corresponding to an increase in wound breaking strength. Adapted from Witte et al. [28].

Dermal Fibroblasts in Wound Healing

Dermal fibroblasts are derived from mesenchymal stem cells [33]. They are cells within the dermis layer that are responsible for depositing connective tissue during wound healing. Fibroblasts can aid the healing process by proliferating, differentiating and increasing collagen production [34]. During tissue repair, fibroblasts can differentiate from their normal quiescent state into myofibroblasts, a proliferative contractile phenotype that secretes collagen, and other factors that constitute the extracellular matrix that they ultimately reside in [34, 35]. Fibroblasts can gain the myofibroblasts phenotype by a TGFβ dominated environment [36], these myofibroblasts also prosses an increased α-SMA protein expression [37]. Upon successful normal wound healing, these myofibroblasts ultimately undergo apoptosis [34]. Impaired wound healing with persistent myofibroblasts can lead to fibroblasts replacing healthy tissue which increases collagen deposition, this process is called Fibrosis [38]. Fibrosis can manifest itself in different ways such as keloids, scleroderma, and radiation dermatitis, which can have severe negative effects on the quality of life of patients [39]. Besides fibrosis, impaired wound healing can also lead to chronic wounds. Classically, chronic wounds are characterized as wounds that are stuck in the inflammation stage [40], and fibrotic wounds are a result of poor remodeling in the maturation stage [38].

However recent evidence has found that fibrosis is also linked with dysregulation of diabetic foot ulcer tissues which was associated with increased expression of inflammatory genes, besides fibrotic genes. [41] These gene expression have been linked to sustained presence of macrophages which are associated with fibroblast proliferation [42]. Therefore, a better classification of chronic wounds would be a non-linear paradigm of overlapping phases, in which coagulation, inflammation, proliferation and remodeling occur at random with no defined timeframe [43].

Photobiomodulation in dermal fibroblasts

PBMT is a promising non-invasive approach to both alleviate symptoms such as pain [44, 45] as well promote wound healing [46] of both acute [47] and chronic wounds [48]. While the exact mechanism of action remains elusive, PBMT has been identified as causing a down regulation of pro-inflammatory cytokines as well as enhancing cell proliferation. One cause for these beneficial effects investigated is the stimulation of the Smad pathway through increased ATP and TGFβ expression due to PBMT [49]. However there are potentially other pathways that are activated after PBMT exposure such as the vascular endothelial growth factor (VEGF) linked pathways, however the exact cellular and molecular mechanisms have not been identified [50]. Moreover, there are insufficient studies available to create definite prescriptive treatment protocols [51]. Results from the limited studies that are available in current literature are outlined below.

Ultraviolet light has been identified as a remedy for skin fibrosis [52] and scleroderma [53], one possible mechanism of which is shown to be through increased expression of interstitial collagenase (MMP1) in fibroblasts [54]. However the lack of penetration depth as well as melanoma skin cancer risks can limit the therapeutic potential of these treatments [55]. Therefore, visible and near-infrared (NIR) light is a more attractive treatment option.

Research has concluded that red light PBMT can modulate key cellular characteristics in fibroblasts associated with skin fibrosis by reducing fibroblast collagen deposition and proliferation [56]. Furthermore, PBMT is also implicated to modulate mechanisms for chronic wounds [48]. However, there is a general lack of clinical trials investigating the efficacy of PBMT [56]. In vitro, red light PBMT has shown to increase MMP1 expression in dermal fibroblasts, which is responsible for extracellular collagen [57]. Other research has shown that, α-SMA is increased in wounded models, but only after increased incubation time post PBMT [58]. Another interesting mechanism is the MicroRNA (miRNA) related cellular cascade leading to skin fibrosis. Research has found that specific anti-fibrotic miRNA are upregulated, and pro-fibrotic miRNA are downregulated following PBMT with high fluence red light [59].

However interestingly, conflicting studies have found that PBMT actually increases fibroblast proliferation [60, 61]. One possible explanation for these conflicts is the variation in study design. For instance, irradiation of fibroblasts with red light PBMT at 16 J/cm2 produced (repairable) DNA damage, while 5 J/cm2 had no effect [62]. Other research has shown that only under dermal tissue oxygen levels (2%) NIR PBMT stimulated fibroblast metabolic activity, and that PBMT had no effect at atmospheric oxygen levels (20%) [63]. They concluded that therefore a significant proportion of problematic interpretations in PBMT results could stem from poor study designs [63].

A synopsis of current molecular and cellular mechanisms and pathways for the function of PBMT is outlined below.

Chromophores & Signaling pathways

Cytochrome C oxidase (COX) is the inner mitochondrial membrane terminal enzyme of the mitochondrial electron transport chain, transferring electrons from four cytochrome c molecules to a single oxygen molecule producing two water molecules. Conversely, four protons are transferred across the mitochondrial membrane which increases its potential. The increased mitochondrial membrane potential (MMP) is utilized by the ATP synthase protein to synthesize adenosine triphosphate (ATP) [64]. COX has

been implicated as a photo acceptor and photo signal transducer of red to NIR regions of the electromagnetic spectrum. PBM has been shown to increase the availability of electrons for ATP, as well as cyclic adenosine monophosphate (cAMP) and ROS production [65]. Nitric oxide (NO) levels are increased as well, either by the release from non-covalently bound heme iron and copper centers of COX or by up-regulation of COX activity as a nitrite reductase. The leading theory on the working of PBM is that increased NO levels compete with oxygen at a ratio for 1:10 thus effectively reducing the necessary enzymatic activity allowing for an influx of oxygen and promoting respiration [66-68].

The most accepted mechanism for PBM, the retrograde mitochondrial signaling pathway as seen in **Figure 2**, for PBM was first proposed by Karu [69], There is communication between the mitochondria and the nucleus, caused by changes in the homeostasis of the downstream mitochondrial ultrastructure.

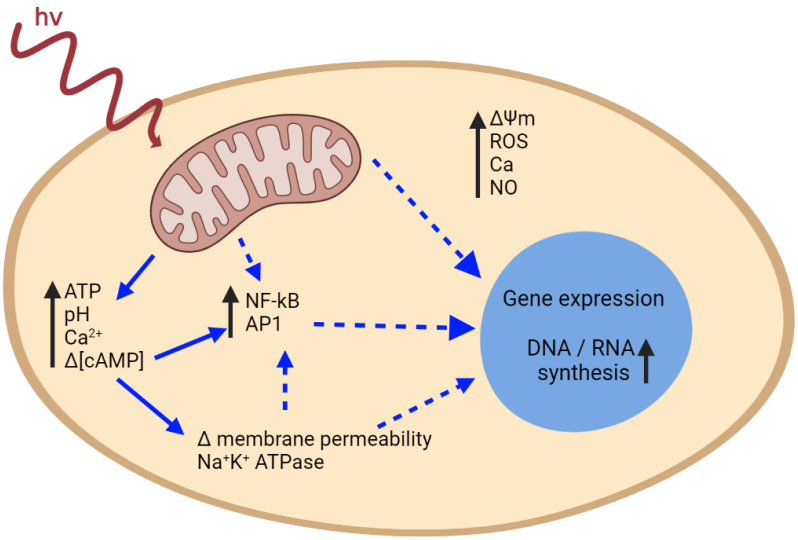

Figure 2. Mitochondrial retrograde signaling pathway. The continuous arrows and dotted arrows represent the main and complementary pathways, respectively.

Light gated ion channels & opsins

Opsins are light sensitive G-protein coupled receptors that have been implicated in mediating cellular signals in response to blue and green light. The most well-known opsin is rhodopsin (OPN2) which is expressed by rod photoreceptors in the retina of mammals and are responsible for dim light recognition and peripheral vision. OPN1 is expressed by cone photoreceptors and can further be categorized into short, medium, and long wavelength (OPN1- SW/MW/LW) and are responsible for color vision [70].

Other opsins found in humans are encephalopsin (OPN3), melanopsin (OPN4) and neuropsin (OPN5) which have been identified in epidermal skin, testes, brain, and retina [71-74]. Opsins function by photoisomerization, where upon absorption of a photon a bound chromophore is changed from cis to trans conformation. This leads to a conformational change in the protein. One of the most important opsins functions of opsins are the role they play in light gated ion channels such as transient receptor potential channels (TRP) [75]. While there are numerous subtypes of TRP which have been identified to mediate sensations such as temperature, pain, taste, such as TRPV1 which responds to allicin (garlic) and capsaicin (chili pepper), TRVP has also been shown to be activated by blue, green and red light [76]. TRP channels

function by mediating the transmission of calcium (Ca^{2+}) ions through the cell membrane into the cytoplasm [77, 78]. Irradiation with blue, green and red wavelengths on human adipose stem cells (hASC) have shown to activate TRPV ion channels, increased intracellular calcium, reduced ATP, increased ROS which increased osteogenic differentiation, while it was also shown to have been inhibited by ion channel blockers [77, 79].

Flavins & flavoproteins

A class of flavoproteins called cryptochromes is widely known to absorb blue light and have been identified to be important in the regulation of the circadian clock in mammals. Cryptochromes rely on flavin adenine dinucleotide (FAD) for absorption of photons [80, 81]. Another flavoprotein, flavin mononucleotide (FMN) is found in complex I of the electron transport chain and is proposed to catalyze the reduction of oxygen (O_2) to superoxide (O_2^-) and thus increase ROS [82, 83]. Conversely, Complex II involves FAD and also absorbs blue light [84] and interestingly blue light has been found to be as effective in increased mitochondrial activity as NIR light [85].

Porphyrins

A group of heterocyclic organic compounds linked to numerous proteins such as hemoglobin, complex IV and cytochrome p450 enzymes [86-88], named porphyrins have an intense absorption peak in the blue light spectrum [89]. 400-420 nm could oxidize heme groups or reduce Cu_B metal centers wound within complex IV [90], which has an absorption peak at 410 nm [91], and has been shown to induce significant increases in cell metabolic and complex IV activity [92]. Likewise, cytochrome p450s (CYPs) are membrane bound proteins that contain heme that is responsive to blue light [93]. The "p450" refers to the maximum absorption peak of 450 nm when bound to carbon monoxide [94]. CYPs are found on the inner mitochondrial membrane and blue light has been shown to increase electron transport chain related genes in keratinocytes, however the irradiation also showed inhibition of cell proliferation and genes related to inflammation [95, 96]. Considering this, porphyrins could offer another explanation to the role of PBM.

Water & heat gated ion channels

Evidence indicates that far infrared light is biologically active [97]. A possible chromophore which can explain biological effects is water since water has an absorption spectrum which peaks around 980nm, and at longer wavelengths, and is by far the most abundant molecule in living organisms. Currently the proposed mechanism entails either photons absorbed by structured interfacial water layers or water clusters [98], however the increase in energy is not sufficient to generate any bulk heating of the tissue and instead is used as a trigger for activation of a sensitive proteins such as heat gated ion channels. The activation of such a channel then allows Ca^{2+} ions to flow into the cytoplasm [99].

The various chromophores are summarized in **Figure 3**.

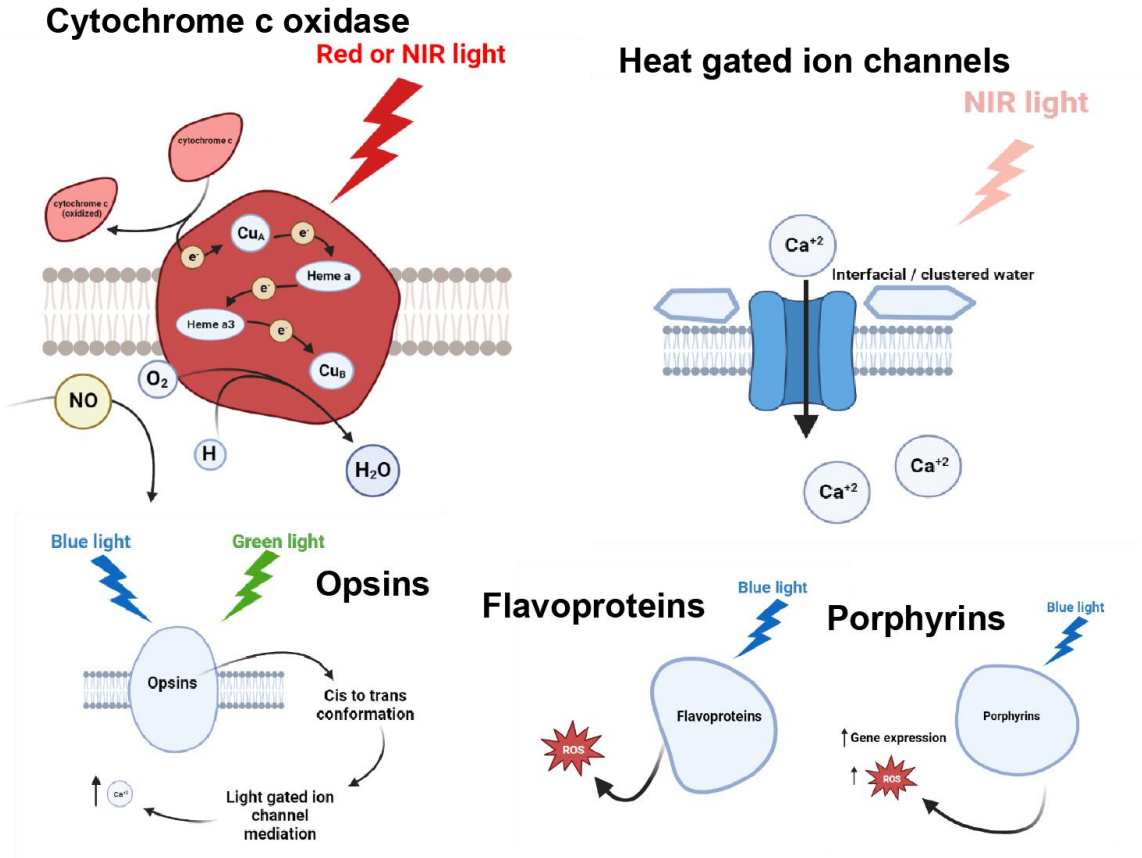

Figure 3. Chromophores responsible for PBM effects and their wavelength. Cytochrome c oxidase absorbs mostly red and infra-red light, heat gated ion channels activated by NIR light absorption of water clusters, opsins absorb mainly blue and green light through cis-retinal chromophore, flavoproteins and porphyrins absorb blue light.

Effects of PBM on cytokines

Nuclear Factor Kappa B (NF-kB) is a transcription factor that plays a key role in regulating expression of numerous genes relating to inflammation, cell survival, stress response and viral infections, among others. NF-kB is redox sensitive and can be directly activated by ROS, or indirectly through TNF and interleukin-1. PBM (λ = 810 nm 0.3 J/cm^2) has been shown to have to activate NF-kB in mouse embryonic fibroblasts [100]. NF-kB is a highly pleiotropic transcription factor that promotes expression of gene products involved with the positive effects of PBM such as pro survival, anti-apoptotic proteins [101] and therefore could explain observed beneficial effects of PBM.

PBM has been shown to reduce pro-inflammatory markers in various cell types [3]. In mouse primary bone marrow derived dendritic cells activated with toll-like receptor (TLR) agonists LPS and CpG oligodeoxynucleotide. A reduction in inflammatory markers MHC class II, CD86, CD11c and IL12 upon treatment with PBM (λ = 810 nm 0.3-3 J/cm^2) was found [102].

Reactive oxygen species (ROS) play a major role in the pathogenesis of inflammation. PBM has been found to modulate oxidative stress depending on the wavelength used, where 660 nm promoted ROS and 970 nm had a reduction of ROS in neutrophil polymorphonuclear granulocytes and keratinocytes in inflamed oral mucosa of cancer patients [103]. However, other studies have found that 660 nm can inhibit

cell death caused by ROS by promoting expression of antioxidant enzyme-related genes [104]. PBM has also shown to have a biphasic dose response or "Arndt-Schulz curve", where ROS production can increase above baseline at higher fluences [105]. These findings show that for clinical settings validation of multiwavelength and dosage protocols are significant.

There are some PBM mechanisms specific for the brain, cerebral blood flow is shown to be increased after transcranial PBM leading to increased tissue oxygenation and increased neurogenesis (formation of new brain cells derived from progenitor cells) and synaptogenesis (formation of new connections between existing brain cells) in mice [106].

The effect on cytokines can vary depending on different mechanisms activated by different wavelength light. 810 nm light was shown to stimulate mitochondrial activity and ATP production, and 980 nm (but not 810 nm) increased cytosolic calcium while decreasing mitochondrial calcium. These effects were blocked by calcium channel blockers, but there was no effect on 810 nm [107].

Macrophage polarization with PBM

Macrophages play a critical role in regenerative processes and tissue repair. These cells can react and perform distinct functions depending on the microenvironment. The M1 macrophage phenotype is a pro-inflammatory cell which can kill pathogens and tumor cells, whereas the M2 phenotype is anti-inflammatory and is involved with clearing cellular debris and tissue repair. The influence of PBM on macrophages is rather interesting. Fernandes et al. [108] activated J774 macrophages to simulate the M1 phenotype and performed PBM (λ =760nm 2.6 J/cm² and 660nm 7.5 J/cm²). Both treatments reduced TNF-α and iNOS expression and TNF-α and COX-2 production, which all indicate a reduction in M1 markers. Von Leden et al. [109] investigated the effect of varying the fluence of PBM (λ =808nm, 0.2, 4, 10 and 30 J/cm²) on primary mouse brain microglia, and cell line BV2 microglia. They found that higher fluences between 4 – 30 J/cm² induced M1 marker expression whereas lower energy densities induced M2 marker expression. In addition, they co cultured microglia with primary neural cultures and demonstrated that PBM has a fluence dose dependent effect on microglial induced neural growth with lower densities having more growth. Souza et al. [110] investigated the effect of PBM on macrophage phenotype in Wistar rats. Muscle injury was induced by liquid nitrogen and PBM (λ =660 nm or 780nm, 25J/cm²) was applied. PBM was able to decrease the number of CD68 M1 macrophages 2 days after injury and increase the number of CD163 M2 macrophages 7 days after injury. However, only PBM using 780nm light was able to increase the number of CD206 M2 macrophages and TGFβ expression, indicating NIR light is more effective in enhancing the repair process. These results show that PBM can optimize the transition from the inflammatory process to the regenerative repair phase facilitating tissue repair.

3. Aim and Objectives

3.1 Aim

In this thesis, the aim is to investigate the viability of PBMT for wound healing by in vitro experimentation, utilizing primary and secondary cells.

3.2 Objectives

The study aims to understand how PBMT affects the different types of cells involved in wound healing. The specific objectives of this thesis are:

1) Determine whether PBMT significantly reduces cell viability, alters collagen production and test how PBMT affects fibroblast mobility and contraction.
2) Investigate how PBMT affects the level of inflammation in vitro on HDF and polarized THP-1 macrophages.
3) Measure how PBMT affects gene and protein expression of factors related to wound healing, through qPCR and WB analysis.
4) Validate if any PBMT induced beneficial effects can be reproduced with primary human skin cell disks.

4. Materials and methods

Cell culture

Human Derman Fibroblasts (HDF cells), originated from mesenchymal cells (Thermo Fisher Scientific) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Lonza), 50 U/mL penicillin (Sigma) and 50 µg/mL streptomycin (Sigma). THP-1 monocytes, obtained from the American Type Culture Collection (ATCC), were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin (Sigma) and 50 µg/mL streptomycin (Sigma).

PBMT Device

The LED array unit (Appendix 1) used to treat cells with PBMT was provided by Harm Jaap Smit and ICAP B.V., Industrieweg 70, Nunspreet, The Netherlands. The properties of the device are shown in **Table 1**.

Table 1. Measured experimental properties of the device used for PBMT developed by ICAP B.V.

Parameter	ICAP device
Wavelength [nm]	660 nm
Spectral bandwidth [nm]	20 nm
Light source	LED
Average radiant power [mW]	3.06 mW
Effective radiant power [mW]	2.00 mW
Polarization	Random
Spot size at target [cm2]	3.80 cm2
Irradiance at target [mW/cm2]	2.81 mW/cm2
Exposure duration	30s/80s/10m
Radiant exposure [J/cm2]	0.13/0.35/2.59 J/cm2
Effective radiant exposure [J/cm2]	0.08/0.21/1.68 J/cm2
Total radiant energy [J]	0.32/0.85/6.40 J
Number of points irradiated	1
Area irradiated [cm2]	3.80 cm2
Number of treatment sessions	1

Cell viability assay

HDF cells were seeded with 5000 cells per well in 96-well plates and subsequently cultured overnight. The following day, the cells were washed with PBS and serum starved overnight by incubating with starvation medium alone, different doses of PBMT was applied and the cells were incubated for 24 h. Cells were then washed and incubated with Alamar blue reagent (Invitrogen), incubated for 4 h and the fluorescent signal was measured using a VIKTORTM plate reader (Perkin Elmer).

Cell migration assay

HDF cells were plated with 10^5 cells per well in 12-well culture plates, incubated overnight and then washed and serum-starved for 24 h. A uniform scratch was made using a 200 µL pipette tip with a custom holder. Afterwards, cells were washed twice and incubated with starvation medium alone (control), or with 5 ng/mL TGFβ. The TGFβ samples were then irradiated with PBMT. Microscopic images were taken at 0 and 24 h to measure the width of each scratch. The images taken for each sample were quantitatively analyzed using ImageJ software (NIH). The area between one side of scratch and the other was measured at time 0 and 24 h for each image to obtain the scratch closure based on the areas that were measured using ImageJ. Graph represents relative wound healing versus TGFβ treated HDF cells.

3D collagen-I gel contraction assay

The 3D collagen-I gel contraction study was performed by following an earlier described protocol [111]. The protocol involves using 5,0 ml of collagen suspension. This suspension was created with 3,0 ml Collagen G1 (5mg/ml, Matrix biosciences), 0,5 ml 10x M199 medium, 85 µl 1N NaOH (Sigma) and sterile water. The collagen suspension was then thoroughly mixed with a confluent (2 x 10^6 cells) T75 flask of HDF cells resuspended in 1 ml DMEM medium. This cell-collagen mixture was then played in with 0,6ml per well in a 24 well plate. The plate was incubated for 1 hour and allowed to gelatinize.

Afterwards, the gel was incubated with 1ml of FBS free medium, with or without 5ng/ml TGFβ and thereafter treated with PBMT. The gels were then detached from the well and pictures were of the well plate were taken with a cellphone camera after 48h of incubation. ImageJ was used to quantitatively analyze the size of the gels by normalizing to the size of the respective well in each picture. The experiments were done in triplicate.

Immunohistochemistry staining

Cells in a 24 well plate were fixed by applying cold 1:1 acetone to methanol solution and incubated at -20°C for 30 minutes. Afterwards, the cells were dried by using a hair dryer and thereafter rehydrated with PBS. The first antibody was then added for 1h at room temperature. The cells were incubated with horseradish peroxidase (HRP) conjugated second antibody for 1h at 21 °C. They were then washed 3x with PBS and incubated with tertiary antibody for 1h at 21 °C. The cells were again washed 3x with PBS and the peroxidase activity was activated by AEC substrate kit (Life Technologies) for 20min. Afterwards the cell nuclei were counterstained with hematoxylin (Sigma). Aquatex mounting medium (Merck) was used to mount the wells and the staining was visualized. Subsequently, pictures were taken using light microscopy (Nikon).

RNA extraction, reverse transcription and real time qPCR

GenElute Mammalian total RNA Miniprep Kit (Sigma) was used to extract the RNA from the cells. The cells were then lysed with 250 µl RNA lysis buffer and 1% 2-Mecraptoethanol (2-ME) per well. The cell lysate was then added into filtration columns. The filtration colums were spun for 5min, and 250 µl of sterile 70% ethanol was added. The lysate/ethanol solution was then transferred to the nuclease binding column and again spun down for 5min. Afterwards, the filtration column was washed with wash solution 1 and 2. The contents were transferred to and Eppendorf and 50 µl elution solution was added. The Eppendorf was spun down for 1 min. The remaining supernatant was collected and the RNA concentration was quantified with a UV spectrophotometer (NanoDrop Technologies). iScript cDNA synthesis kit (Bio-rad) was used to reverse transcribe the RNA. qPCR was then done by using 2x SensiMix SYBR and Fluorescein kit (Bioline) and pre-tested gene specific primers (Sigma). PCR was done using Biorad CFX384, with 95 °C 10min and 40 cycles of 95 °C/15 s, 72 °C/15s, and 58 °C/15s. Finally, the threshold values were normalized to the reference gene 18s. The fold induction were calculated with the 2-ΔΔCt method.

Western Blot analysis

Cell lysis buffer (Cell Signaling Technology) with 1x DDT was used to lyse the HDF cells by incubation for 20min. Afterwards, the cell lysate was transferred to an Eppendorf. 40 µl of the lysate was transferred to another Eppendorf and boiled in a dry bath. SDS-PAGE and 10% Tris-glycine gels (Life Technologies) were added. The proteins were then transferred to a PVDF membrade, which were developed by attaching primary, secondary and tertiary antibodies. Pierce ECL region (Thermo Fisher Scientific) was used to develop the bands. Pictures were then taking using FluorChem Imaging System (ProteinSimple). The band intensity of each protein band was quantitatively analyzed with ImageJ. The analysis was normalized to the β-actin protein expression.

Biopsy sample

To assess the effect of PBMT on primary human skin samples, skin biopsies with consent of the patients were obtained from Medisch Spectrum Twente Enschede (Appendix 2). The biopsies were transported at 4°C in the supplemented DMEM culture medium as described above. Upon arrival, disks were created using a sterile biopsy punch. The disks were washed in PBS and then placed individually in a 16 well plate with either only starvation medium or starvation medium and 5 ng/mL TGFβ. The disks were then treated with PBMT or control and after incubation the RNA extraction and qPCR protocol was followed to assess the gene expression.

Graphs and statistical analyses

GraphPad Prism version 8 was used to create all graphs for each quantitative analysis. The analysis are shown with mean ± standard error of the mean (SEM). The quantitative statistical analyses were also carried out in the GraphPad Prism software package. The comparison between different groups were done by using one-way analysis of variance (ANOVA) with the Bonferroni post hoc test. Obtained statistical differences between every two groups were calculated using a two tailed unpaired t test. Significant differences are denotated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5. Results

5.1 PBMT mediated inhibition of TGFβ induced collagen gel contractility and cell migration of HDF cells

When stimulated with TGFβ, HDF cells become highly migratory and contractile cells [112, 113]. TGFβ, is of particular importance in all phases of wound healing, as it exerts effects by regulating cell proliferation, differentiation, ECM production and immune response modulation [114]. Therefore, the effects of PBMT on migration and contraction were investigated. Contractility of HDF cells was examined by a 3D collagen gel contraction assay, and it was found that PMBT decreased TGFβ induced contraction (**Figure 4 A, C**). Further, a scratch assay was performed to assess HDF cell migration. it was observed that TGFβ induced migration of HDF cells was dose dependently inhibited by increasing exposure time of PBMT (**Figure 4 B, D**). The effects of PBMT on HDF cell viability was also analyzed and no significant differences was observed with different exposure times (**Figure 4 E**).

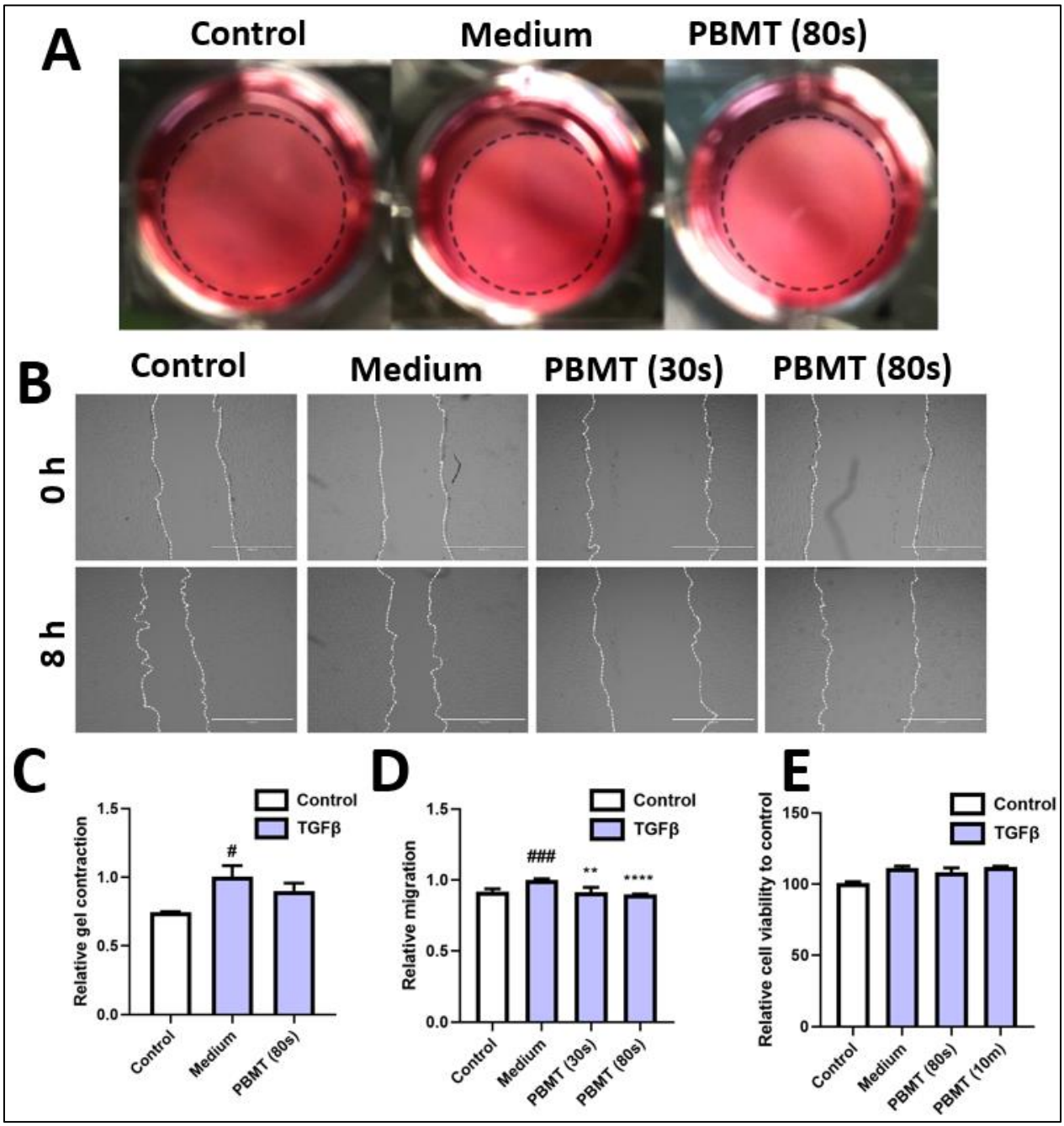

Figure 4. Efficacy of PBMT on contractility and migration of TGFβ activated HDF cells. **(A)** Representative images (after 48h) and **(C)** quantitative analysis of 3D collagen gel contraction by control and TGFβ activated HDF cells treated with medium alone or PBMT (80 sec), n=3. **(B)** Representative images (after 0h and 8h) and **(D)** quantitative analysis of migration by control and TGFβ activated HDF cells treated with medium alone or PBMT (30sec and 80 sec). n=3. (E) Relative cell viability of control and TGFβ activated HDF cells treated with medium alone or PBMT (80 sec and 10 min). n=3. Graphs represent mean ± SEM, # $P < 0.1$, ### $P < 0.001$, represents significance versus control; ** $P < 0.01$, **** $P < 0.0001$ represent significance versus TGFβ activated cells.

5.2 PBMT effects on collagen and α-SMA expression in fibroblast-mediated matrix remodeling of HDF cells

Collagen and α-SMA protein play a critical role in fibroblast-mediated matrix contraction and remodeling, which are of critical importance in wound healing [115, 116]. To investigate the effect of PBMT on Collagen and α-SMA protein expression, an immunohistochemical staining assay was performed (**Figure 5**). The assay revealed that TGFβ activated HDF cells significantly increased collagen I expression compared to control. Following PBMT for 80 seconds on TGFβ activated HDF cells, Collagen I was inhibited, while the effect trends to baseline control, a longer exposure of 10 minutes showed that the effect was lost, suggesting over exposure can lead to nullifying the beneficial response. Results on α-SMA expression was mostly inconclusive.

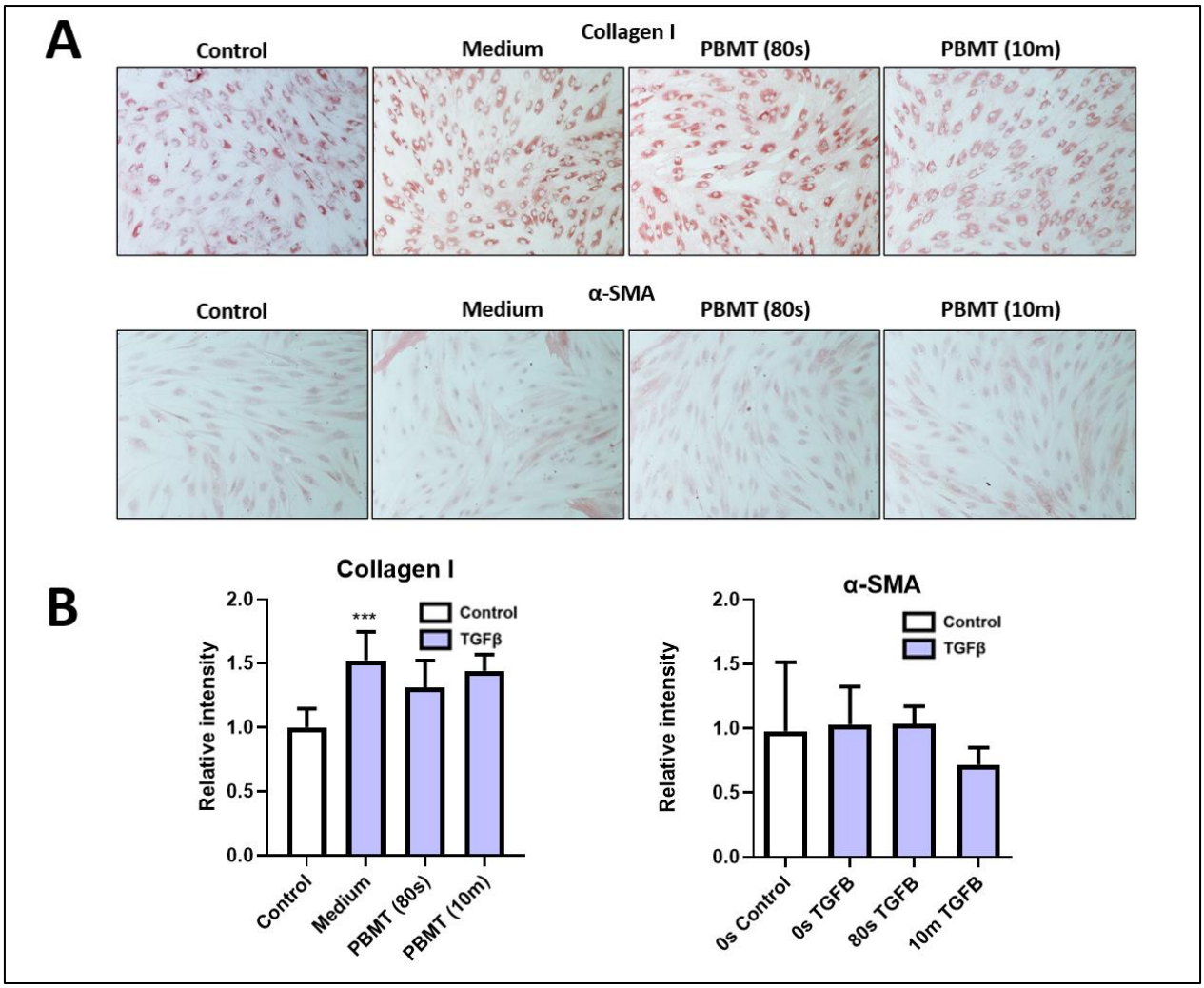

Figure 5. Effects of PBMT on collagen I and α-SMA expression of TGFβ activated HDF cells. **(A)** Representative images (magnification = 40x) and **(B)** quantitative analysis of immunohistochemical staining for Collagen I and α-SMA in control and TGFβ activated HDF cells with medium alone or PBMT (80 sec and 80 sec). n=2. Graphs represent mean ± SEM, *** $P < 0.001$, represents significance versus control.

5.3 Validation of PBMT effects on TGFβ-induced protein expression in HDF cells via Western Blot analysis

The PBMT mediated response of TGFβ treated HDF cells was confirmed by western blot analysis (**Figure 6**). It was confirmed that TGFβ activated HDF cells significantly increased collagen I expression compared to control which was dose dependently inhibited by PBMT treatment for collagen. Interestingly, α-SMA was increased for TGFβ medium and further by PBMT, however there was no statistical significance. In contrast to the immunohistochemical staining assay (**Figure 5**), the inhibition of the beneficial effect at 10 minutes of PBMT exposure was not found in the western blot assay, however the direct comparison with TGFβ medium only was not significant.

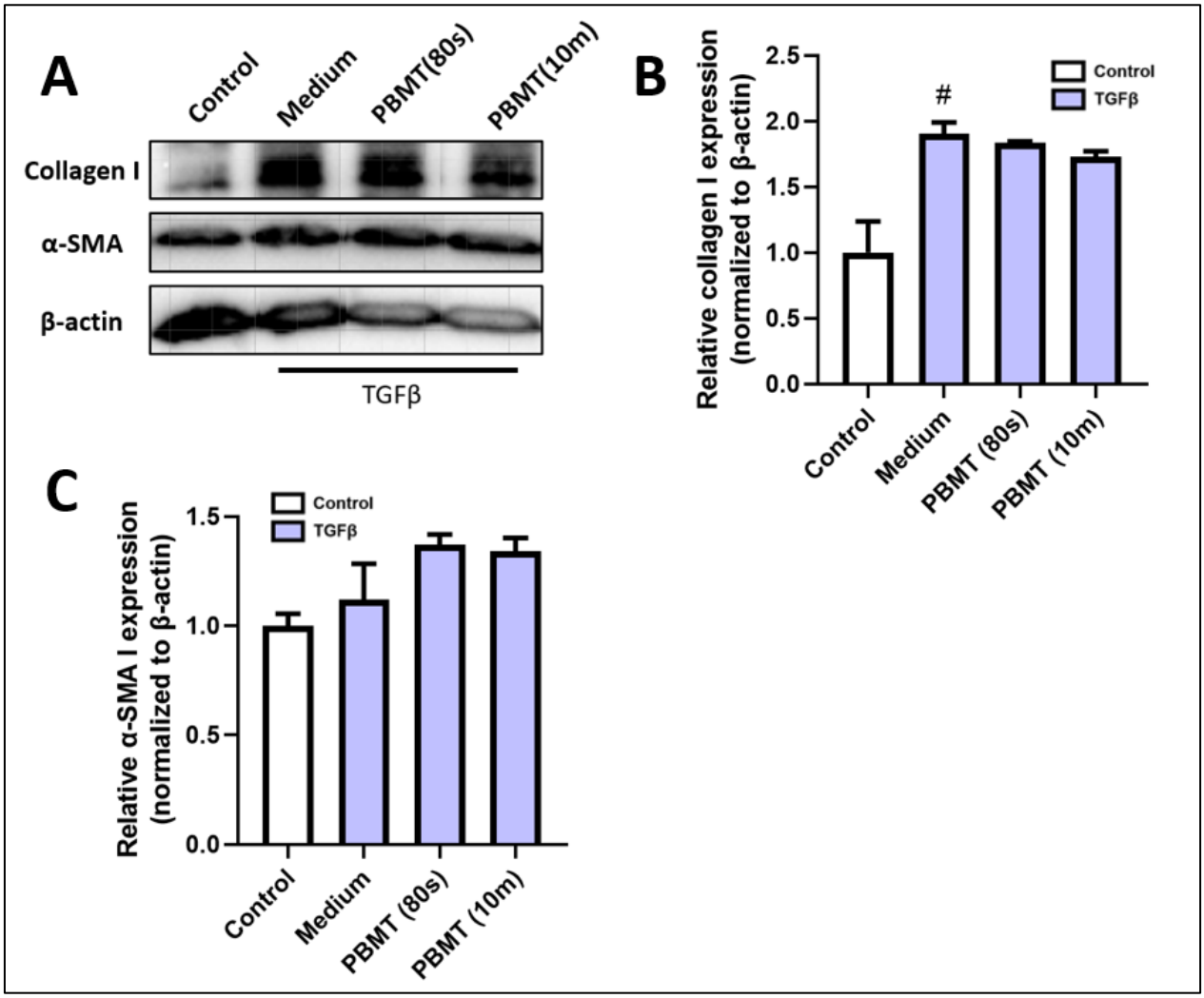

Figure 6. Western blot analysis of PBMT on collagen I and α-SMA expression of TGFβ activated HDF cells. **(A)** Representative images of western blot depicting bands for collagen I, α-SMA, and β-actin, and (B) quantitative analysis for collagen, **(C)** α-SMA examined in control and TGFβ activated HDF cells treated with medium alone or PBMT (80 sec and 10 min), n=2. Graphs represent mean ± SEM, # $P < 0.1$, represents significance versus control.

5.4 ECM related gene expression of HDF cells following PBMT

mRNA expression levels were examined for ECM related genes COL1A1, ACTA1, MMP2, MMP9, TIMP1, CTGF, COL3A1 and nitric oxide synthesis gene iNOS in HDF cells with and without TGFβ and PBMT (**Figure 7**). In line with expectations, treatment with TGFβ containing medium increased all ECM related genes tested except for MMP9. Following treatment with PBMT, the gene expression for COL1A1, ACTA1, MMP9, CTGF, iNOS showed a response towards baseline control values. These findings suggest a regulatory effect of PBMT on the expression of key genes associated with ECM remodeling in HDF cells under conditions of TGFβ stimulation, which could have a beneficial impact on wound healing [117], however there was no statistical significance found.

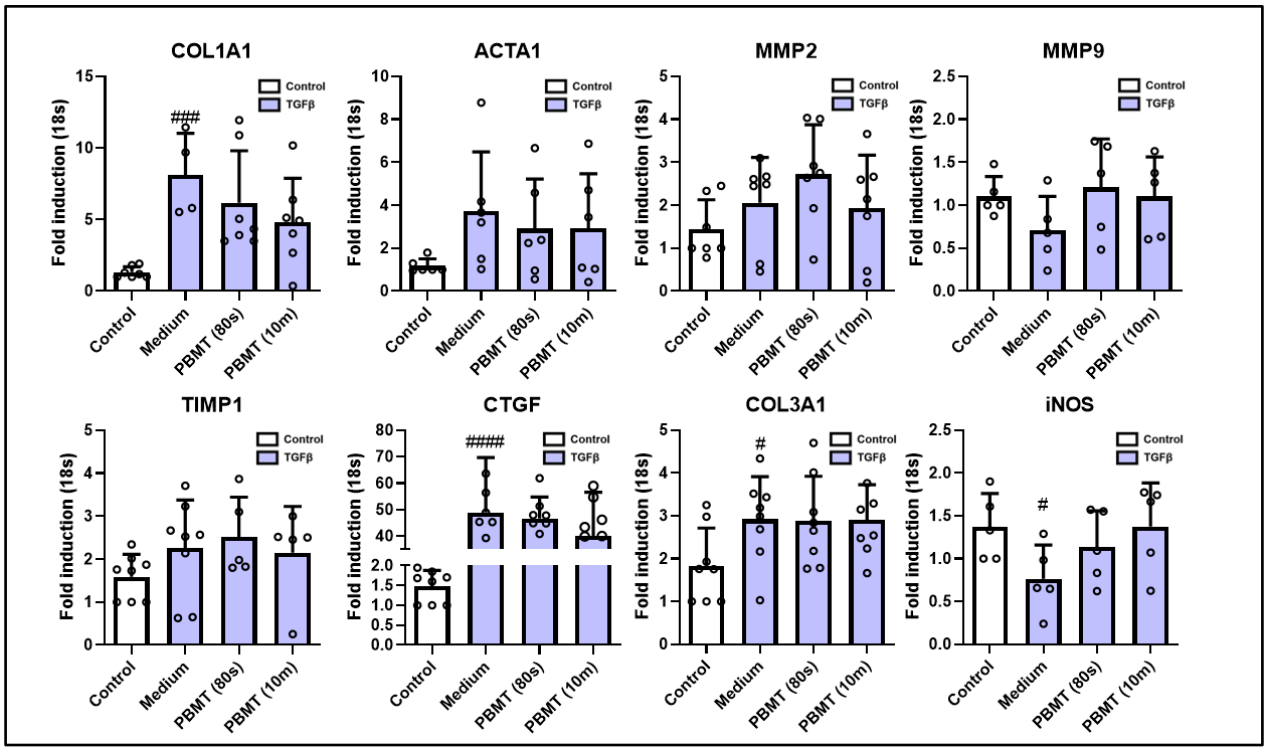

Figure 7. Effects of PBMT on gene expression of TGFβ activated HDF cells. qPCR analysis of COL1A1, ACTA1, MMP2, MMP9, TIMP1, CTGF, COL3A1 and iNOS in TGFβ activated HDF cells treated with medium alone or PBMT (80 sec, 10min). n=5. Graphs represent mean ± SEM, # $P < 0.1$, ### $P < 0.001$, #### $P < 0.001$, represents significance versus control. Hollow circles represent individual data points.

5.5 Effect of PBMT on gene expression of THP-1 monocytes, and polarized M1 and M2 macrophages

THP-1 monocytes were polarized to M1 and M2 macrophage phenotypes, and thereafter treated with PBMT. **Figure 8** shows the subsequent gene expression for inflammatory and immunoregulatory relevant genes iNOS, CCL2, TNF-α, IL-1β, CD163, IL-10, and Col1. While the results are largely statistically inconclusive, a general trend can be seen of upregulation of each gene following PBMT on each macrophage phenotype. Macrophages are sources of ECM components during skin repair; thus, upregulation of ECM genes, as well as immunoregulatory genes, can prove beneficial for wound healing [118].

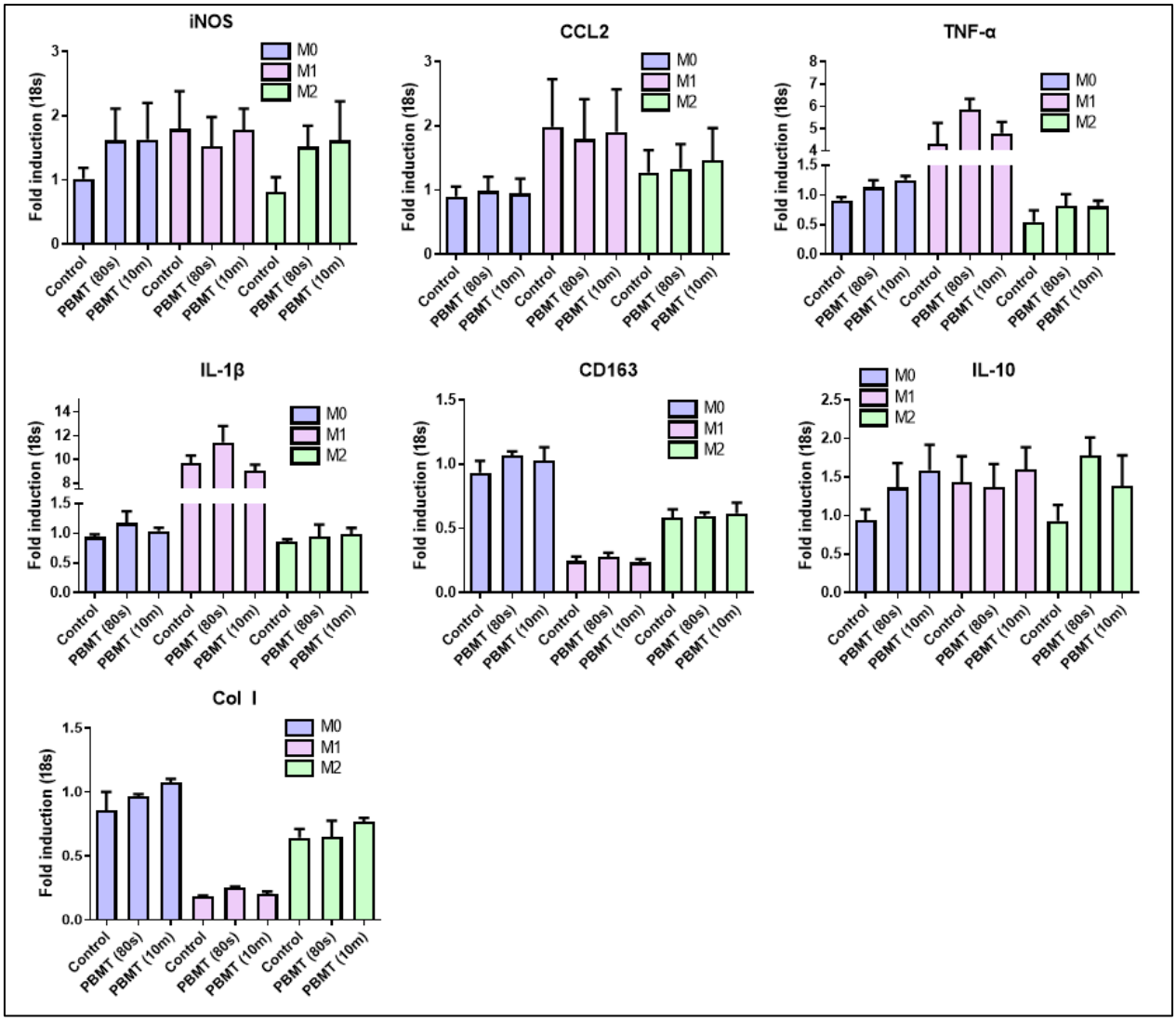

Figure 8. Effects of PBMT on gene expression of polarized THP-1 cells. qPCR analysis iNOS, CCL2, TNF-α, IL-1β, CD163, IL-10 and COL I, in THP-1 monocytes (M0) and THP-1 polarized M1 and M2 macrophages, treated with medium alone or PBMT (80 sec, 10min). n=4. Graphs represent mean ± SEM.

5.6 Effect of PBMT on gene expression of primary human skin

Finally, COL1A1, α-SMA, COL3, TIMP1 and IL-1β gene expression of TGFβ activated primary human skin cell disks treated with and without PBMT was analyzed (**Figure 9**). In contrast to expectations and previous findings, TGFβ decreased gene expression for all tested genes except IL-1β. Following treatment with PBMT, the gene expression for all tested genes trend towards control baseline untreated with TGFβ. This again shows a regulatory effect of PBMT on expression of key genes for ECM remodeling and immunoregulatory response, following TGFβ activation. Although the findings are preliminary, this shows that PBMT can have beneficial implications for wound healing [117].

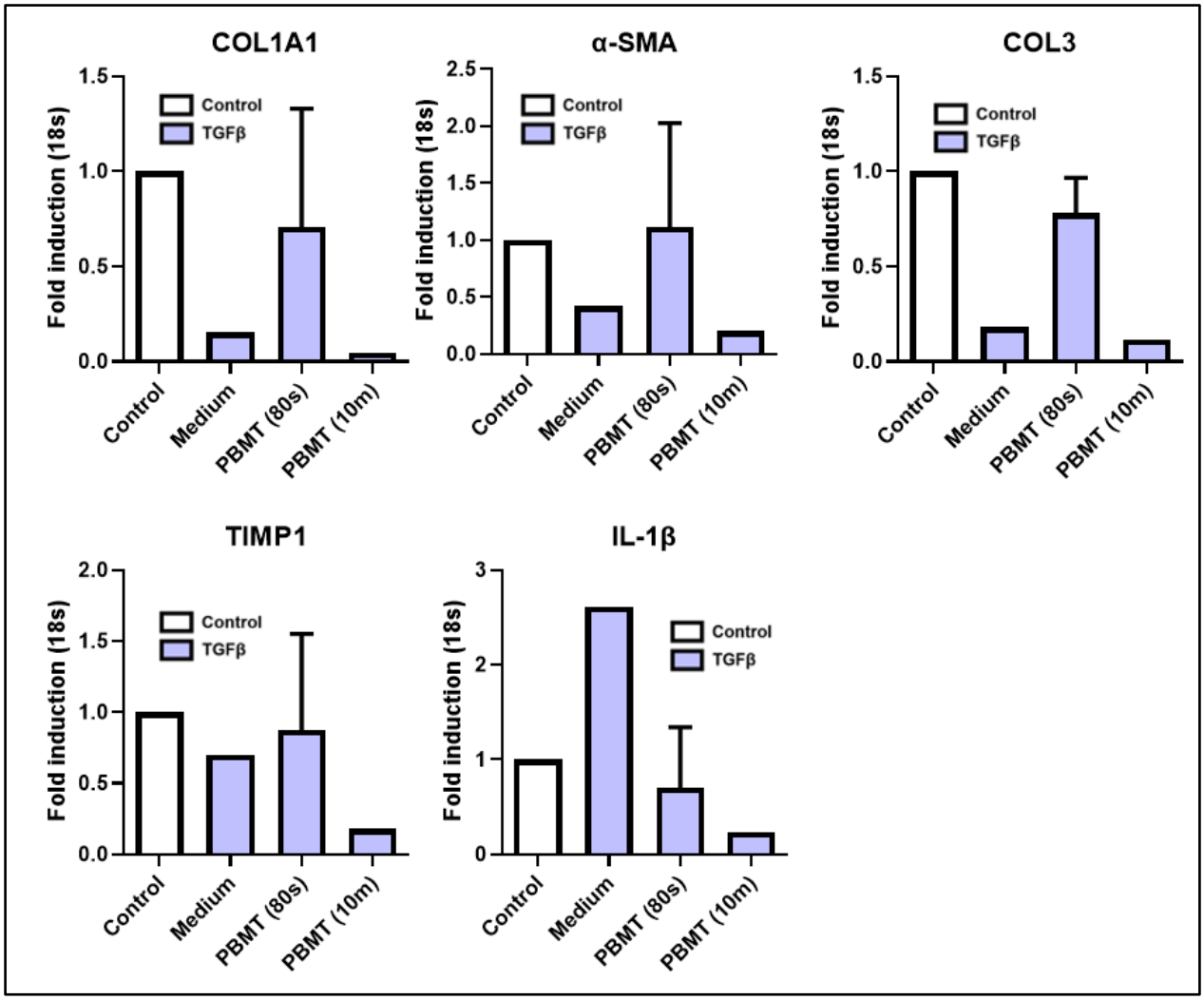

Figure 9. Effects of PBMT on gene expression of TGFβ activated primary human skin cell disks. qPCR analysis of COL1A1, α-SMA, COL3, TIMP1, and IL-1β in TGFβ activated primary human skin cell disks treated with medium alone or PBMT (80 sec, 10min). n=1, 2. Graphs represent mean ± SEM.

6. Discussion

The viability of PBMT for wound healing was evaluated by testing for inflammation, cellular migration, collagen synthesis and gene expression in a series of experiments utilizing HDF, THP-1 and primary human skin cells.

Previous studies have found that PBMT can induce an overall reduction in inflammation [119-121], the modulation of which is of critical importance for optimizing the wound healing process [122]. Moreover, PBMT has been found to selectively modulate ECM-modifying enzymes in human annulus fibrosus cells [123], as well as remodel the ECM of HDF cells by upregulating collagen I, TIMP 1, TIMP 2 while downregulating MMP2 and MMP9; suggesting proliferative matrix remodeling after treatment [124]. Interestingly, Mokoena et al. [58] have shown that PBMT is effective in differentiating fibroblasts into myofibroblasts in diabetic wounded cells, the success of which is vital for proper wound healing. In their study, the differentiation occurred with increased α-SMA while bypassing the activation of the TGFβ/Smad pathway, suggesting an alternative mechanism.

The wavelength of PBMT plays a critical role in the mechanism of attenuation of the wound repair process. Mignon et al. [125] found that human fibroblasts showed both cytotoxic and inhibitory changes when exposed to high doses of blue light, which was subsequently eliminated by refreshing the potentially ROS containing medium. Low doses of blue light significantly increased collagen production by papillary fibroblasts, whereas high doses significantly slowed the closure of scratch assay. This suggests that seeking optimal treatment parameters for each stage in the wound healing process can be of great importance. Indeed Chellini et al. [124] concluded that NIR PBMT in combination violet-blue light, if properly conducted by respecting the sequence of events of the wound healing process, the healing process could be significantly enhanced.

While previous studies have been contradictory by showing either an increase or decrease in metabolic activity, which can further be dependent on parameters such as wavelength, this study showed that red wavelength light is nontoxic for HDFs and decreased overall contractility and migration (**Figure 4**). This can be explained by an overall reduction in inflammation and fibroblast activation, by inhibiting inflammation and fibrotic responses, PBMT can indirectly lead to a decrease in contractility and migration [3].

Similarly, there was an overall reduction collagen I expression in the PBMT treated HDFs (**Figure 5**), which falls in line with the previous findings. However, at a higher exposure time (10min) the effect was trending towards an increase in collagen I, suggesting an increase inflammation. This would imply that there is an optimal dosage under the 10-minute mark. Following the same line, a decrease in α-SMA would be expected, as it is downstream from TGFβ signaling. However, a positive correlation between α-SMA and PBMT was shown in the more comprehensive western blot analysis (**Figure 6**). As discussed earlier, this counterintuitive effect is also found by Mokoena et al. [58] who showed that the TGFβ/Smad pathway is bypassed by another mechanism. The lack of distinction in α-SMA expression in the immunohistochemical staining assay could be due to protein localization or accessibility of antibody binding epitopes. The modulation of α-SMA has been directly linked to the mechanical properties of the

ECM in wound healing context [126], which would indicate that PBMT can even manage the repair process through mechanical cellular forces.

Although many observations throughout this study were not statistically significant, a general trend can be seen, most of which are consistent with previous studies. Therefore, we will discuss these results in the context of existing literature, highlighting potential implications and areas for further investigation.

On the gene expression level (**Figure 7**), expression of COL1A1 expectedly decreased after PBMT, this decrease however was found to be dose dependent even at 10 minutes. Interestingly, the gene expression for ACTA1 which encodes for α-SMA, showed an overall decrease following PBMT. The discrepancy between mRNA expression and protein expression highlights the complexity of cellular responses, only examining dosage on the mRNA level does not give a full picture, and effects such as mRNA turnover, protein stability, or other feedback mechanisms can play a role [127]. Previous research has found that α-SMA is upregulated following PBMT indicating increased myofibroblast activation, but only after extended incubation periods which was not the case in this study [58]. Investigating the role of the incubation period after PBMT could therefore also be interesting research.

MMP2, MMP9, responsible for ECM degradation and remodeling, and inhibitor TIMP1, showed a dose dependent response whereby at 80 seconds there was an increase which was diminished again at 10 minutes of exposure. Gene expression for CTGF and iNOS showed a decreasing and increasing dose dependent response respectively, and COL3A1 showed no response to PBMT. COL3A1 was expected to be downregulated following PBMT, as is reported in literature in ventricular septal samples [128]. Deviation from this expectation could be due to the cellular context of HDF in which fibroblasts involved in early wound healing stages might maintain COL3 expression [129]. The remaining results coincide with previous research which has found that PBMT can modulate ECM remodeling enzymes, where the effect that either stimulates or inhibits the gene response is highly sensitive to fluence and wavelength, as well as cell type and other factors [130]. Souza et al. [131] found that PBMT with 660 nm LED in combination with photosensitizer aluminum chloride phthalocyanine increased MMP9 secretion of HDFs, however in their study PBMT alone had no effect. Similarly, Da Ré Guerra et al. [132] found that PBMT with 830 nm promoted MMP9 in Wistar rat tendon, and conversely, Alves et al. [133] found that PBMT with 808 nm reduced MMP9 in Wistar rat knees. Moreover, the upregulation for MMP2 and MMP9 is coherent with the results found in earlier studies that upregulate MMP1 [57]. The effect of PBMT on TIMP1 protein expression was found to be cell context specific, whereby diabetic hypoxic wounded HDFs showed an upregulation in TIMP1 whereas normal hypoxic wounded HDFs showed a downregulation following PBMT [134]. New research has found that PBMT upregulated iNOS gene expression, however it downregulated the protein expression in Wistar rat joints [135], they suggest this is due to the iNOS activation pathways which are related to an increase in oxidative balance through an increase in systemic antioxidant enzymes, which reduce protein levels.

These differences highlight that ECM remodeling is a complex process that is influenced by multiple parameters, including wavelength and exposure duration, as well as the specific cellular context. This suggests therefore that to properly regulate the healing process, the specific parameters of PBMT in combination with the cellular context should be precisely optimized.

Macrophages play a critical role in wound healing, whereby macrophage-based therapies show a promising beneficial effect on hard to heal wounds [136]. The effect of PBMT on the gene expression of

monocytes differentiated to M1 and M2 phenotype (**Figure 8**) shows a modulation of key markers, indicating that PBMT can influence macrophages in both M1 and M2 states. Specifically, in M1 macrophages, PBMT downregulated pro-inflammatory markers iNOS, CCL2 as well as anti-inflammatory marker IL-10 while upregulating pro-inflammatory markers TNF-α, IL-1β and anti-inflammatory marker CD163 as well as COL 1. This effect again was inhibited after a prolonged exposure of 10 minutes giving more evidence that there is an optimal dosage of PBMT. In the context of wound healing, the observed reduction of iNOS and CCL2 suggests that PBMT can mitigate the inflammatory capacity of M1 macrophages, potentially reducing tissue damage associated with chronic inflammation [137].

Interestingly, while PBMT downregulated these markers, it simultaneously upregulated other pro-inflammatory markers TNF-α and IL-1β which are key cytokines that drive the inflammatory process. The upregulation of these markers shows that PBMT can enhance specific parts of the immune response to possibly enhance the initial defense mechanism during the early stages of wound healing. Since PBMT can be modulated in multiple ways to obtain different outcomes, the dual modulation of PBMT on macrophages suggests that a finely tuned therapy can be valuable to regulate the immune response for optimal wound healing. The inhibition of the effects of PBMT at prolonged exposure of 10 minutes further drives the concept of a biphasic dose response.

For the M2 polarized THP-1 cells, the effect of PBMT was similarly nuanced. M2 macrophages are generally associated with anti-inflammation and tissue repair [138]. The effect of PBMT showed an increase in all the markers tested, with the strongest effects for iNOS and IL-10 genes. This upregulation of pro-inflammatory markers in M2 macrophages indicates that PBMT could enhance wound healing by promoting M2 macrophage polarization and activity.

While not significant, results for both HDF as well as macrophages mostly align with studies of colleagues within the research group, as shown in **Table 2**. HDF cell migration, as well as HDF and macrophage (RAW) gene expression results are corroborated, whereby PBMT showed similar results for collagen-1 α-SMA, TIMP1, and MMP gene expression in HDF cells and iNOS and IL-6 in RAW cells. Interestingly, even the dose dependent responses of TIMP1 and the MMP family were found. It is important to realize, however, that their studies were also not statistically significant. These findings suggest that while PBMT has an effect, the effect is minimal and thus studies require a large sample size to obtain statistically significant results. Therefore, further studies are needed to validate the findings.

Table 2. Comparison with results from research from other members of the Medical Cell BioPhysics (MCBP) group [139, 140].

Author	Study	Result after PBMT	Statistically significant	Aligns with this study's results
K. A. Booij	HDF cell migration	Reduced	No	Yes
	HDF gene expression of:			
	Collagen -1	Reduced	No	Yes
	ACTA2	Reduced	No	Yes
	TIMP1	30s increased, 10min reduced	No	Yes
	MMP3	30s increased, 10min reduced	No	Yes
J. Oude Nijhuis	RAW gene expression of:			
	iNOS	Increased	No	Yes
	IL-6	Increased in M2	No	Yes
	TNF-α	Reduced	No	No

Unlike immortalized cell cultures, which provide controlled conditions, biopsy samples preserve the complex structure of human skin with the diversity of the various cell types and components of the extracellular matrix [141]. This makes them valuable in evaluating the efficacy and understanding the mechanisms of how PBMT can work in a real-world context of wound healing. The results in **Figure 9** show that PBMT influences gene expression in skin biopsy. PBMT helped to restore the levels of gene expression that had been altered due to treatment with TGFβ. These findings further support that PBMT may regulate fibrosis and inflammation which can translate into an improved healing response. Another key observation is that the effect of PBMT on human skin biopsy depends on time and fluence, whereby longer exposure times reduce the efficacy of the treatment.

However, contrary to expectations TGFβ did not increase collagen and a-SMA gene expression as opposed to the previous in vitro experiments. One explanation is that the complex interaction between different cell types in the biopsy can react differently to TGFβ stimulation [142]. This interaction effect could be strengthened by the limited or incompatible nutrient supply, since the diverse cell types react differently to the culture medium [143]. Furthermore, the low sample size (n=1) may have also influenced the results since individuals are different from one another and can react differently to treatment [144], therefore a larger sample size is needed. Finally, we used skin biopsy from healthy skin, whereas skin from (chronic) wounds have a microenvironment with different concentrations of microorganisms, such as bacteria, which can affect the wound healing process [145].

In this study we have not quantitatively considered oxygen levels experienced by the cells, which have been reported to significantly impact the efficacy of PBMT [63]. Optimizing the study design for oxygen levels could have increased the effectiveness of PBMT in each analysis which could have led to significance in the results. Overall, the results seem to generally fall in line with the literature studies as well as the results from previous research within the MCBP group. Specific deviations such as α-SMA decreases after PBMT could be explained by the variations in study design.

7. Future Recommendations

Based on the findings, there are several areas where further research is needed to fully understand and optimize the use of PBMT in wound healing. In future studies, different wavelengths, doses and exposure times should be tested to determine the best conditions for fibroblast activation, polarization of macrophages and collagen synthesis. The LED properties of future studies should be carefully evaluated, since the effectiveness of PBMT is highly dependent on wavelength, exposure time and fluence [146]. Moreover, any future in vitro studies should carefully consider tertiary study parameters such as oxygen levels experienced by the cells [63].

Current studies [147], including this one, focus on the short-term effects of PBMT. It is important to investigate the impact of multiple sessions over a period of days or weeks to evaluate the effectiveness of the treatment on wound healing. Future studies should take into account the different stages of wound healing, whereby the wound healing stages from inflammation to proliferation may require a different treatment [148, 149]. MMPs are especially interesting since they can modulate the wound healing response [150, 151] and have been found to be directly influenced by PBMT in this study. PBMT is uniquely positioned for wound healing by leveraging the dose-dependent response in which the inflammation and proliferation stage can be controlled by changing the parameters of PBMT.

While in vitro and biopsy studies provide useful information, testing PBMT in human clinical trials is ultimately necessary. Future studies should also investigate how PBMT interacts with blood flow and other immune cells. The advantages of PBMT in that it is non-invasive, non-toxic and painless, which makes it relatively easy and cost-effective to implement in clinical trials. Moreover, PBMT can be used in outpatient settings such as post-surgical home therapy, making it a practical option for patients. The effectiveness of PBMT can further be investigated on differences in acute or chronic wounds, as well as different wound types such as burns and surgical wounds.

Because the barrier towards human clinical trials is lower for PBMT as compared to traditional drug-based therapies, it could be more effective to first critically determine in which scenarios PBMT is effective and only afterwards reverse engineer the mechanisms. The methodology to discover the underlying mechanisms would therefore follow an inverse or parallel drug development process as opposed to a traditional approach where mechanistic studies precede clinical applications. By prioritizing human clinical trial efficacy first, future research can identify which patient populations and treatment parameters have the strongest beneficial effects. Mechanistic studies can then be refined to focus on the most relevant pathways instead of exploring all cellular interactions since the wound healing process is so complex. This approach would also accelerate clinical adoption. Furthermore, going straight to human trials would skip the complicated process of study design optimization, such as oxygen levels, cell confluency, and other in vitro limitations. Moreover, the incubation period after PBMT is a factor rarely investigated, however it has been found to be impactful in α-SMA expression [58], and therefore it is an interesting avenue for further research.

Furthermore, future study design will have to consider patient-specific variances such as differences between ethnic skin types [152, 153], sex, and anatomic location of the wound [154].

8. Conclusion

In this thesis, the effects of photobiomodulation therapy (PBMT) on wound healing were investigated in vitro. This was done specifically through investigating the effects on inflammation, cell migration, collagen production and gene expression. These effects were studied in human dermal fibroblasts (HDF), THP-1 monocytes and primary human skin cells.

Most of the results were not statistically significant, however the experiments suggested that PBMT affected collagen contraction and fibroblast migration induced by TGFβ, in addition to affecting the expression of key extracellular matrix genes. Protein expression analysis confirmed the modulation of collagen and α-SMA. Furthermore, gene expressions in polarized THP-1 monocytes showed that PBMT modulated both proinflammatory and anti-inflammatory markers, this suggests that it can play a role in immune system modulation, critical for wound healing. The lack of statistical significance shows the need for further investigations with larger sample sizes. The study with primary human skin samples encountered further limitations due to the small sample size. Despite these limitations, the preliminary results show that PBMT has a high potential for wound healing applications, however more research is needed.

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References

1. Mester, E., B. Szende, and P. Gartner, *[The effect of laser beams on the growth of hair in mice].* Radiobiol Radiother (Berl), 1968. **9**(5): p. 621-6.
2. Agrawal, M.R.H.M.V.P.d.S.T., *Handbook of low-level laser therapy*. 2017, Singapore: Pan Stanford Publishing.
3. Hamblin, M.R., *Mechanisms and applications of the anti-inflammatory effects of photobiomodulation.* AIMS Biophys, 2017. **4**(3): p. 337-361.
4. Serrage, H., et al., *Under the spotlight: mechanisms of photobiomodulation concentrating on blue and green light.* Photochem Photobiol Sci, 2019. **18**(8): p. 1877-1909.
5. Wang, Y., et al., *Photobiomodulation (blue and green light) encourages osteoblastic-differentiation of human adipose-derived stem cells: role of intracellular calcium and light-gated ion channels.* Sci Rep, 2016. **6**: p. 33719.
6. Wang, L., S.L. Jacques, and L. Zheng, *MCML--Monte Carlo modeling of light transport in multi-layered tissues.* Comput Methods Programs Biomed, 1995. **47**(2): p. 131-46.
7. Chen, Y., et al., *Clinical evidence of photobiomodulation therapy (PBMT) on implant stability and success: a systematic review and meta-analysis.* BMC Oral Health, 2019. **19**(1): p. 77.
8. Dodd, E.M., et al., *Photobiomodulation therapy for androgenetic alopecia: A clinician's guide to home-use devices cleared by the Federal Drug Administration.* J Cosmet Laser Ther, 2018. **20**(3): p. 159-167.
9. Juanita j. Anders, P. *Photobiomodulation by ASLMS*. 2016 [cited 2021 14/02/2021]; Available from: https://www.aslms.org/for-the-public/treatments-using-lasers-and-energy-based-devices/photobiomodulation.
10. Rarokar, N., et al., *Phototherapy: a critical review.* Photophysics and Nanophysics in Therapeutics, 2022: p. 3-14.
11. de Freitas, L.F. and M.R. Hamblin, *Proposed Mechanisms of Photobiomodulation or Low-Level Light Therapy.* IEEE J Sel Top Quantum Electron, 2016. **22**(3).
12. Shinde, A.V., C. Humeres, and N.G. Frangogiannis, *The role of α-smooth muscle actin in fibroblast-mediated matrix contraction and remodeling.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2017. **1863**(1): p. 298-309.
13. Oliveira, A.C.d., et al., *Quality of life of people with chronic wounds.* Acta Paulista de Enfermagem, 2019. **32**: p. 194-201.
14. Kirsner, R.S. and W.H. Eaglstein, *The wound healing process.* Dermatologic clinics, 1993. **11**(4): p. 629-640.
15. Ellis, S., E.J. Lin, and D. Tartar, *Immunology of wound healing.* Current dermatology reports, 2018. **7**: p. 350-358.
16. Palta, S., R. Saroa, and A. Palta, *Overview of the coagulation system.* Indian journal of anaesthesia, 2014. **58**(5): p. 515-523.
17. MacLeod, A.S. and J.N. Mansbridge, *The innate immune system in acute and chronic wounds.* Advances in wound care, 2016. **5**(2): p. 65-78.
18. Wilgus, T.A., S. Roy, and J.C. McDaniel, *Neutrophils and wound repair: positive actions and negative reactions.* Advances in wound care, 2013. **2**(7): p. 379-388.
19. Briggaman, R.A., et al., *Degradation of the epidermal-dermal junction by proteolytic enzymes from human skin and human polymorphonuclear leukocytes.* Journal of Experimental Medicine, 1984. **160**(4): p. 1027-1042.
20. Pirilä, E., et al., *Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wounds of different etiologies.* Wound Repair and Regeneration, 2007. **15**(1): p. 47-57.

21. Nwomeh, B.C., et al., *MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers.* Journal of Surgical Research, 1999. **81**(2): p. 189-195.
22. Adams, D.O., *Molecular interactions in macrophage activation.* Immunology Today, 1989. **10**(2): p. 33-35.
23. Nathan, C., *Neutrophils and immunity: challenges and opportunities.* Nature reviews immunology, 2006. **6**(3): p. 173-182.
24. Brancato, S.K. and J.E. Albina, *Wound macrophages as key regulators of repair: origin, phenotype, and function.* The American journal of pathology, 2011. **178**(1): p. 19-25.
25. Landén, N.X., D. Li, and M. Ståhle, *Transition from inflammation to proliferation: a critical step during wound healing.* Cellular and Molecular Life Sciences, 2016. **73**: p. 3861-3885.
26. Tonnesen, M.G., X. Feng, and R.A. Clark. *Angiogenesis in wound healing*. in *Journal of investigative dermatology symposium proceedings*. 2000. Elsevier.
27. Widodo, A., P.S. Rahajoe, and R.T. Astuti, *TGF-β expression and wound tensile strength after simple interrupted suturing and zip surgical skin closure (IN VIVO study).* Annals of Medicine and Surgery, 2020. **58**: p. 187-193.
28. Witte, M.B. and A. Barbul, *General principles of wound healing.* Surgical Clinics of North America, 1997. **77**(3): p. 509-528.
29. SMIT, H.J., *A five-level model for wound analysis and treatment.* Wounds UK, 2018. **14**(4).
30. Nunan, R., K.G. Harding, and P. Martin, *Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity.* Disease models & mechanisms, 2014. **7**(11): p. 1205-1213.
31. Alvarez, O.M., et al., *Incorporating wound healing strategies to improve palliation (symptom management) in patients with chronic wounds.* Journal of Palliative Medicine, 2007. **10**(5): p. 1161-1189.
32. Lee, K., S. Lee, and J. Kim, *Wound Pain Management: The Present and the Future.* Journal of Wound Management and Research, 2024. **20**(3): p. 199-211.
33. Shamis, Y., et al., *Fibroblasts derived from human embryonic stem cells direct development and repair of 3D human skin equivalents.* Stem cell research & therapy, 2011. **2**: p. 1-12.
34. Darby, I.A. and T.D. Hewitson, *Fibroblast differentiation in wound healing and fibrosis.* International review of cytology, 2007. **257**: p. 143-179.
35. Darby, I.A., et al., *Fibroblasts and myofibroblasts in wound healing.* Clinical, cosmetic and investigational dermatology, 2014: p. 301-311.
36. Werner, S., T. Krieg, and H. Smola, *Keratinocyte–fibroblast interactions in wound healing.* Journal of investigative dermatology, 2007. **127**(5): p. 998-1008.
37. Agarwal, C., et al., *Healing and normal fibroblasts exhibit differential proliferation, collagen production, α-SMA expression, and contraction.* Annals of biomedical engineering, 2006. **34**: p. 653-659.
38. Andrews, J.P., et al., *Keloids: The paradigm of skin fibrosis—Pathomechanisms and treatment.* Matrix Biology, 2016. **51**: p. 37-46.
39. Bock, O., et al., *Quality of life of patients with keloid and hypertrophic scarring.* Archives of dermatological research, 2006. **297**: p. 433-438.
40. Zhao, R., et al., *Inflammation in chronic wounds.* International journal of molecular sciences, 2016. **17**(12): p. 2085.
41. Januszyk, M., et al., *Characterization of diabetic and non-diabetic foot ulcers using single-cell RNA-sequencing.* Micromachines, 2020. **11**(9): p. 815.
42. Shook, B.A., et al., *Myofibroblast proliferation and heterogeneity are supported by macrophages during skin repair.* Science, 2018. **362**(6417): p. eaar2971.
43. Falanga, V., et al., *Chronic wounds.* Nature Reviews Disease Primers, 2022. **8**(1): p. 50.

44. Chow, R.T. and P.J. Armati, *Photobiomodulation: implications for anesthesia and pain relief.* Photomedicine and Laser Surgery, 2016. **34**(12): p. 599-609.
45. González-Muñoz, A., et al. *Efficacy of photobiomodulation therapy in the treatment of pain and inflammation: a literature review*. in *Healthcare*. 2023. MDPI.
46. Kuffler, D.P., *Photobiomodulation in promoting wound healing: a review.* Regenerative medicine, 2016. **11**(1): p. 107-122.
47. Moradi, A., et al., *An improvement in acute wound healing in mice by the combined application of photobiomodulation and curcumin-loaded iron particles.* Lasers in medical science, 2019. **34**: p. 779-791.
48. Leyane, T.S., S.W. Jere, and N.N. Houreld, *Cellular signalling and photobiomodulation in chronic wound repair.* International journal of molecular sciences, 2021. **22**(20): p. 11223.
49. Mokoena, D., et al., *Role of photobiomodulation on the activation of the Smad pathway via TGF-β in wound healing.* Journal of Photochemistry and Photobiology B: Biology, 2018. **189**: p. 138-144.
50. Kasowanjete, P., S.S.D. Kumar, and N.N. Houreld, *A review of photobiomodulation on PI3K/AKT/mTOR in wound healing.* Journal of photochemistry and photobiology, 2024. **19**: p. 100215.
51. Krenitsky, A., R.I. Ghamrawi, and S.R. Feldman, *Phototherapy: a Review and Update of Treatment Options in Dermatology.* Current Dermatology Reports, 2020. **9**(1): p. 10-21.
52. Gambichler, T. and L. Schmitz, *Ultraviolet A1 phototherapy for fibrosing conditions.* Frontiers in Medicine, 2018. **5**: p. 237.
53. Hassani, J. and S.R. Feldman, *Phototherapy in scleroderma.* Dermatology and Therapy, 2016. **6**: p. 519-553.
54. Gruss, C., et al., *Induction of interstitial collagenase (MMP-1) by UVA-1 phototherapy in morphea fibroblasts.* The Lancet, 1997. **350**(9087): p. 1295-1296.
55. Maiorino, A., et al., *Melanoma and non-melanoma skin cancer in psoriatic patients treated with high-dose phototherapy.* Journal of Dermatological Treatment, 2016. **27**(5): p. 443-447.
56. Mamalis, A., D. Siegel, and J. Jagdeo, *Visible red light emitting diode photobiomodulation for skin fibrosis: key molecular pathways.* Current dermatology reports, 2016. **5**: p. 121-128.
57. Austin, E., et al., *Transcriptome analysis of human dermal fibroblasts following red light phototherapy.* Scientific Reports, 2021. **11**(1): p. 7315.
58. Mokoena, D.R., et al., *Photobiomodulation at 660 nm stimulates fibroblast differentiation.* Lasers in surgery and medicine, 2020. **52**(7): p. 671-681.
59. Mamalis, A., et al., *MicroRNA expression analysis of human skin fibroblasts treated with high-fluence light-emitting diode-red light.* Journal of biophotonics, 2019. **12**(5): p. e201800207.
60. Chaves, M.E.D.A., et al., *Effects of low-power light therapy on wound healing: LASER x LED.* Anais brasileiros de dermatologia, 2014. **89**(4): p. 616-623.
61. de Sousa, A.P.C., et al., *Effect of LED phototherapy of three distinct wavelengths on fibroblasts on wound healing: a histological study in a rodent model.* Photomedicine and laser surgery, 2010. **28**(4): p. 547-552.
62. Mbene, A.B., N.N. Houreld, and H. Abrahamse, *DNA damage after phototherapy in wounded fibroblast cells irradiated with 16 J/cm2.* Journal of Photochemistry and Photobiology B: Biology, 2009. **94**(2): p. 131-137.
63. Mignon, C., et al., *Photobiomodulation of human dermal fibroblasts in vitro: decisive role of cell culture conditions and treatment protocols on experimental outcome.* Scientific reports, 2017. **7**(1): p. 2797.
64. Wu, S., et al., *Cancer phototherapy via selective photoinactivation of respiratory chain oxidase to trigger a fatal superoxide anion burst.* Antioxid Redox Signal, 2014. **20**(5): p. 733-46.

65. Karu, T.I., *Multiple roles of cytochrome c oxidase in mammalian cells under action of red and IR-A radiation.* IUBMB Life, 2010. **62**(8): p. 607-10.
66. Lane, N., *Cell biology: power games.* Nature, 2006. **443**(7114): p. 901-3.
67. Pannala, V.R., A.K. Camara, and R.K. Dash, *Modeling the detailed kinetics of mitochondrial cytochrome c oxidase: Catalytic mechanism and nitric oxide inhibition.* J Appl Physiol (1985), 2016. **121**(5): p. 1196-1207.
68. Poyton, R.O. and K.A. Ball, *Therapeutic photobiomodulation: nitric oxide and a novel function of mitochondrial cytochrome c oxidase.* Discov Med, 2011. **11**(57): p. 154-9.
69. Karu, T.I., *Mitochondrial signaling in mammalian cells activated by red and near-IR radiation.* Photochem Photobiol, 2008. **84**(5): p. 1091-9.
70. Castellano-Pellicena, I., et al., *Does blue light restore human epidermal barrier function via activation of Opsin during cutaneous wound healing?* Lasers in surgery and medicine, 2019. **51**(4): p. 370-382.
71. Blackshaw, S. and S.H. Snyder, *Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain.* J Neurosci, 1999. **19**(10): p. 3681-90.
72. Haltaufderhyde, K., et al., *Opsin expression in human epidermal skin.* Photochem Photobiol, 2015. **91**(1): p. 117-23.
73. Kojima, D., et al., *UV-sensitive photoreceptor protein OPN5 in humans and mice.* PLoS One, 2011. **6**(10): p. e26388.
74. Nissilä, J., et al., *P-780-The abundance and distribution of melanopsin (OPN4) protein in human brain.* European Psychiatry, 2012. **27**(S1): p. 1-1.
75. Poletini, M.O., et al., *TRP channels: a missing bond in the entrainment mechanism of peripheral clocks throughout evolution.* Temperature (Austin), 2015. **2**(4): p. 522-34.
76. Gu, Q., et al., *Stimulation of TRPV1 by Green Laser Light.* Evid Based Complement Alternat Med, 2012. **2012**: p. 857123.
77. Wang, Y., et al., *Red (660 nm) or near-infrared (810 nm) photobiomodulation stimulates, while blue (415 nm), green (540 nm) light inhibits proliferation in human adipose-derived stem cells.* Scientific reports, 2017. **7**(1): p. 1-10.
78. Smani, T., et al., *Functional and physiopathological implications of TRP channels.* Biochim Biophys Acta, 2015. **1853**(8): p. 1772-82.
79. Wang, Y., et al., *Photobiomodulation (blue and green light) encourages osteoblastic-differentiation of human adipose-derived stem cells: role of intracellular calcium and light-gated ion channels.* Scientific reports, 2016. **6**(1): p. 1-9.
80. Bouly, J.P., et al., *Cryptochrome blue light photoreceptors are activated through interconversion of flavin redox states.* J Biol Chem, 2007. **282**(13): p. 9383-9391.
81. Sancar, A., *Regulation of the mammalian circadian clock by cryptochrome.* J Biol Chem, 2004. **279**(33): p. 34079-82.
82. Yang, M.Y., C.J. Chang, and L.Y. Chen, *Blue light induced reactive oxygen species from flavin mononucleotide and flavin adenine dinucleotide on lethality of HeLa cells.* J Photochem Photobiol B, 2017. **173**: p. 325-332.
83. Cheon, M.W., et al., *Low level light therapy by Red–Green–Blue LEDs improves healing in an excision model of Sprague–Dawley rats.* Personal and ubiquitous computing, 2013. **17**(7): p. 1421-1428.
84. Osborne, N.N., C. Nunez-Alvarez, and S. Del Olmo-Aguado, *The effect of visual blue light on mitochondrial function associated with retinal ganglions cells.* Exp Eye Res, 2014. **128**: p. 8-14.
85. Serrage, H.J., et al., *Differential responses of myoblasts and myotubes to photobiomodulation are associated with mitochondrial number.* J Biophotonics, 2019. **12**(6): p. e201800411.

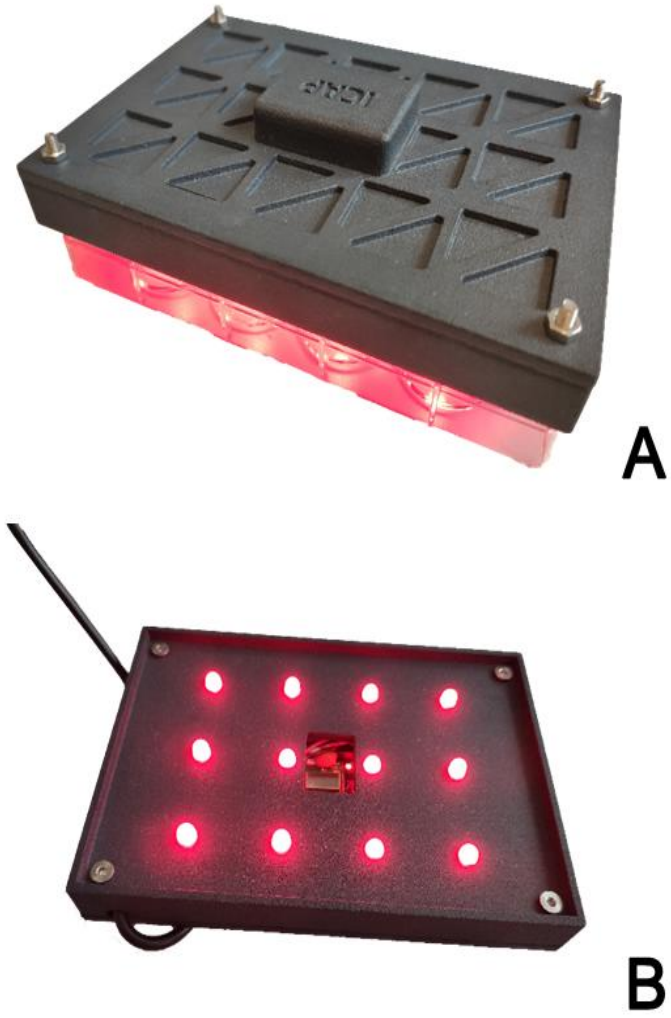
86. Marengo-Rowe, A.J., *Structure-function relations of human hemoglobins.* Proc (Bayl Univ Med Cent), 2006. **19**(3): p. 239-45.
87. Veerman, E.C., et al., *The reaction of cytochrome aa3 with (porphyrin) cytochrome c as studied by pulse radiolysis.* Biochim Biophys Acta, 1982. **680**(2): p. 134-41.
88. Ener, M.E., et al., *Photooxidation of cytochrome P450-BM3.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18783-6.
89. Koren, K., et al., *Strongly Phosphorescent Iridium(III)-Porphyrins - New Oxygen Indicators with Tuneable Photophysical Properties and Functionalities.* Eur J Inorg Chem, 2011. **2011**(10): p. 1531-1534.
90. Karu, T., *Primary and secondary mechanisms of action of visible to near-IR radiation on cells.* J Photochem Photobiol B, 1999. **49**(1): p. 1-17.
91. Ankri, R., R. Lubart, and H. Taitelbaum, *Estimation of the optimal wavelengths for laser-induced wound healing.* Lasers Surg Med, 2010. **42**(8): p. 760-4.
92. Buravlev, E.A., et al., *Are the mitochondrial respiratory complexes blocked by NO the targets for the laser and LED therapy?* Lasers Med Sci, 2015. **30**(1): p. 173-80.
93. Muller-Enoch, D., *Blue light mediated photoreduction of the flavoprotein NADPH-cytochrome P450 reductase. A Forster-type energy transfer.* Z Naturforsch C J Biosci, 1997. **52**(9-10): p. 605-14.
94. Werck-Reichhart, D. and R. Feyereisen, *Cytochromes P450: a success story.* Genome Biol, 2000. **1**(6): p. REVIEWS3003.
95. Hannemann, F., et al., *Cytochrome P450 systems--biological variations of electron transport chains.* Biochim Biophys Acta, 2007. **1770**(3): p. 330-44.
96. Becker, A., et al. *Impact of blue LED irradiation on proliferation and gene expression of cultured human keratinocytes*. in *Mechanisms for Low-Light Therapy X*. 2015. International Society for Optics and Photonics.
97. Inoue, S. and M. Kabaya, *Biological activities caused by far-infrared radiation.* Int J Biometeorol, 1989. **33**(3): p. 145-50.
98. Ho, M.W., *Illuminating water and life: Emilio Del Giudice.* Electromagn Biol Med, 2015. **34**(2): p. 113-22.
99. Damodaran, S., *Water at Biological Phase Boundaries: Its Role in Interfacial Activation of Enzymes and Metabolic Pathways.* Subcell Biochem, 2015. **71**: p. 233-61.
100. Chen, A.C., et al., *Low-level laser therapy activates NF-kB via generation of reactive oxygen species in mouse embryonic fibroblasts.* PLoS One, 2011. **6**(7): p. e22453.
101. Tergaonkar, V., *NFkappaB pathway: a good signaling paradigm and therapeutic target.* Int J Biochem Cell Biol, 2006. **38**(10): p. 1647-53.
102. Chen, A.C., et al., *Effects of 810-nm laser on murine bone-marrow-derived dendritic cells.* Photomed Laser Surg, 2011. **29**(6): p. 383-9.
103. Rupel, K., et al., *Photobiomodulation at multiple wavelengths differentially modulates oxidative stress in vitro and in vivo.* Oxidative medicine and cellular longevity, 2018. **2018**.
104. Heo, J.C., et al., *Photobiomodulation (660 nm) therapy reduces oxidative stress and induces BDNF expression in the hippocampus.* Sci Rep, 2019. **9**(1): p. 10114.
105. Huang, Y.Y., et al., *Biphasic dose response in low level light therapy - an update.* Dose Response, 2011. **9**(4): p. 602-18.
106. Hamblin, M.R., *Photobiomodulation for traumatic brain injury and stroke.* J Neurosci Res, 2018. **96**(4): p. 731-743.
107. Wang, Y., et al., *Photobiomodulation of human adipose-derived stem cells using 810nm and 980nm lasers operates via different mechanisms of action.* Biochim Biophys Acta Gen Subj, 2017. **1861**(2): p. 441-449.

108. Fernandes, K.P., et al., *Photobiomodulation with 660-nm and 780-nm laser on activated J774 macrophage-like cells: Effect on M1 inflammatory markers.* J Photochem Photobiol B, 2015. **153**: p. 344-51.
109. von Leden, R.E., et al., *808 nm wavelength light induces a dose-dependent alteration in microglial polarization and resultant microglial induced neurite growth.* Lasers Surg Med, 2013. **45**(4): p. 253-63.
110. Souza, N.H.C., et al., *Photobiomodulation and different macrophages phenotypes during muscle tissue repair.* J Cell Mol Med, 2018. **22**(10): p. 4922-4934.
111. Akcora, B.O., G. Storm, and R. Bansal, *Inhibition of canonical WNT signaling pathway by beta-catenin/CBP inhibitor ICG-001 ameliorates liver fibrosis in vivo through suppression of stromal CXCL12.* Biochim Biophys Acta Mol Basis Dis, 2018. **1864**(3): p. 804-818.
112. Postlethwaite, A.E., et al., *Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta.* J Exp Med, 1987. **165**(1): p. 251-6.
113. Sumiyoshi, K., et al., *Smads regulate collagen gel contraction by human dermal fibroblasts.* Br J Dermatol, 2003. **149**(3): p. 464-70.
114. Kiritsi, D. and A. Nystrom, *The role of TGFbeta in wound healing pathologies.* Mech Ageing Dev, 2018. **172**: p. 51-58.
115. Ehrlich, H.P. and T.K. Hunt, *Collagen Organization Critical Role in Wound Contraction.* Adv Wound Care (New Rochelle), 2012. **1**(1): p. 3-9.
116. Shinde, A.V., C. Humeres, and N.G. Frangogiannis, *The role of alpha-smooth muscle actin in fibroblast-mediated matrix contraction and remodeling.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(1): p. 298-309.
117. Potekaev, N.N., et al., *The Role of Extracellular Matrix in Skin Wound Healing.* J Clin Med, 2021. **10**(24).
118. Etich, J., et al., *Gene Expression Profiling of the Extracellular Matrix Signature in Macrophages of Different Activation Status: Relevance for Skin Wound Healing.* Int J Mol Sci, 2019. **20**(20).
119. Gupta, A., et al., *Superpulsed (Ga-As, 904 nm) low-level laser therapy (LLLT) attenuates inflammatory response and enhances healing of burn wounds.* J Biophotonics, 2015. **8**(6): p. 489-501.
120. Huang, Y.Y., et al., *Low-level laser therapy (LLLT) reduces oxidative stress in primary cortical neurons in vitro.* J Biophotonics, 2013. **6**(10): p. 829-38.
121. Langella, L.G., et al., *Photobiomodulation therapy (PBMT) on acute pain and inflammation in patients who underwent total hip arthroplasty-a randomized, triple-blind, placebo-controlled clinical trial.* Lasers Med Sci, 2018. **33**(9): p. 1933-1940.
122. Shukla, S.K., et al., *Pharmacological control of inflammation in wound healing.* J Tissue Viability, 2019. **28**(4): p. 218-222.
123. Hwang, M.H., et al., *Photobiomodulation on human annulus fibrosus cells during the intervertebral disk degeneration: extracellular matrix-modifying enzymes.* Lasers Med Sci, 2016. **31**(4): p. 767-77.
124. Chellini, F., et al., *In vitro evidences of different fibroblast morpho-functional responses to red, near-infrared and violet-blue photobiomodulation: Clues for addressing wound healing.* Applied Sciences, 2020. **10**(21): p. 7878.
125. Mignon, C., et al. *Photobiomodulation of distinct lineages of human dermal fibroblasts: a rational approach towards the selection of effective light parameters for skin rejuvenation and wound healing*. in *Mechanisms of Photobiomodulation Therapy XI*. 2016. SPIE.
126. Goldberg, M.T., et al., *TNF-α suppresses α-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing.* Journal of Investigative Dermatology, 2007. **127**(11): p. 2645-2655.

127. Perl, K., et al., *Reduced changes in protein compared to mRNA levels across non-proliferating tissues.* BMC Genomics, 2017. **18**(1): p. 305.
128. Feliciano, R.D.S., et al., *Photobiomodulation therapy's effects on cardiac fibrosis activation after experimental myocardial infarction.* Lasers Surg Med, 2022. **54**(6): p. 883-894.
129. Devos, H., et al., *Reviewing the Regulators of COL1A1.* Int J Mol Sci, 2023. **24**(12).
130. da Silva Sergio, L.P., et al., *Photobiomodulation on extracellular matrix.* Laser Physics, 2023. **33**(3): p. 033001.
131. Souza, C., et al., *Synergistic effect of photobiomodulation and phthalocyanine photosensitizer on fibroblast signaling responses in an in vitro three-dimensional microenvironment.* J Photochem Photobiol B, 2021. **222**: p. 112256.
132. Da Re Guerra, F., et al., *Low level laser therapy accelerates the extracellular matrix reorganization of inflamed tendon.* Tissue Cell, 2017. **49**(4): p. 483-488.
133. Alves, A.C., et al., *Effect of low-level laser therapy on metalloproteinase MMP-2 and MMP-9 production and percentage of collagen types I and III in a papain cartilage injury model.* Lasers Med Sci, 2014. **29**(3): p. 911-9.
134. Ayuk, S.M., H. Abrahamse, and N.N. Houreld, *Photobiomodulation alters matrix protein activity in stressed fibroblast cells in vitro.* J Biophotonics, 2018. **11**(3).
135. Bomfim, F., et al., *Photobiomodulation effects on synovial morphology, iNOS gene, and protein expression in a model of acute inflammation.* Acta Cir Bras, 2024. **39**: p. e392024.
136. Minutti, C.M., et al., *Tissue-specific contribution of macrophages to wound healing.* Semin Cell Dev Biol, 2017. **61**: p. 3-11.
137. Kashfi, K., J. Kannikal, and N. Nath, *Macrophage Reprogramming and Cancer Therapeutics: Role of iNOS-Derived NO.* Cells, 2021. **10**(11).
138. Bansal, R., et al., *Macrophages in liver disease*. 2020, Frontiers Media SA. p. 1754.
139. Nijhuis, J.O., *The Effects of Photobiomodulation on Macrophage-driven Inflammation and Cytokine Storms.* 2021.
140. Booij, K.A., *The effects of Photobiomodulation (PBM) on the in vitro (diabetic) woundhealing process.* nd.
141. Rodrigues, A.D. and A. Rowland, *Profiling of drug-metabolizing enzymes and transporters in human tissue biopsy samples: a review of the literature.* The Journal of Pharmacology and Experimental Therapeutics, 2020. **372**(3): p. 308-319.
142. Van Bruaene, N., et al., *TGF-β signaling and collagen deposition in chronic rhinosinusitis.* Journal of Allergy and Clinical Immunology, 2009. **124**(2): p. 253-259. e2.
143. Vangipuram, M., et al., *Skin punch biopsy explant culture for derivation of primary human fibroblasts.* Journal of visualized experiments: JoVE, 2013(77): p. 3779.
144. Evans, W.E. and J.A. Johnson, *Pharmacogenomics: the inherited basis for interindividual differences in drug response.* Annual review of genomics and human genetics, 2001. **2**(1): p. 9-39.
145. Haalboom, M., et al., *Wound swab and wound biopsy yield similar culture results.* Wound Repair and Regeneration, 2018. **26**(2): p. 192-199.
146. Rupel, K., et al., *Photobiomodulation at multiple wavelengths differentially modulates oxidative stress in vitro and in vivo.* Oxidative medicine and cellular longevity, 2018. **2018**(1): p. 6510159.
147. Oyebode, O., N.N. Houreld, and H. Abrahamse, *Photobiomodulation in diabetic wound healing: a review of red and near-infrared wavelength applications.* Cell Biochemistry and Function, 2021. **39**(5): p. 596-612.
148. Kimura, S. and T. Tsuji, *Mechanical and immunological regulation in wound healing and skin reconstruction.* International Journal of Molecular Sciences, 2021. **22**(11): p. 5474.
149. Peña, O.A. and P. Martin, *Cellular and molecular mechanisms of skin wound healing.* Nature Reviews Molecular Cell Biology, 2024. **25**(8): p. 599-616.

150. Rohani, M.G. and W.C. Parks, *Matrix remodeling by MMPs during wound repair.* Matrix biology, 2015. **44**: p. 113-121.
151. Kandhwal, M., et al., *Role of matrix metalloproteinase in wound healing.* American journal of translational research, 2022. **14**(7): p. 4391.
152. Wesley, N.O. and H.I. Maibach, *Racial (ethnic) differences in skin properties: the objective data.* American journal of clinical dermatology, 2003. **4**: p. 843-860.
153. Rawlings, A.V., *Ethnic skin types: are there differences in skin structure and function? 1.* International journal of cosmetic science, 2006. **28**(2): p. 79-93.
154. Darlenski, R. and J.W. Fluhr, *Influence of skin type, race, sex, and anatomic location on epidermal barrier function.* Clinics in dermatology, 2012. **30**(3): p. 269-273.

Appendix



Appendix 1. PBMT Device. (A) shows the device on top of a 16 well plate and (B) shows the LED array side to be exposed to the 16 well plate.

Appendix 2. Human skin biopsy sample preparation. (A) shows the biopsy, (B) shows the preparation of skin cell disks utilizing a hole punch, and (C) shows the cell disks in a 16 well plate.