The molecular and cellular effects of polarized photobiomodulation on human fibroblasts *in vitro*

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Abstract

Photobiomodulation (PBM) is a widely-used clinical therapy used to treat a myriad of different conditions, including in the treatment of wounds. Despite the utility of this therapy, the underpinning mechanisms of its biological effects remain unclear. The leading hypothesis is centred on mitochondrial cytochrome C oxidase (CcO). It posits that the photons emitted during PBM interact with CcO, in the process displacing Nitric Oxide, which allows oxygen to interact with CcO more readily, hence improving cellular metabolism. However, more contemporary research has shown that PBM can improve cellular biological functions in the absence of CcO.

Beyond, the fundamental mechanisms of PBM there also remains debate concerning the optimum light exposure and treatment protocols of PBM. Variables such as wavelength, power, irradiation time, beam area, radiant energy, fluence, polarization state, pulse parameters and treatment cycles, are all factors which can influence the outcome of PBM. Of these, polarization—the property of light that specifies the direction of the oscillating electric field—is an intriguing variable to investigate. There is a small, but growing body of research that demonstrates that polarized PBM (P-PBM), when compared to otherwise matched non-polarized PBM (NP-PBM) may increase the biological efficacy of this therapy. Despite these promising results, more research is needed to elucidate the mechanistic changes that polarization can influence in the field of PBM. Therefore, this project aims to model the molecular and cellular effects of P-PBM *in vitro*, in a cell type known to be critical in the wound healing response, namely fibroblasts. Specifically, this project will compare the biological effects of P-PBM and NP-PBM on fibroblast cells in a model which represents the oxidative stress conditions found in chronic wounds.

Firstly, in this thesis, a custom light source and stage is designed, constructed and profiled showing good intra-experiment reliability. From here the optimum light irradiation and cell culture parameters were determined through a series of pilot studies utilising multiple cellular viability, proliferation and apoptosis measurements.

Using the aforementioned protocols, the effect of P-PBM compared to NP-PBM is profiled through cellular proliferation, migration, mitochondrial membrane potential and apoptosis studies. These results showed that largely, P-PBM exerts greater cell proliferative, metabolic and protective effects, when compared to NP-PBM and appropriate controls.

Finally, the transcriptome of human dermal fibroblasts in response to PBM is profiled. This analysis demonstrated a number of differentially expressed genes related to both the

mitochondria and extracellular matrix, as well as multiple significantly enriched ontological pathways.

In sum, this project demonstrates that P-PBM in this setting, can exert a greater biological effect compared to otherwise matched NP-PBM and experimental controls, which has future applications in the treatment of wounds. Additionally, it demonstrates that PBM appears to influence multiple parts of the mitochondria, in addition to CcO, better shaping the fundamental underpinnings of PBM.

Student Declaration

I, Nicholas Tripodi, declare that the PhD thesis entitled *'The molecular and cellular effects of polarized photobiomodulation on human fibroblasts in vitro'* is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

24th January, 2023

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Preface

The work performed in this thesis was done so at Victoria University, Melbourne, Australia within the specified PhD candidature period (February 2020 – February 2023). Although there were many collaborators involved in this thesis, the candidate was the lead on all areas of project conceptualisation, design and planning, as well as data collection, manuscript, and thesis preparation. The specific roles of each person involved in collaboration is listed in the 'Details of included papers' section below, with detailed contribution roles being listed in Appendix II.

This thesis is submitted in a 'thesis with publication' format, as per Victoria University's guidelines. Therefore, each chapter is set out with its own Introduction, Methods, Results, Discussion and Conclusion section, despite not all chapters being published as a peer-reviewed journal article. Additionally, there is a context and prefacing section before each main chapter to state how each chapter fits into the thesis narrative, as well as a general introduction and discussion chapter and the beginning and end respectively.

There was no external party engaged in the preparation of this thesis, which was the sole responsibility of the candidate and the supervisory team.

Inconsistencies in chapter formatting are due to requirements of the journals to which the chapters are submitted or published.

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DETAILS OF INCLUDED PAPERS: THESIS WITH PUBLICATION

Please list details of each scholarly publication and/or manuscript included in the thesis submission. Copies of published scholarly publications and/or manuscripts submitted and/or final draft manuscripts should also be included in the thesis submission.

This table must be incorporated in the thesis before the Table of Contents.

Chapter No.	Publication Title	Publication Status • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	Publication Details • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
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4	The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: A systematic review and meta-analysis of randomized control trials	Published	Tripodi, N., Feehan, J., Husaric, M., Sidiroglou, F., & Apostolopoulos, V. (2021). The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: a systematic review and meta-analysis of randomized
7	The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: potential applications to wound healing	Published	Tripodi, N., Sidiroglou, F., Fraser, S., Husaric, M., Kiatos, D., Apostolopoulos, V., & Feehan, J. (2022). The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: Potential
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Declaration by [candidate name]: Nicholas Tripodi

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Date: 12/12/22

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List of Abbreviations

µM	Micromole
ADP	Adenosine diphosphate
AT	Achilles tendinopathy
ATP	Adenosine triphosphate
BP	Biological Process
BrdU	Bromodeoxyuridine/5-bromo-2'-deoxyuridine
CC	Cellular Component
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CcO	Cytochrome c oxidase
CCTs	Controlled clinical trials
CD	Cluster of differentiation
CI	Confidence Interval
COL1A1	collagen type-I alpha
CW	Continuous Wave
DASH	Disabilities of the arm, shoulder, and hand measure
DEGs	Differentially Expressed Genes
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EdU	ethynyl-2'-deoxyuridine
EGF	Endothelial growth factor
ESWT	Extracorporeal shock wave therapy
Exc	Exercise
FAD	Flavin adenine dinucleotide
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide
HFFF2	Human Caucasian foetal foreskin fibroblast
HILT	high-intensity laser therapy
HRQoL	Health-related quality of life
HSP	Heat shock proteins
IL	Interleukin
J	Joules
J/cm^2	Joules per centimetre square
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LD	Laser Diode
LED	Laser Emitting Diode
LED	Light emitting diode
LET	lateral elbow tendinopathy
LLLT	Low level laser therapy
MD	Mean Difference

MF	Molecular Function
mM	Milimoles
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mW	Milliwatt
NAD	Nicotinamide adenine dinucleotide
NC	Negative Control
NF-κB	nuclear factor kappa B
NGS	Next-generation sequencing
NIR	Near-infrared light
NK	Natural Killer
nm	Nanometer
NO	Nitric Oxide
NP-PBM	Non-polarized photobiomodulation
NPPBMT	Non-polarized photobiomodulation therapy
O/Intervention	Other Intervention
O2	Oxygen
O_2	Oxygen
OD	Optical density
PBM	Photobiomodulation
PBMT	Photobiomodulation therapy
PC	Positive Control
PC2	Physical Containment Level 2
PI	Propidium iodide
P-PBM	Polarized photobiomodulation
PPBMT	Polarized photobiomodulation therapy
PROMS	Patient reported outcome measures
PRTEE	Patient reported tennis elbow evaluation
PT	Patella tendinopathy
PW	Pulsed Wave
QDASH	Quick disabilities of the arm, shoulder, and hand measure
QoL	Quality of life
RCTs	Randomized controlled trials
ROS	Reactive oxygen species
RT	Rotator cuff tendinopathy
SAS	Subacromial syndrome
SCI	Spinal cord injuries
SDQ	Shoulder disability questionnaire
SMD	Standardized mean difference
SPADI	Shoulder pain and disability index
TNFα	Tumour necrosis factor-α
US	Ultrasound
USD	United States Dollars

VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
VEGF	Vascular epithelial growth factor
VISA-P	Victoria institute of sport assessment-patella tendon
W	Watt
WALT	World association for laser therapy
WST-8	(2-(2-methoxy- 4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt)
ΔΨ	Mitochondrial membrane potential

Chapter 1: General Introduction

Chronic wounds: An overview

Skin, the largest organ in the human body is susceptible to a number of chronic and debilitating conditions. Of these, there is growing concern regarding the rising burden of chronic wounds. Chronic wounds are defined as a wound that has not receded to its previous, or an acceptable level of anatomical and functional integrity within three months, or beyond the normal expected rate of tissue healing time for a given area/condition [1]. Current rises in the incidence of chronic wounds is in part due to the ageing population, as well as higher rates of important causative co-morbidities such as: diabetes, obesity, and vascular pathology [2]. It has been reported that the global incidence of chronic wounds from all aetiologies is 2.21 in every 1000 people [3], with approximately 4.5 million sufferers in the United States alone [4]. Despite these alarming numbers however, true numbers are likely higher due to difficulty in collecting this data in developing nations [3]. Given the scale of their impact, it stands to reason that its economic burden of chronic wounds is also of great concern. In the United States alone it is estimated to cost the health system roughly $20 billion USD annually [1], while in the United Kingdom, it accounts for a staggering 5.5% of total expenditure by the National Health Service [5]. In Australia, over 420,000 people suffer from chronic wounds, with a cost of more than $3 billion annually [6].

Not only are chronic wounds responsible for a heavy burden on the population at large, they cause significant personal quality of life (QoL) challenges to those afflicted. Research demonstrates that those living with chronic wounds have a significantly decreased health-related quality of life (HRQoL) compared to the general population [7]. Specifically, they report decreases in multiple HRQoL domains, including: physical role and function, bodily pain, general health, vitality, social function, mental health, and emotional role [8]. Additionally, the severity of the wound(s) appears to correlate with negative HRQoL scores [8]. It is also important to note that many individuals with chronic wounds also suffer from other co-morbidities, which further negatively affecting HRQoL. This information is vital for policy makers and clinicians alike as low QoL scores can negatively affect patient adherence to critical lifestyle interventions, and overall patient outcomes [9].

Wound healing physiology and the role of fibroblasts

Wound healing is a complex process involving many biological substrates, typically divided into four distinct phases: haemostasis, inflammation, proliferation, and tissue remodelling (Figure 1) [10-13]. Upon initial injury, vascular injury will often occur, with the body will attempt to halt the bleeding in the haemostasis phase. The affected blood vessels will immediately constrict in an effort to prevent further blood loss, through both the intrinsic and

extrinsic clotting pathways, as well as platelets activation, contribute to the clot formation process [10, 11].

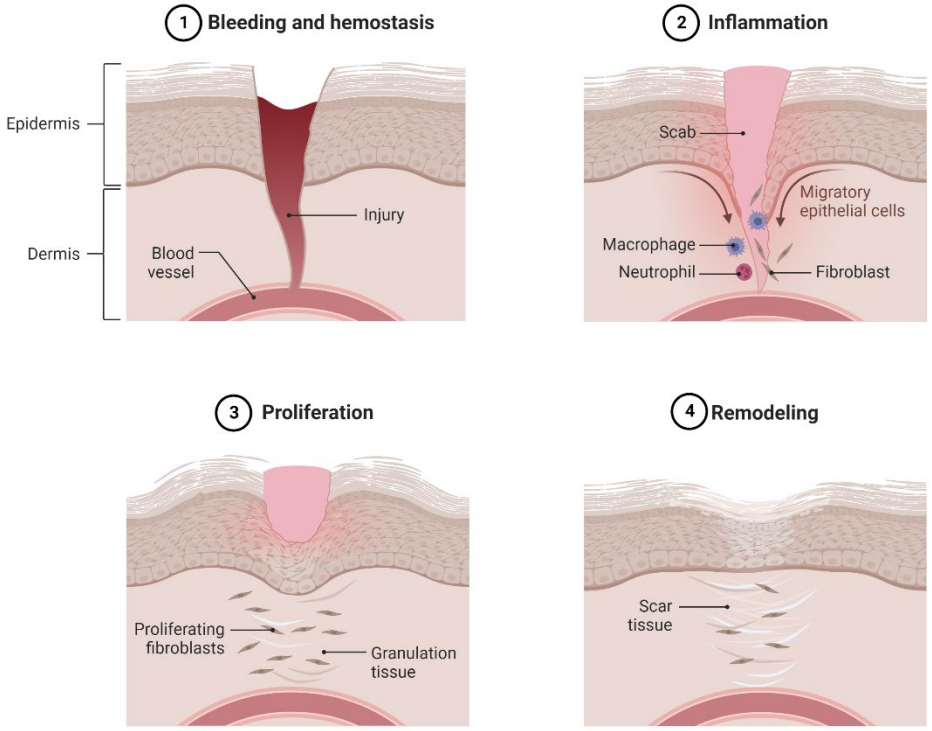

Figure 1: The four key phases of wound healing. Created with biorender.com

Next is the inflammation phase, which is initiated by various chemical signalling mechanisms induced upon tissue damage and from the haemostasis process. Firstly, neutrophils arrive at the wound site within the first hour and contribute to clearing debris and bacteria. Soon after local, and circulating, monocyte-derived macrophages assist in regulating the immune response, while also contributing to angiogenesis and formation of granulation tissue. Lymphocytes also ultimately become present, contributing to the regulation of the immune response and in assisting in the structural integrity of the healing wound [12, 14]. It is important to note that the inflammation stage will last as long as it takes to clear the foreign matter from the wound.

The proliferation phase begins once haemostasis is achieved, and most of the wound is clear from foreign matter. There are a number of simultaneously occurring processes that make up this phase. Angiogenesis—the formation of new blood vessels occurs, assisting with nutrition and delivery of necessary substances required for tissue healing [13, 15]. Fibroblast migration plays an important role in this phase, where they function to stabilise and eventually assist in closure of the wound. Epithelialisation is also an important process within this stage, where epithelial cells attempt to form a covering over the wound connecting

through to the layers below and assist in restoring the structural integrity of the area [13, 15]. Lastly in the proliferation stage comes wound retraction, which commences after approximately seven days post-injury, with the closure rate being dependent on the complexity of the wound itself. Here, is where the wound starts to close over, primarily due to the contraction of myofibroblasts. The final stage of wound healing is the remodelling stage [10, 12]. This long-lasting stage consists of the gradual formation and maturation of scar tissue and normal epithelium. Although there is a strong attempt by the body to heal the tissue to a pre-injury level, primarily by replacing type 1 collagen with type 3 collagen, most wounds will only reach up to 80% of their original tensile strength [10].

Of the many cells and growth factors that orchestrate wound healing, fibroblasts play a critical role, and are particularly important during the proliferation phase of wound healing [13, 16]. Early in the wound healing process the clot formation promotes chemotaxis of fibroblasts to the site of the injury, and trigger rapid proliferation [10, 17]. At the wound site fibroblasts function closely with the ECM [16]. Integrins—the fibroblast cell surface receptors attach to both the ECM and intracellular cytoskeleton where they cluster and form cell complexes [18]. From here the fibroblasts produce various ECM proteins, which ultimately produce collagen locally at the wound site [10, 13, 17]. This initial laying down of tissue slowly replaces the haemostatic blood clot, and is known as granulation tissue [10, 13]. This is a critical point in the process, as the collagen matrix is the main structural component of all connective tissues. Once sufficient protein is laid down, some of the fibroblasts differentiate into a myofibroblast phenotypes, and in response to sufficient mechanical tensioning, start to close the wound by actin and myosin contracture within the cells themselves [17-19]. It is also important to note that not only is mechanical tension required for wound closure, but the mechanical stimuli promotes further collagen and ECM protein production, and an inhibition of matrix metalloproteinases (MMPs), which are responsible for the degradation of various ECM proteins [17, 18]. Notably, the reverse process also occurs in the absence of sufficient mechanical tension, highlighting the importance of correct amount of tissue stress to optimise wound healing.

Wound treatments

The scale of burden that chronic wounds bring has naturally led to the development of many treatment options and protocols. Debridement—the removal of non-viable tissue—is performed early in the management of chronic wounds, and is a key part of good wound bed preparation [20]. This can be done through means such as: surgical (scissors or scalpel), mechanical (gauze, hydrotherapy, pads, etc.), autolytic (endogenous enzymes) and enzymatic (collagenase ointment) [20]. Simultaneously, it is also critical to prevent infection

and subsequent seqeulae that this can bring. This can be achieved by cleansing the wound with either saline or dilute vinegar, and following this, treating the wound with topical antimicrobial agents if indicated [20]. Once the wound bed has been prepared and sterilized, dressings are applied to the wound to aid in moisture balance and prevention of further or new infection. This commonly involves the application of gauze, ideally with moisture-retentive properties. Alternatively, clinicians may opt to employ negative pressure vacuum closure, which has strong efficacy in the treatment of chronic wounds [4, 20]. More recently, there are a number of advanced, bioengineered dressings that can be used in place of standard dressings. These agents mirror some of the structural and functional properties of human skin, to assist in the healing process. The three main classes of these agents are the epidermal, dermal, and multilayer types [4, 20]. Despite the wise use of these advanced dressings, there is the need for more research to investigate their effectiveness when compared to more traditional wound dressings [4, 20].

Photobiomodulation as a treatment for wounds

With the high cost of many novel wound healing management agents, scientists and clinicians alike have started to investigate alternative, lower-cost primary and adjunctive treatments for wound healing. Phototherapy—the use of light for therapeutic purposes—is one of these treatments. Phototherapy can be traced back to the ancient Egyptians, and their sun god Ra. It was believed that through Ra's power, by exposing themselves to direct sunlight, the worshippers could increase their energy and vitality [21]. In modern times, a wide-range of phototherapeutic devices are available to treat a variety of conditions, ranging from skin lesions to neurodegenerative diseases. There are many different forms of phototherapeutic devices available to clinicians currently, which all utilise light at various wavelengths and intensities to irradiate the target tissue(s) [22]. Of these, photobiomodulation (PBM), is the most widely-known and used [23]. PBM, formerly known as low-level laser therapy (LLLT), uses low-intensity (<1 Watt) light within the visible and/or near-infrared (most commonly red and near-infrared) to exert its effects [22, 24]. The current theories and evidence surrounding the fundamental biological mechanisms of PBM are discussed in greater detail in the subsequent chapters, but in short—further research is needed to elucidate the full spectrum of biological influence that exerts [25].

Despite the lack of clarity around the fundamental mechanisms of PBM, there exists a body of clinical work that demonstrates PBM can be effectively used as a low-cost and non-invasive therapy for a number of clinical conditions, of which wound healing is one [26]. PBM has been shown to be an effective therapy in the management of chronic wounds such as burns, venous ulcers, pressure ulcers, and diabetic foot ulcers [27], as well as for reducing

post-surgical scarring [28]. However, many of these studies were of low-quality, with inadequate controls and high heterogeneity. This is compounded by the absence of a universal set of PBM treatment standards and protocols for wound healing [26], and when combined with the debate around the fundamental mechanisms PBM, the need for more *in vitro* and clinical research into PBMs effect on wound healing is clear.

The application of PBM is applicable to the 'Goldilocks principle,' where too little energy will not create any detectible biological effect, while too much can cause negative biological effects. This is known as the biphasic dose response or Arndt-Schulz effect [24, 29]. In addition to the energy factor, there remains several under-investigated application physical properties of light to consider. These include: wavelength; irradiance; pulse structure; coherence; and polarization [29]. The polarization of light presents as an interesting variable to investigate [30]. Normally, light travels in all different orientations, however polarized light differs by being filtered, so its constituent electric and magnetic fields have a uniform orientation. Although some evidence exists showing that polarized PBM (P-PBM) may induce different or more pronounced cellular effects when compared to otherwise matched, non-polarized light [31], there remains a significant gap in the current knowledge base regarding its true effects.

Project aims, goals and thesis structure

Building on from this evidence, this project aims to model the molecular and cellular effects of P-PBM *in vitro,* in a cell type known to be critical in the wound healing response, namely fibroblasts. Specifically, this project will compare the biological effects of polarized and non-polarized PBM irradiation on fibroblast cells. This project will meet these aims across a number of coherently scaffolded chapters which will now be outlined:

- Chapter 2 is a comprehensive review of the effects that PPBM can have across *in vitro*, animal, and clinical studies, specifically when compared to non-polarized PBM (NPPBM).

- Chapter 3 is a systematic review which profiles the specific effects of all forms of PBM on human dermal fibroblasts *in vitro*, the cell line used for all experiments in this thesis.

- Chapter 4 is a systematic review and meta-analysis that investigates the clinical effect of PBM in treating tendinopathy in humans. Although separate from *in vitro*

studies, which this thesis focuses on, it is useful to investigate the clinical effects of PBM on a condition which involves a specialised fibroblast cell—the tenocyte.

- Chapter 5 and 6, using the relevant literature from chapters 2-4, details both the irradiation parameter set up and characterisation, and the cellular viability, proliferation and apoptosis pilot studies that were used to inform the methodology for the remainder of the thesis.

- Chapter 7 details how P-PBM and NP-PBM compares across a number of cellular measures which include: cellular viability, proliferation, mitochondrial membrane potential and apoptosis, and discusses the potential applications to wound healing.

- Chapter 8, the final data chapter profiles the effects of P-PBM and NP-PBM on the transcriptome of human dermal fibroblasts

- Chapter 9 and 10, the general discussion and conclusion respectively, tie all the work together, and discuss and contextualise its practical significance, limitations, and applications to future work.

Thesis significance

By improving the knowledge surrounding the fundamental mechanisms of PBM, and phototherapy more broadly, it is hoped that this thesis can contribute to a set of accepted evidence-based protocols for the *in vitro* application of PBM. Furthermore, it will contribute to transformative and cost-effective ways of treating chronic, preventable illness in Australia and abroad, which will ultimately help ease a significant economic and humanistic burden plaguing our society.

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Chapter 2: Good, better, best? The effects of polarization on Photobiomodulation Therapy

Chapter context and preface

This chapter is a literature review that focussed on identifying all the literature on monochromatic polarized PBM across *in vitro*, animal, and clinical studies. This was important as it identified the gaps in the literature and helped informed the subsequent project plan of this thesis. Although it was published as a narrative review, the systematic search strategy that was used identified all the available literature on the topic.

Chapter 2: Declaration of Co-Authorship and Co-Contribution

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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Surname: Tripodi First name: Nicholas

Institute: Institute for Health and Sport Candidate's Contribution (%): 70

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 17/2/20

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted] 2/12/22

Signature Date

3. CO-AUTHOR(S) DECLARATION

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Good, better, best? The effects of polarization on Photobiomodulation Therapy

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Abstract

Photobiomodulation Therapy (PBMT) is a widely adopted form of phototherapy used to treat many chronic conditions that effect the population at large. The exact physiological mechanisms of PBMT remain unsolved; however, the prevailing theory centres on changes in mitochondrial function. There are many irradiation parameters to consider when investigating PBMT, one of which is the state of polarization. There is some evidence to show that polarization of red and near-infrared light may promote different and/or increased biological activity when compared to otherwise identical non-polarized light. These enhanced cellular effects may also be present when the polarized light is applied linear to the tissue direction. Herein, we synthesize the current experimental and clinical evidence pertaining to polarized photobiomodulation therapy; ultimately, to better inform future research into this area of phototherapy.

Keywords

Low-level light therapy, photobiomodulation, polarized light therapy, polarization

1. Introduction

Phototherapy encompasses a broad spectrum of therapeutic modalities, all designed to bring about a positive biological effect. The earliest documented evidence of phototherapy dates back to the ancient Egyptians, who worshipped the sun god Ra. Through Ra's perceived power, the worshippers would expose themselves to direct sunlight to increase their energy levels and vitality [1]. In more recent times, a diverse group of phototherapeutic devices have been developed aimed at treating of a range of conditions, spanning from skin lesions to neurodegenerative diseases. These include: UV therapy, commonly used to treat dermatological conditions such as psoriasis, acne, vitiligo and lichen planus [2, 3]; polarized light therapy, which is used to treat musculoskeletal and dermatological conditions [4, 5]; and broad-spectrum fluorescent light-boxes, which are used to treat seasonal affective disorder [6-10]. Amongst all the phototherapies used clinically, Photobiomodulation Therapy (PBMT), appears to be the most widely used and accepted. PBMT is a system of phototherapy that uses low-intensity, non-destructive laser and/or light emitting diode (LED) to create a therapeutic effect [11]. This type of phototherapy dates back to the 1960s, and like many scientific breakthroughs, was discovered by mistake. Whilst working at Semmelwies University in Budapest, Hungary, Endre Mester assessed whether laser could cause cancer in mice. To his surprise, not only did the mice exposed to lasers not develop cancer, the experimental wound inflicted on them healed faster [11]. From this point onward, the medical application of lasers and LEDs has slowly grown, as has the evidence base [12]. PBMT has also been referred to as 'cold-laser', 'soft-laser', 'low-level laser/laser therapy' or 'biostimulation' [11, 13]. All of these use red and/or near-infrared (NIR) light commonly to create a biological effect. The known efficacious wavelengths that have been investigated range between 600nm-1000nm [12], thus spanning both red and NIR. The full mechanistic effects of PBMT are currently not clear, but its effects are known to occur at both the cellular and molecular level [14].

PBMT has been shown to be clinically effective across a range of pathologies, many of which cause a significant burden to global health services and society more broadly. Given the theorized biological effects of PBMT on cellular factors related to tissue healing, research has been completed that shows PBMT can accelerate the healing of chronic diabetic ulcers [15]. PBMT has also been shown to assist in the treatment of various dermatological conditions such as psoriasis [16], hypertrophic scars and keloids [17] and may have the capacity to modulate various acne-inducing pathways [18]. PBMT has also been used in treating conditions associated with the nervous system. Another key focus of clinical research into PBMT is that of the treatment of pain. Multiple trials have shown PBMT to be effective in promoting analgesia in patients with diagnosed neuropathic pain [19] as well as

both chronic and acute low back and neck pain [20] [21] [22]. Trials have also found PBMT to be of benefit in the treatment and management of various forms of osteoarthritis [23, 24] and tendinopathy [25, 26]. Finally, PBMT can also be applied to the sporting population. In fact, PBMT can provide immediate pain relief in sports injuries [27] and when used before exercise, can cause a significant performance improvement in both strength and endurance sports [28].

Despite plausible biological mechanisms and widespread use, there is still more research needed to better quantify the biological effects of PBMT and develop an accepted set of evidence-based guidelines for its use [14]. The application of PBMT is a delicate balance; too little energy will not create any detectible effect and too much can cause negative effects. This is known as the biphasic dose response or Arndt-Schulz effect [12, 13]. There can be a number of variables manipulated that can contribute to the summation of PBMT dosage, which include: wavelength, irradiance, pulse structure, coherence and polarization [12]. Light waves normally travel across all different planes. Light can be polarized by blocking or absorbing specific planes of light propagation, so the remaining photons travel in a specified plane or planes. There are three main types of polarization: linear polarization, where light travels in a single plane only; circular polarization, where light travels in two distinct linear planes that are perpendicular to one another; and elliptical polarization, where the light travels in an elliptical fashion, by combining two linear segments of light at different amplitudes [29]. Research suggests that linear or circular polarization may induce different or more pronounced cellular effects when compared to otherwise identical, non-polarized light, potentially being more pronounced, when polarized light aligns parallel to its target tissue [30, 31]. Currently, there is a small amount of evidence documenting the effects of polarized PBMT (PPBMT) and fewer still comparing non-polarized PBMT (NPPBMT) and PPBMT. Given that red and NIR light has the largest underpinning body of evidence, it makes sense to investigate the differences between polarized and non-polarized light within this spectrum, before expanding to polychromatic polarized light sources. Therefore, this review will synthesize the current experimental and clinical evidence surrounding narrow-band, monochromatic PPBMT (600-1000nm), ultimately to better inform this potential area of advancement within the field of PBMT, and help to inform other, broader-spectrum phototherapy research.

2. Review Methodology

Searches were conducted using CINAHL (Cumulative Index to Nursing and Allied Health Literature), MEDLINE, PUBMED, The Cochrane Library and Google Scholar. The following

search terms were used: low-level light therapy; photobiomodulation; photobiomodulation therapy; low-level laser therapy; polarization; polarized light; polarized PBMT; polarized low-level light therapy; polarized low-level laser therapy; polarized laser; polarized laser irradiation; polarized light therapy; polarized phototherapy; polarized photobiomodulation; polarized photobiomodulation (Figure 1). American and English spellings were used for all terms. Studies from all years were included. The inclusion criteria were peer reviewed original research, reviews and case studies related to the search topics. Studies that examined non-polarized light only, polychromatic light, or light outside of the 600-1000nm range were omitted. Non-English articles that were not able to be translated were excluded. Initial search identified 7590 entries. After exclusion of duplicates and conference abstract titles, an abstract analysis was used to identify potential items. Full-text analysis of all papers was performed to assess appropriateness for inclusion in this review. Reference lists of included articles were also used to locate additional relevant articles. In total 16 number of studies were found related to red and NIR PPBMT (Figure 1). No ethical approval was required for this review.

Figure 1: Summary of search strategy and paper exclusion

3. A primer on light-tissue interactions

Light is made up of packets of energy known as photons, which constantly travel at the speed of light throughout the known universe. The more photons in number, the brighter the light is. The perceived colour of light is determined by its wavelength on the electromagnetic spectrum. Visible light to humans, is generally defined as a wavelength between 400-700nm. When light interacts with living tissue, it can be absorbed, reflected or transmitted [14]. Generally, only a small amount of light is reflected from biological tissue, this is said to follow Snell's law, which describes the change in direction of a light wave as it transitions between two media. Most light however, is absorbed. Light absorption by biological tissue is characterised by the absorption coefficient (μ_a). It is also important to consider the scattering of light within tissue, which is the precursor to light absorption. Scattering is described by the scattering coefficient (μ_s). To determine total light attenuation (μ_t)—the reduction in the intensity of light due to absorption and scattering—the scattering coefficient is added to the absorption coefficient. Hence, total light attenuation is expressed as:

$$\mu_t = \mu_s + \mu_a$$

Focussing on the components of light attenuation, an 'optical window' model has been develop to explain the relatively high levels of light penetration of red and NIR light [12]. As wavelengths get closer to the blue end of the spectrum, light is absorbed and scattered more readily in biological tissue. Additionally, at wavelengths greater then 1150nm, water starts to absorb a significant amount of light energy. PBMT, demonstrated mainly for wavelengths from 600-1000nm, exploits this optical range by generating maximum light penetration and minimum light attenuation [14]. It is important to note that this optical window refers to *in vivo* applications, and may explain why otherwise wavelengths of light show positive effects *in vitro*, yet don't translate to human and animal studies. Considering polarisation in this context, it may represent a method of achieving improved light penetration in biological tissues within the 600-1000nm range.

4. PBMT mechanisms of action

As there is scant mechanistic evidence pertaining to PPBMT we will prelude this review by describing the current theoretical mechanisms of NPPBMT (Figure 2). At a cellular level, PBMT appears to interact principally with the mitochondria [32]. The functions of the mitochondria are well known and are being increasingly investigated as a source of pathology [33]. Within mammalian mitochondria, cytochrome c oxidase (CCO)—an enzyme of the mitochondrial respiratory chain, which assists in the transfer of electrons from CCO to

molecular oxygen [34]— has been shown to absorb red and NIR light, which then affects its structure and/or function [35]. This molecular photoacceptor is known as a chromophore [36]. When red and NIR light interacts with the CCO chromophore it increases its available energy and thus, increases the mitochondrial ability to generate adenosine triphosphate (ATP) [14]. The precise mechanism of how PBMT affects CCO remains unknown, but the current prevailing theory is based on the interplay between, nitric oxide (NO), oxygen and CCO [12]. It has been shown that NO competes with oxygen to interact with CCO, resulting in lowered cellular respiration and decreased ATP production [37]. Polychromatic light has been demonstrated to acutely reverse the inhibition of CCO by NO [38]. Moreover, exogenous NO has been shown to directly inhibit the functional cellular effects of PBMT *in vitro* [39]. These processes inform this mechanistic theory of PBMT whereby red and NIR light causes the dissociation of NO from CCO at a mitochondrial level, resulting in a higher rate of cellular respiration and increased ATP production [40].

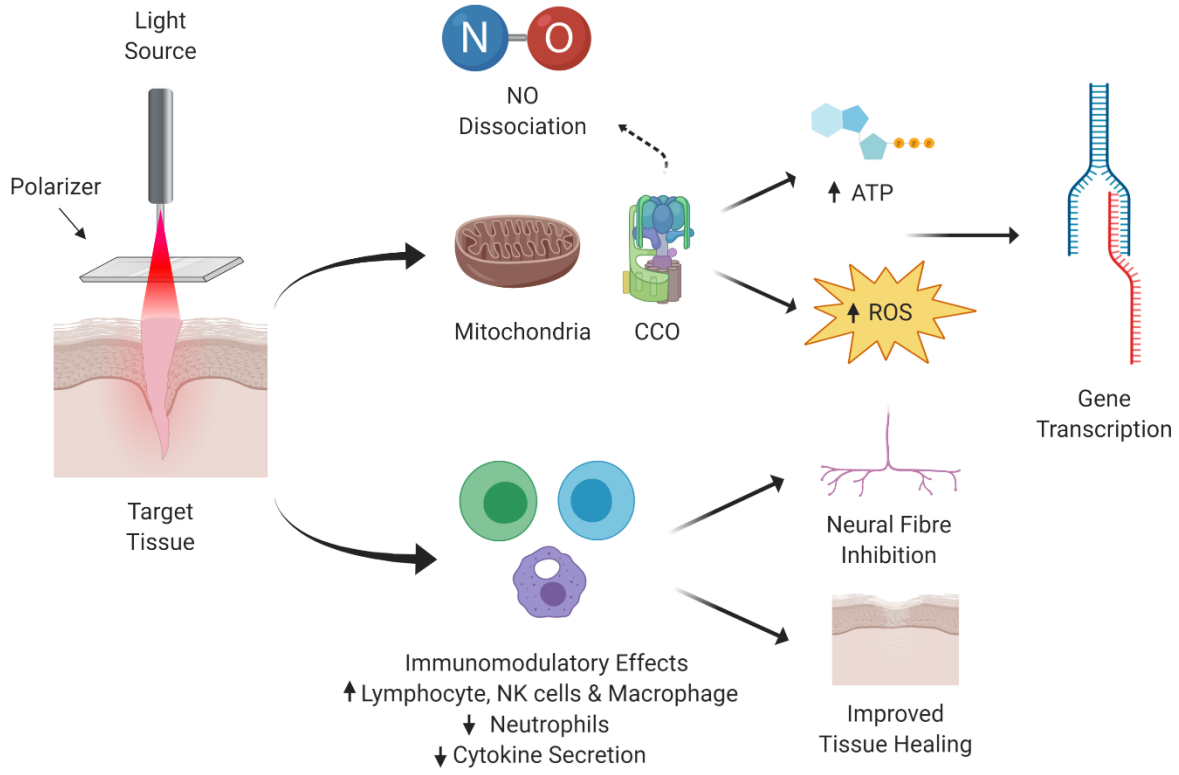

Figure 2: Proposed Mechanisms of PBMT Diagram

PBMT appears not only to affect mitochondrial function, it has also been shown to have an effect on cellular reactive oxygen species (ROS) [14]. ROS are molecules that are important in redox signalling, oxidative stress, cell signalling, enzyme activation, regulation of cell cycles, and protein synthesis [14, 41, 42]. During many cellular processes, a portion of the

oxygen metabolised is converted to ROS. PBMT promotes the metabolism of oxygen, presumably through its effects on the mitochondria, which can lead to an increase ROS production [14]. This has been demonstrated *in vitro* with PBMT changing the redox potential of a cell towards greater oxidation [43] and increasing ROS generation within the cell [44]. ROS can also activate nuclear factor kappa B (NF-κB)[45]. NF-κB is a transcription factor that can activate a number of genes, including those coded for cytokine and chemokine release, cell adhesion, cell surface receptors, anti-apoptosis and cellular proliferation[46, 47]. PBMT has been shown to increase NF-κB, presumably through the generation of ROS[45]. NF-κB is generally considered pro-inflammatory and PBMT anti-inflammatory. On face value this does not appear to compatible, however, it is proposed that both ROS and NF-κB may play a role in the dose-response relationship in PBMT. In the right amount NF-κB can cause reduced apoptosis, and increased cell proliferation and migration—responses thought to be beneficial in tissue healing [48]. Overexposure though, causes an undesired increase in ROS and NF-κB, that could potentially cause the downturn in cellular function when tissue is overexposed to PBMT [48]. More generally ROS can cause the modulation of DNA transcription and thus, may activate genes that play stimulatory or protective roles within the cell [14, 42, 47]. These changes in gene expression have been demonstrated across multiple cell lines. For example, *in vitro* experiments on fibroblasts have shown that PBMT promotes upregulation of multiple genes involved in DNA repair (MPG), inflammation (LENG5), growth and proliferation (CDK5R1) and metabolism (CANX) [49-51]. Similar changes to key genes involved in adaptation and healing have also been shown in muscle and tendon tissue *in vitro* and *in vivo* [52-57]. PBMT is also thought to play a major role in regulating the immune system by modulating many key cells affecting the immune system. Specifically, PBMT has been shown to alter M1-related cytokine and chemokine expression via mitochondrial biogenesis and histone modification [58] and to enhance proliferation of peripheral blood mononuclear cells [59]. Additionally, PBMT can cause increased macrophage proliferation and altered differentiation [60], an increase in CD45 lymphocytes and natural killer cells[61] and interestingly, a decrease in the number of neutrophils in areas of inflammation [62]. These immune changes are key mechanisms across other forms of phototherapy [4] and further, are fundamental in producing the pain suppressing effects of PBMT. PBMT is known to modulate multiple substances related to the inflammatory drivers of nociception, which include: Prostanoids (prostaglandins, leukotrienes, eicosanoids); Kinins; Serotonin; Histamine; Cytokines; Neuropeptides; ROS; and ATP [63]. Additionally, PBMT can decrease nociceptive input by inhibiting A and C neural fibres by decreasing axonal flow, thought to work in conjunction with the aforementioned molecular changes [64-66]. It is currently thought that PPBMT works via the same pathways as NPPBMT, however, these effects may be enhanced through polarization (Figure 2).

5. PPBMT *in vitro* Experiments

The effect of PPBMT has been evaluated in both connective tissue and immune cell lines with the aim of quantifying PPBMT's effect on tissue healing and the immune response. Collagen is the most abundant protein in mammals and plays a critical role in the wound healing process [67]. One study measured the effect of the polarization angle on NIH/NT3 fibroblasts. It specifically measured vascular endothelial growth factor (VEGF) secretion, differentiation to myofibroblasts and collagen organization after irradiation with a 800nm polarized light. Cells were irradiated at a 0°, 45°, 90° and 135° polarization angle for 6 minutes daily, for 6 days. This was compared against both a population that was exposed to light polarized in all orientations and a non-irradiated control. The results demonstrated increased cell viability, VEGF secretion and myofibroblast differentiation in all irradiated groups and compared to the non-irradiated control. In addition, the degree of polarization influenced collagen organization. The 0°-135° samples showed increased collagen alignment at 30° and 130°. This contrasts the 'all degree' and control sample that demonstrated peaks at 110° and 180°. However, as there was no NPPBMT sample, this study could not demonstrate a clear advantage of PPBMT [68].

Further, the effects of PPBMT and NPPBMT on Wharton's jelly derived mesenchymal stem cells was assessed. Following a 24-hour incubation period, the cells were irradiated once for 2, 4 or 6 minutes. There was a NPPBMT, PPBMT and control (non-irradiated) group. Cells that were irradiated for 6 minutes showed significantly increased levels of proliferation from the control group, however no significant difference was observed between the PPBMT and NPPBMT group. Furthermore, it was clear that cell counts and colony formation were both significantly higher after PPBMT when cells were plated at higher confluency (500 cells, per 35 mm well). However, scratch wound assays showed no significant improvement in wound closure rates in any group [69]. A limitation of this study includes that only one round of irradiation was performed; other analogous studies have shown that multiple doses of PLLLLT tend to show better outcomes compared to NPPBMT [31, 70]. Nevertheless, this study does provide evidence of some small advantage of PPBMT over NPPBMT.

In addition, the effects of PPBMT on the immune system have been studied. A study found that linearly PPBMT and NPPBMT caused an immunosuppressive effect, in terms of cellular proliferation, on human lymphocytes when compared to a halogen irradiated control sample. In addition, the immunosuppressive effect of the linear PPBMT was found to be 20% greater

than the NPPBMT sample [71]. A major limitation of this study was a lack of exact protocol reporting, making replication impossible.

Despite the previous experiments showing possible advantages of PPBMT over NPPBMT there are studies casting doubt on the increased efficacy of PPBMT over NPPBMT. One study investigated the effects of irradiating HeLa cells with linearly polarized red laser light (637nm). The experiment contained four trial groups; three groups were irradiated with a 99.4%, 60.9% and 34.2% polarization coefficient respectively, whilst a non-irritated group was used as a control. Despite the number of cells adhering to the glass surface (a measure of their biological activity) being significantly higher in the irradiated groups, there was no difference between the two experimental groups. This led to the conclusion that degree of polarization had no additional effects [72]. That said, the absence of comparison to a 0% polarization and the high exposure radiation intensity could have been confounding factors in the study.

6. PPBMT Animal Models

There have been a few studies showing positive effects of PPBMT on wound healing in animal models. One experiment measured the effects of PPBMT on the healing of artificially induced wounds in mice. The mice were irradiated with either linear or perpendicular PPBMT (632.8nm), with the angle of polarization being relative to their spinal cord. Each mouse had their own control wounds that were not irradiated. The results demonstrated that the irradiated wounds healed faster than the non-irradiated wounds and additionally, that parallel polarization caused faster and more complete healing compared to perpendicular [73]. The same research group used a similar methodology to assess collagen birefringence in skin repair in response to PPBMT (632.8nm). The results demonstrated that the wounds irradiated with parallel PPBMT with respect to the rats spinal cord showed higher birefringence, indicative of a higher degree of collagen organisation and therefore wound healing, when compared to perpendicular polarization [74]. Researchers have also studied the differences in light-tissue interaction between healthy and healing rat skin. An experiment found that in the first three days of healing, the polarized laser lost significantly more intensity when passing through the healing tissue when compared to the non-irradiated, injured control as well as healthy tissue. The authors suggested that this effect was possibly due to the large number of inflammatory cells and debris in the healing tissue [75]. A similar methodology to assess collagen birefringence in healthy rat tendons. One Achilles tendon was irradiated with PPBMT and the other no exposed to light as a control. The PPBMT was orientated parallel relative to the tendon. It was found that the irradiated

tendon exhibited enhanced collagen alignment relative to the control and the authors suggested that this effect may be applicable in the treatment of pathological tendons [76]. However, there was no comparison to non-parallel PPBMT or NPPBMT and therefore it is uncertain if the reported effects are due to the incident polarisation or PBMT more broadly.

The effects of PPBMT on healing of rabbit tissue was also noted. A comparison of parallel, perpendicular and 45-degree PPBMT relative to the wound against a non-irradiated controls was assessed. It was clear that, the fastest healing wounds were those irradiated with the parallel polarized light, followed by the perpendicular and 45 degree light respectively [70]. Despite positive results, as there were only four animals examined in this experiment, making the results less reliable - more wound models could have been used for a stronger result. PPBMT has also been shown to have an effect on the viscoelastic properties of soft tissues. A soft tissue sample was taken from the pleura of an animal and irradiated with PPBMT either perpendicular or parallel to the direction of tissue stretch. Tissue viscoelasticity was assessed via displacement sensor and stretch load cell before and after radiation. The results showed that the sample irradiated parallel to the stretch direction exhibited the greatest increase in viscoelastic capacity. The authors hypothesized that this effect could be due to changes in collagen organisation, however no direct mechanistic evidence of this was reported, nor was the type of animal sample [77].

There has also been a combined *in vivo* and *in vitro* study conducted on would healing in mice. Researchers took NIH3T3 fibroblast cells from wild mice and irradiated them with a 627nm LED device at varied intensities. The experiment used five groups: an unlit control, a non-polarized light, and three types of polarized light: linearly polarized, right circularly polarized and left circularly polarized. *In vitro*, the linearly and right circularly polarized group demonstrated the greatest cellular proliferation. The authors suggested these changes were due to an increase in the irradiation absorbance value. The most efficacious intensity was reported to be between 2 and 8 J/cm^2. *In vivo*, a full thickness skin defect of 20mm in diameter was created in mice. These wounds were irradiated using the same protocols as the *in vitro* study. It was found that the linearly and right circularly polarized light demonstrated the best healing effect at 7 days post-injury. Additionally, the right circularly polarized light promoted significantly increased expression of the type 1 procollagen mRNA compared to the control. However, there was no significant difference in type 3 procollagen mRNA expression between groups [30]. Interestingly, the authors did note a small temperature change 0.1°C per/min. The authors were confident that this small change did not influence their results, however analysis of heat-shock proteins would have been pertinent here to support this claim.

The effects of PPBMT on spinal cord injuries (SCI) have also been noted. One protocol induced an artificial spinal cord contusion using a with a weight-drop device. Before the injury site was surgically repaired the contusion was irradiated with either parallel or perpendicular PPBMT relative to the spinal cord. These rats were compared to a control group that was injured but did not receive any irradiation. The spinal cord was re-exposed and irradiated for five consecutive days. The results demonstrated that both irradiated groups recovered faster from the injury, with the parallel polarization group demonstrating a significantly better functional evaluation compared to the perpendicular group. Both irradiated groups also demonstrated a significantly smaller cavity formation induced by the contusion compared to control and that parallel polarization caused an approximate 40% greater light transmission through the spinal cord, compared to perpendicular irradiation. Interestingly, they also showed that there were no significant differences between irradiated and control groups in spinal cord ATP content. This contradicts the key proposed mechanism of PBMT in which it acts on mitochondrial synthesis of ATP, implicating other biological mechanisms at play generating a therapeutic effect. The authors hypothesized that the improved functional recovery of the parallel irradiation was due to more efficient tissue light propagation [31]. However, the light penetration was measured on a healthy rat spinal cord, limiting its application to SCI. Given that other research has found that light penetration through injured tissue is less than in healthy tissue [75], the findings would be more applicable if demonstrated on injured spinal cord tissue. All these studies demonstrate the plausible effects of PPBMT in animal wound healing but raise further questions about the underpinning mechanisms of PPBMT and the optimum dosage at different stages in healing processes.

7. Limitations

While the research above paints a thought-provoking picture of the efficacy and mechanisms of PPBMT, there remain many key limitations and questions. Firstly, there are conflicting findings pertaining to the light-tissue interactions of polarized light. Human and animal tissue exhibits anisotropic mechanical behaviour, meaning that their mechanical properties can vary in a three-dimensional space throughout the body. This is thought to be mainly due to the variation of collagen fibres in tissues [78, 79]. A key limiting factor in the transmission of light through tissues is scattering, particularly in the dermis due to collagen fibre density and its three-dimensional structure [80]. One study found the orientation of polarization that causes the least light scattering in human skin is correlated to the alignment of collagen tissue, and may have significant implications for phototherapy [80]. Another study found that in denser biological tissues, linearly polarized light is maintained better than circularly polarized light [81]. Furthermore, it has been shown that the more superficial layers of the

skin (epidermis, papillary dermis) allow penetration of polarized light with only a small amount of depolarization [82].

There is also conflicting evidence regarding the effects of PPBMT *in vitro*. One study found no change in cell function with PPBMT and have suggested that polarization does not change the efficacy of PBMT [72]. However, as this study used HeLa cells, which are not linearly cylindrical structured like collagen fibres or axons, a hypothesis might be that the morphology of a specific cell renders them susceptible to PPBMT. Polarized light penetration can also be affected by the anisotropic nature of the skin and can be depolarized after about 1mm [76]. However, evidence has shown that polarized light can penetrate healthy human skin to at least 1.2mm with only marginal depolarization [83]. Furthermore, it has been demonstrated in animal nerve tissue that PPBMT applied perpendicular to the axis of the white matter tracts caused a significant increase in light penetration when opposed to perpendicular PPBMT [84]. In an attempt to model *in vivo* circulatory conditions one study looked at the amount of depolarization through animal tissue with and without fluid flow through the tissue. The results demonstrated that polarization was largely unaffected when passing through static tissue or, when the fluid flow was parallel to the polarization direction. Polarization was partially lost when flow was perpendicular to the polarization direction and when the rate of fluid movement was increased [85]. Considering all this, in conjunction with the known effects of PPBMT in animal models [31, 70] it seems plausible that polarized light aligned parallel to cylindrical, or linear biological microstructures such as myofibrils, axons or collagen fibres [79] may represent a more efficacious method to administer PBMT. With the advancement of 3D cell culture and 3D bioprinting, the potential advantages of PPBMT may be able to be quantified *in vitro*, representing a cost saving and ethical advantage over traditional animal research. However, more *in vitro* research is required to confirm this, and to reveal whether any advantages of PPBMT found *in vitro*, would persist *in vivo*.

Secondly, most of the experiments did not compare PPBMT to NPPBMT and further, did not use a light control outside the 600-1000nm range, only a non-irradiation control. Therefore, it is impossible to confidently state whether the reported effects of PPBMT are significantly different from NPPBMT or even polychromatic, visible light sources. It is also unclear if the reported increases in efficacy are due to the increased penetration of PPBMT or if they are caused by the increase in relative irradiation intensity caused by the polarization effect. Thirdly, it remains unclear if the plane polarized light emitted by some helium-neon (he-ne) lasers is a factor to consider when interpreting the findings within this field [86]. Few, if any, PBMT research using he-ne lasers report their polarization state. Given that there is a potential biological difference caused by this effect, any future research using he-ne lasers, should report if they emit plane polarized light or not, and how that light is orientated to the

target tissue. Finally, to our knowledge, there have been no human studies conducted that compare PPBMT and NPBMT, making clinical generalization of the relative efficacy impossible based on the current evidence.

8. Conclusion

PBMT has been shown to be an efficacious system of phototherapy for treating varied common conditions that affect the population. Its proposed mechanisms are centred on increasing available ATP and changes in gene expression. The polarization of PBMT presents as an interesting variable to investigate further. Some evidence has shown when compared to NPPBMT, PPBMT can cause quicker and more organised wound healing and that it may be able to penetrate biological tissue more effectively when applied in a parallel orientation relative to the tissue being irradiated. However, more detailed mapping of cellular and molecular responses to the therapy is required to show a clear differentiation between PPBMT and NPPBMT, and other phototherapy modalities more broadly. Future research should be directed at ascertaining more detailed mechanistic evidence *in vitro* and *in vivo*, as well as comprehensively examining light-tissue interactions. Overall, PPBMT appears to be a promising advancement in phototherapy, though more research is needed to validate these claims to allow for its clinical utilization.

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Chapter 3: The effects of photobiomodulation on human dermal fibroblasts *in vitro*: A systematic review

Chapter context and preface

Once the cell line (HFFF2) to be used in this experiment was identified, a systematic review was undertaken to determine the effects of PBM on this cell type, and other human dermal fibroblast cells. The aim of this was to determine the range of effects the PBM can exert on these cells, and to inform the experimental protocols of this thesis. This publication specifically profiled the effect of PBM on human dermal fibroblasts in the domains of cellular viability, proliferation, ATP production and mitochondrial effects, and also protein and gene expression.

Chapter 3: Declaration of Co-Authorship and Co-Contribution

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Nicholas Tripodi Digitally signed by Nicholas Tripodi Date: 2020.06.10 12:16:53 +10'00' | 10.6.20

Signature **Date**

3. CO-AUTHOR(S) DECLARATION

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The effects of photobiomodulation on human dermal fibroblasts *in vitro*: A systematic review

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Abstract

Photobiomodulation (PBM) is reported to impart a range of clinical benefits, from the healing of chronic wounds to athletic performance enhancement. The increasing prevalence of this therapy conflicts with the lack of understanding concerning specific cellular mechanisms induced by PBM. Herein, we systematically explore the literature base, specifically related to PBM (within the range 600-1070nm) and its influence on dermal fibroblasts. The existing research in this field is appraised through five areas: cellular proliferation and viability; cellular migration; ATP production and mitochondrial membrane potential; cellular protein expression and synthesis; and gene expression. This review demonstrates that when fibroblasts are irradiated *in vitro* within a set range of intensities, they exhibit a multitude of positive effects related to the wound healing process. However, the development of an optimal *in vitro* framework is paramount to improve the reliability and validity of research in this field.

Keywords: *low level light therapy; photobiomodulation; cellular proliferation; cellular viability; cellular protein expression; gene expression*

1. Introduction

Chronic wounds are a significant burden to the global healthcare system, costing upwards of 30 billion USD per annum [1, 2]. In the United States alone, chronic wounds affect approximately 6.5 million people and account for up to 25 billion USD of healthcare expenditure annually [3]. With an aging population, the prevalence of chronic wounds is expected to increase [4], posing a significant challenge to healthcare systems globally. Historically, wound healing interventions have consisted of standard medical procedures such as: surgical debridement, topical antibiotics and skin substitutes (e.g. peptide coated mesh) [5, 6]. As medical technology has advanced, lesser-known and lower-cost therapies, such as phototherapy have emerged, offering novel treatments for a variety of conditions. Phototherapy has long been proposed to aid in tissue healing since its inception in the mid-20th century [7]. Since this time, laser, and more recently LED, collectively known as photobiomodulation (PBM), has been applied to a growing variety of wound and skin conditions [8]. However, the lack of an accepted set of optimal parameters has led to inconsistency in reported PBM experimental outcomes, resulting in a disparate range of procedural standards and results [9].

Wound healing is a complex physiological phenomenon the body undergoes in response to tissue damage. The interrelated and dynamic nature of tissue healing can be broken down into three fundamental phases: inflammatory, proliferative and remodelling [10]. The inflammatory phase consists of vascular and cellular cascades in response to damage. Local vasodilation results in blood and extravasated fluid entering the extracellular space, inhibiting local lymphatic function. This influx causes the cardinal signs of inflammation: pain, redness, heat and edema. Simultaneous to this influx, hemostasis begins with platelet aggregation, growth factor released and chemotaxis of immune cells (primarily neutrophils and monocytes). Following the inflammatory phase, which may continue for up to two weeks, is the proliferative stage. This stage is characterised by three specific functions: re-epithelisation (barrier creation); angiogenesis (blood vessel regeneration); and fibroplasia (formation of granulation tissue). The proliferation phase can take weeks to months to complete, after which remodeling returns the tissue to its original histological state, or as close to it as the specific injury allows. Remodelling is typified by the replacement of type III collagen for type I. Type III collagen is predominantly secreted by fibroblasts during the proliferative phase of healing, whereas type I collagen is the typical pre-injury phenotype that lends itself to greater dermis strength [11, 12]. It is important to note that while each of the tissue healing phases has a specific function, they occur in a contiguous and overlapping fashion [13].

Fibroblasts are the most abundant cell type found in connective tissue [14]. They exist in a quiescent state until stimulated by chemoattractants released by tissue damage, at which point they begin proliferating within the fibrin clot, which it degrades by secreting extracellular matrix (ECM) factors [15]. From here, when wounded, they undergo differentiation to myofibroblasts, which is initially triggered by transforming growth factor-β1-3. This differentiation is crucial as the increased actin content of the myofibroblast increases the migration and wound contracting ability of the cells [15]. Regardless of the tissue, myofibroblasts contribute significantly to the ECM via tensional forces that assist in remodelling the ECM [15]. The ECM is a key player in cell replication, influencing not only cell structure and shape, but proliferation, migration, survival and differentiation [16]. As the wound is closing and once the wound ECM has a similar tensile strength to the tissues surrounding it, they undergo apoptosis [15]. In sum, fibroblasts and the ECM directly influence each other in a symbiotic relationship, which occurs throughout all body systems [14].

PBM was initially developed by Hungarian physician Endre Mester in 1967 when he noticed an unexpected acceleration of hair regrowth whilst studying the effects that laser light exposure caused cancerous cell growth in rats [17]. Naturally, light therapy has evolved significantly since its origins and has expanded immensely in its application. PBM has been used successfully in the treatment of dermatological conditions, non-healing wounds, scarring, ulcers, musculoskeletal conditions, chronic pain, analgesia and immune modulation [18-22]. Despite its increasing use, many of the underlying physiological mechanisms of PBM remain unknown [8, 23, 24], prompting the necessity for further investigation.

The most common forms of PBM use wavelengths of 600nm to 1070nm to create a therapeutic effect [7]. At low intensities, changes observed in exposed tissues are believed to be attributable to photochemical, rather than thermal effects—hence the term ‘Cold Laser’ [19]. While the wavelength is the primary characteristic of PBM, other variables that can have an effect on its application include: fluence, polarization and pulse structure [8, 23]. While the many of the mechanisms of PBM are still unclear, there are a number of documented cellular and molecular effects. Tissues exposed to PBM have been shown to have altered mitochondrial metabolism, specifically increasing the efficiency of cytochrome C oxidase (COX), and hence, stimulating adenosine triphosphate (ATP) production and

generating reactive oxygen species (ROS) [18]. This increased efficiency is thought to occur by PBM promoting the disassociation of nitric oxide (NO) from COX, therefore allowing increased oxygen reduction [8]. These processes are thought to produce subsequent effects on gene expression, cell signalling, cell cycle regulation, enzyme activation and downstream protein synthesis [18]. Additional proposed effects include modulation of calcium, potassium and sodium ion transportation, which are vital for cellular physiology, analgesia and immunomodulation [25, 26].

2. Objectives

This review systematically examines the current evidence describing the effects of PBM on dermal fibroblasts *in vitro,* with a focus on cellular viability and proliferation, cellular migration, ATP production and mitochondrial membrane potential, protein expression and synthesis, and gene expression. We aim to collate the demonstrated photobiological effects, as well as to summate the effects of differing fluence on these changes. We also analyze the strengths and weaknesses of the existing literature, suggesting ways in which novel research can be directed, and ultimately contribute to the development of a widely accepted experimental standard for future *in vitro* PBM research, to facilitate effective clinical translation.

3. Methods

This review was conducted according to the PRISMA statement guidelines [27]. The search strategy used for this review is located in Appendix 1. Databases that were searched for in this review were: PUBMED; EMBASE; CINAHL; SCOPUS; and web of science. The search was completed in January 2020, and updated in October 2020. Studies from all years were included. Inclusion criteria were: use of low-intensity (<1 Watt) red and near-infrared PBM/LLLT (600nm-1070nm), use of *in vitro* models with human dermal fibroblasts tissue (primary or cell lines), and investigation of at least one of: cellular viability; cellular proliferation; cellular migration; ATP production; mitochondrial membrane potential; protein expression and synthesis; and gene expression**.** Studies that examined polychromatic light, or light outside of the 600-1070nm range were excluded. Non-English articles that were not able to be translated were also excluded. The initial search yielded 4,929 results. Once duplicates were removed, title and abstract screening was performed to identify appropriate studies by two of the authors, with a third resolving any conflicts. From there, full texts of included studies were assessed for eligibility by two of the authors, with a third resolving any

conflicts. There were 112 full-text articles were assessed for eligibility. Review of titles, abstracts and text led to the ultimate inclusion of 46 studies in the qualitative synthesis (Figure 1). The data extracted from the papers were cell line, irradiation parameters (light source, fluence, power, total exposures) and experimental results. Studies that reported fluence in mW/cm^2 were converted to J/cm^2, so that consistent inter-study comparisons could be made. As no risk of bias assessment for this type of *in vitro* research has been developed, none was used.

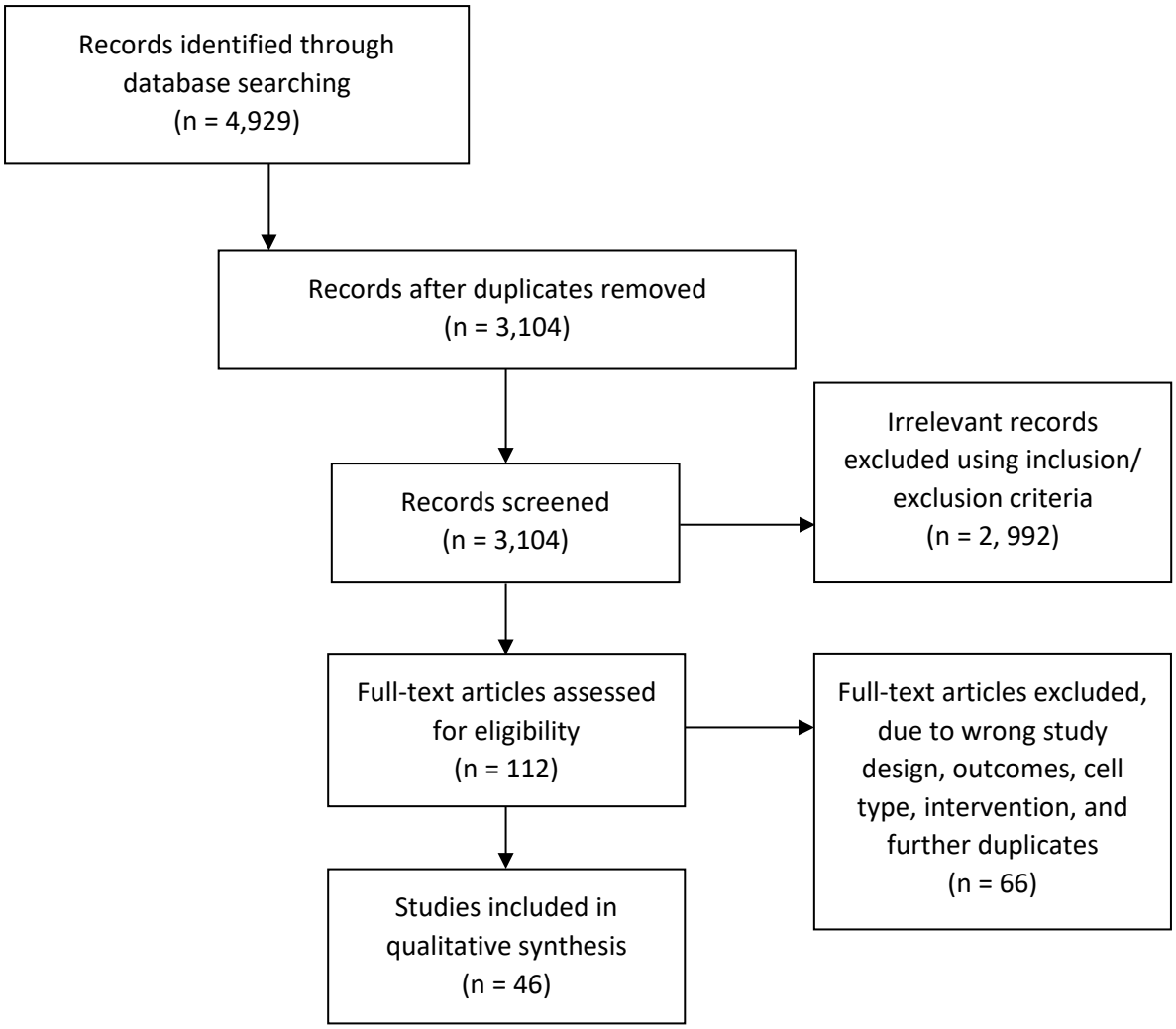

Figure 1: Literature search process according to the PRISMA guidelines

4. Results

4.1 Cellular Viability

There have been several investigations into the cellular viability and proliferation effects of PBM, using a variety of assay methods, namely: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), WST-8 and Vision Blue assays. The literature regarding optimal fluence levels for dermal fibroblasts gives a mixed picture. Doses of 0.5, 1, 5 and 5.5 J/cm^2 demonstrate increased viability when compared to non-irradiated controls [28-33], while similar fluences of 1.5, 2.5, 3, 5, 6, 10, 12, 15, 16, 20, or 25 J/cm^2 showed no change [30, 34-40], and doses of 0.5 and 10 J/cm^2 have even shown a decrease in viability [34]. Interestingly, a higher dose of 30J/cm^2 have been shown to increase viability [41], seemingly contradicting dose-response principles in PBM. This inconsistency in experimental wavelength and fluence selection casts uncertainty on the optimal parameters for PBM, and emphasizes the demand for homogeneity in application and reporting. While the existing evidence suggests PBM exposure increases cellular viability, the outcomes appear dependent on multiple variables including cell condition, wavelength, fluence and duration (Figure 2).

4.2 Cellular Proliferation

Common measures of PBM-induced cell proliferation include: Neutral Red, Trypan Blue, Bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU), ethynyl-2'-deoxyuridine (EdU), Propidium iodide (PI) and Methylene blue assays. In contrast to viability, the literature describes a clearer dose-response effect with doses of 0.45, 0.5, 0.75, 1, 2, 2.5, 3, 3.16, 3.61, 4, 5 and 10 J/cm^2 demonstrating increased proliferation [28, 29, 32, 42-53]. Unlike cellular viability, evidence regarding the positive effects of PBM on proliferation are relatively consistent. There are only two studies showing unchanged proliferation at fluences of 2.4, 2.5, and 4 J/cm^2 [44, 54], and higher doses of 10, 16 and 20 J/cm^2 show either unchanged, or decreased proliferation [38, 44, 45, 48, 49]. This suggests that proliferation may be a more sensitive and accurate measure than viability when measuring the cellular effects of PBM *in vitro* (Figure 2).

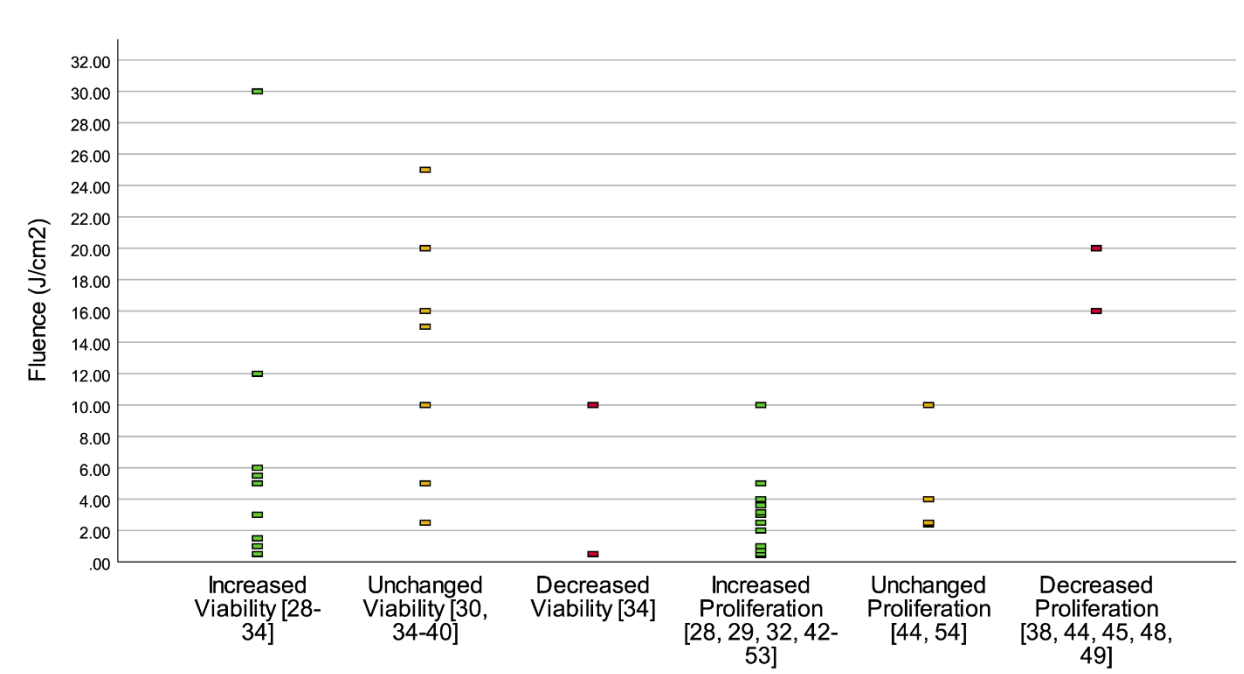

Figure 2: Graphical summary of PBM's effect on dermal fibroblast cellular viability and proliferation

4.3 Cellular Migration

The assessment of wound convergence via a scratch wound assay is an accurate and affordable measurement of cellular migration, as well as proliferation and hypertrophy, and hence, is commonly employed to determine the efficacy of interventions *in vitro* [55]. Given the *in vitro* and *in vivo* focus on effects of PBM on wound healing, wound migration studies have proven popular within PBM basic science research [42]. The literature on this aspect of PBM research illustrates that fluences of 0.5, 3 and 5 J/cm^2 appear to promote faster and more complete cellular migration measured via scratch closure [28, 30, 37, 40, 43, 45, 53, 56-58], while a higher fluence of 16 J/cm^2 caused a slowing of wound convergence compared to non-irradiated controls [30, 45, 58]. Despite existing investigations into PBM and wound migration appear positive, there is still a lack of clarity surrounding the precise parameters required to induce effective change to healing in human fibroblasts *in vitro*.

4.4 ATP Production and Mitochondrial Membrane Potential

The major proposed cellular and molecular mechanisms of PBM are centered on increases in bioavailable ATP and overall mitochondrial function [8]. Bioluminescence assays are the predominant method to assess changes in ATP level in fibroblasts and give a mixed picture of the optimum irradiation parameters for PBM in this setting. Fluences of 0.5 or 5 J/cm^2 have shown significant differences in ATP levels [44, 59, 60], while fluences of 2.5, 5, 15, or 16 J/cm^2 have shown no differences in ATP levels compared to controls [35, 44, 48, 56, 58, 59]. Contrasting this, other analogous work demonstrated that a fluence of 5, 10 or 16 J/cm^2 resulted in a decrease in ATP production [44, 48, 58, 60]. To further confuse the area, even higher doses of 45, 90 and 180 J/cm^2 have been demonstrated to cause no significant changes in ATP compared to non-irradiated controls [41], highlighting that ATP levels may not be well correlated to the PBM dose-response relationship.

Researchers have also investigated how PBM effects the mitochondrial membrane potential of dermal fibroblasts. One study described no changes to the mitochondrial membrane potential of fibroblasts under normal cell culture conditions after an irradiation of 5 J/cm^2, compared to a non-irradiated control, but showed a significant increase in mitochondrial membrane potential in wounded, hypoxic and acidotic cells exposed to the same treatment [56]. Another study found that both continuous wave (CW) and pulsed wave (PW) lasers created a dose-dependent decrease in mitochondrial membrane potential at a fluence of either 15 or 45 J/cm^2, while a fluence of 5J/cm^2 created a slight increase in in PW mode, and slight decrease in CW mode, however, neither change reached statistical significance [61]. Interestingly, another study found PBM at 3 J/cm^2 caused a decrease in mitochondrial membrane potential compared to a non-irradiated control [39]. These results provide conflicting evidence as to an optimum dose response effect in this domain, again highlighting the need for greater consistency between PBM parameters in *in vitro* experiments.

4.5 Protein Expression and Synthesis

Increased cytokine expression is characteristic of accelerated wound closure and healing post-trauma [12]. While pro-inflammatory cytokines are beneficial in the short term, persistent production may delay wound healing overall [12]. Broadly speaking, the protein expressed in response to PBM in human dermal fibroblasts can be divided into inflammatory or matrix and cytoskeleton proteins. Of the matrix and cytoskeleton proteins, a fluence of 5 J/cm^2 has been demonstrated to upregulate CD90, extra domain A fibronectin (EDA-FN), α-smooth muscle actin (α-SMA), TGF-β1, p-Smad2/3, all crucial in fibroblast differentiation [62] and has demonstrated the inhibitory effect of TIMP1 on matrix metallopeptidases (MMPs),

by simultaneously increasing TIMP1 and decreased MMP-3 and -9 [62]. Additionally 2, 2.5 or 5 J/cm^2 can increase the synthesis of epidermal growth factor (EGF) [35], basic fibroblast growth factor (bFGF) [30, 48, 58, 60, 63] and also collagen, type 1, alpha 1 [31, 64].Conversely, a higher dose of 16 J/cm^2 has been shown to decrease the production of bFGF [30, 48].

PBM has been demonstrated to influence the activity of macrophages and monocytes in their production of important cytokines such as tumor necrosis factor-α (TNFα), interleukin 6 (IL-6) and interleukin 8 (IL-8) amongst others [65]. A dose of 5J/cm^2 has been shown to decrease the amount of TNFα, interleukin 1 beta (IL-1b), while having no effect on interleukin 6 (IL-6) [51], while conversely, another study found that 3 doses of PBM at a fluence of 3 J/cm^2 significantly increased TNFα, IL-1b, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) [39]. Other studies however, found that doses of 0.5, 1, 2, and 5 J/cm^2 stimulated the release of IL-6 [29, 45, 63], with conflicting research also demonstrating that fluences of 3, 4, 5, and 16 J/cm^2 causing no change to its expression [45, 52]. One of these studies also found that fluences of 3, 4, 5 J/cm^2 did not affect the levels of IL-1b, TNFα, and IL-6, while fluences of 6 and 8 J/cm^2 upregulated these pro-inflammatory cytokines [52]. Researchers have also found that fluences as little as 0.5 J/cm2 can affect cytokines and growth factors involved in cell communication and proliferation such as BDNF and FGF 6 and 7 [40]. Interestingly though, higher irradiation levels of 45, 90 and 180 J/cm^2 have been shown to increase the expression of heat shock proteins (HSP) 27, 60, 70 and 90 [41] which commonly suggests a stress response. These studies suggest that PBM appears to modulate cellular cytokine secretion, however, inconsistency between assessment methods—mainly from differences in cell culture conditions—presents a barrier to identification of the precise immunomodulatory effects that occur following irradiation [66] (Table 1).

Protein	Stimulatory Fluence - J/cm2	Neutral Fluence - J/cm2	Inhibitory Fluence - J/cm2
HSP 27, 60, 70, 90	45, 90, 180 [41]	-	-
TGF-β1	-	5 [63]	-
pTGF-β1R1	-	5 [63]	-
p-Smad2/3	-	5 [63]	-
Thy-1 (CD90)	-	-	5 [63]
EDA-FN	5 [63]	-	-

α-SMA	5 [63]	-	-
COL1A1	5 [31, 63]	-	-
TIMP1	5 [63]	-	-
MMP3	-	-	5 [63]
MMP 9	-	-	5 [63]
EGF	5 [36]	5 [36]	
bFGF	2 [64], 2.5 [48] 5 [30, 48, 58, 60], 16 [60]		16 [30, 44]
IL-6	0.5, 1, 2 [64], 5 [29, 45], 6, 8 [52]	3, 4 [52] 5 [51, 52], 16 [45]	-
IL-1b	3[39], 6, 8 [52]	3, 4, 5 [52]	5 [29, 51]
TNF-a	3 [39], 6, 8 [52]	3, 4, 5 [52]	5 [29, 51]
NF-κB	3 [39]		
BDNF, **Eotaxin-3, FGF6, FGF7, Fractalkine, Fit3-ligand, and GCP2**	0.5 [40]		

Table 1: A summary of the known protein expression in human dermal fibroblasts by PBM

4.6 Gene Expression

As the PBM research field grows, investigations into changes in gene expression and the transcription factors that govern this expression have been at the forefront of recent studies. Multiple *in-vitro* studies have been conducted to determine the regulation of genes involved in the wound healing process in response to PBM. While many genes likely undergo changes when irradiated, much of the current literature has focused on the modulation of genes related to cell proliferation and wound healing such as collagen type-I alpha (*COL1A1*), vascular epithelial growth factor (*VEGF*), and various MMPs encoding genes. Multiple studies have shown that a dose of 0.1, 0.88, 1.5, 3, 4 or 5J/cm^2 can significantly modulate various genes related to wound healing and the ECM, in both normal, and healthy cell cultures [46, 64, 67-74], and furthermore a single study has shown modulation of similar genes at a fluence of 20 J/cm^2 [38]. Interestingly, research at a higher fluence of 45, 90 and 180 J/cm^2 has demonstrated the upregulation genes encoding for HSP 27 and 90 again

suggestive of a stress response [41]. One study has also investigated the effects of PBM on mitochondrial energy metabolism genes, demonstrating that a fluence of 5 J/cm^2 can upregulate genes related to mitochondrial complexes I, IV and V such as *NDUFA11, COX6C, ATP5F1*, reinforcing the mechanistic mitochondrial hypothesis of PBM [75]. Researchers have also investigated the effect of PBM on fibroblast genes that predispose to cancer, demonstrating that a fluence as little as 0.67J/cm^2 can affect a range of cancer genes such as *BRCA 1* and *2* [76]. While evidence appears to demonstrate an ability for PBM to alter the transcriptional profile of fibroblasts, translation of this into functional outcomes is problematic, with none of the studies investigating the downstream cellular effects of these changes (Table 2).

Fluence	Increased Gene Expression		Unchanged Gene Expression		Decreased Gene Expression		Ref
0.1 J/cm^2	*NCAM1*[#]		*COL11A*[#] *COL6A1*[#] *CD44*[#]	*MMP11*[#] *CTGF*[#]	-	-	[73]
0.67 J/cm^2	*RUNX1*[¨] *PDGFRA*[¨] *EHBP1*[¨] *GPC3*[¨] *AXIN2*[¨] *KDR*[¨] *GLMN*[¨] *MSMB*[¨] *EPHB2*[¨] *MSR1*[¨] *KIT*[¨]		*ERCC5*[#¨] *PDE11A*[#] *CD96*[#¨] *GPC3*[#] *MSG6*[#] *DKC1*[#] *TP5*[#] *HFE*[#] *NF1*[#¨] *EXT1*[#] *EPCAM*[#¨] *FANCD2*[#¨] *KIT*[#] *BUB1B*[#¨] *POLH*[#] *ESCO2*[#¨] *ANTXR2*[#] *FANCA*[¨] *MET*[¨] *BRCA2*[¨] *BARD1*[¨] *RECQL4*[¨] *FANCI*[¨] *XRCC3*[¨]	*MSH6*[¨] *PTCH2*[¨] *GALNT12*[¨] *ERCC6*[¨] *DIS3L2*[¨] *RAD51B*[¨]; *TMC6*[¨] *MSR1*[¨] *PDE 11A*[¨] *KDR*[¨] *RET*[¨] *BMPR1A*[¨] *EPHB2*[¨] *RUNX1*[¨] *PDGFRA*[¨] *EHBP1*[¨] *EPHB2*[¨] *SDHC*[¨] *TSC1*[¨] *MSR1*[¨] *ATM*[¨] *BLM*[¨] *BRIP1*[¨]	*NF1*[#] *NTRK1*[#] *MSR1*[#] *ANTXR1*[#] *ERCC5*[#] *FLCN*[#] *TP53*[#¨]	*GPC3*[¨] *TMC6*[¨] *PTCH1*[¨] *DKC1*[¨]	[76]

				BRCA1¨			
0.88 J/cm²	*CDK5R1#* *PDGFC#* *BCR#* *DAG1#* *P38Beta2#* *SRF#* *SEPW1#* *ATOX1#* *RIPK1#* *SSI-1#* *CANX#* *ZMPSTE24#* *BCAT2#* *AHCY#* *TOR1B#* *PSMB3#* *PPIH#* *APOC3#* *LYPLA2#* *NDUFB2#* *ETFB#* *ATP5H#* *ABC1#* *KCNG1#* *SCN4A#* *KCNJ13#* *DAG1#* *ARHD#* *MYH9#* *RANBP9#* *FMOD#* *TIP39#*	*CEACAM3#* *CDH12#* *OC81537#* *ADRM1#* *MPG#* *APRT#* *NUDT1#* *GCN5L1#* *GAS41#* *LOC51131#* *LENG5#* *AMSH#* *PENK#,* *GC20#* *PDE6D#* *AD-017#* *PELP1#,* *DSCR3#* *MPG#* *KERA#* *DUSP5#* *FLJ22625#* *KIAA0076#* *FKBP1A#* *MGC4251#* *YF13H12#* *FLJ20186#* *MCG13033#* *FLJ12886#* *KIAA0202#* *FUBP#* *KIAA0332#*	-	**-**	*CCNH#* *KNSL1#* *CUL1#* *HSPA1A#* *CASP6#* *STIP1#* *ELL2#* *CCT2#* *PAMCI#* *HDLBP#* *ENO3#* *ALDOA#* *NR2F2#* *CLIC4#* *ASNA1#* *ARPC2#* *LRRFIP1#* *TPM4#* *KRTHA1#* *FBN1#*	*MMP10#,* *CDH13#* *ZNF74#* *ZNF7#* *TSN#* *SEP2#* *ELF1#* *CSRP1#* *DDXL#* *PTTG1IP#* *LPP#* *YWHAB#* *RBMS2#* *PPP4R1#* *G3BP#* *PTMS#* *RES4-22#* *SERPINE 1#* *TRIP10#* *SF3B2#*	[74]
1.5 J/cm²	*COL 1#* *TGF-B#* *TIMP1#*	*TIMP2#* *IL-6#*	*COL3#*		*MMP1#* *MMP2#* *HSP70#*		[72]
4 J/cm²	*FGF#* *VEGF#* *TGF-β1#* *TGFβR1#* *TGFβR2#*	*ACTA1#* *FN1#* *DCN#* *DDR2#* *MMP2#*	*TGFβR3#*		*CTGF#*	*ELN#*	[71]

5 J/cm²	*ACTC1*[#] *ADAMTS1*[#*] *ADAMTS8*[#*¤] *ADAMTS13*[*] *ATP4B*[^] *ATP5F1*[*] *ATP5G2*[^] *CD40LG*[#] *CD44*[#*] *CDH1*[#*¤] *CFS2*[#] *CNTN1*[*¤] *COL1A1*[#*¤ ¯»] *COL4A1*[#] *COL4A2*[#] *COL4A3*[#] *COL5A1*[#*] *COL5A3*[#] *COL6A1*[#] *COL6A2*[#] *COL7A1*[*] *COL8A1*[#*] *COL11A1*[¤] *COL12A1*[#*] *COL14A1*[#¤] *COX6B2*[¤] *COX6C*[*¤] *CSF3*[#] *CTNNA1*[*] *CTNNB1*[#] *CTNND1*[#] *CTNND2*[¤] *CTSG*[#] *CTSL2*[#]	*ITGA3*[#*] *ITGA4*[#] *ITGA5*[#*] *ITGA6*[#*] *ITGA7*[*] *ITGA8*[*¤] *ITGAL*[*¤] *ITGAM*[*] *ITGAV*[#*] *ITGB2*[*] *ITGB3*[#*] *ITGB4*[*¤] *KAL1*[#*] *LAMA3*[#¤] *MMP2*[#*] *MMP3*[¤] *MMP7*[#¤] *MMP8*[*] *MMP9*[#¤] *MMP9* *MMP11*[#*¤] *MMP13*[¤] *MMP14*[#] *MMP15*[#*] *NCAM1*[#*] *NDUFA11*[*] *NDUFS7*[*] *PDGFA*[#] *PECAM1*[*] *PLAT*[#] *PLG*[#] *PPA1*[¤] *RAC1*[#] *SELE*[*¤] *SELL*[*¤] *SELP*[*]	*ADAMTS13*[#¤] *CD44*[*] *CDH1*[*¤] *CLEC3B*[#*¤] *CNTN1*[#*¤] *COL1A1*[#¤] *COL4A2*[¤] *COL6A1* *COL6A2* *COL7A1* *COL8A1*[¤] *COL11A1*[#*] *COL12A1*[¤] *COL14A1*[#*] *COL15A1*[#*¤] *COL16A1*[#*] *CTGF*[*¤] *CTNNA1*[*¤] *CTNNB1*[#*¤] *CTNND1*[*] *CTNND2*[¤] *ECM1*[¤] *HAS1*[#¤] *ICAM1*[#*¤] *ITGA1*[¤] *ITGA2*[#*] *ITGA3*[*] *ITGA4*[#*¤] *ITGA6*[#¤] *ITGA7*[#*¤] *ITGA8*[#¤] *ITGAL*[#¤]	*ITGB5*[#*¤] *ITGB5*[#] *LAMA1*[*] *LAMA2*[*¤] *LAMA3*[*] *LAMB1*[#*¤] *LAMB3*[#*] *LAMC1*[#*] *MMP3*[#*¤ ¯»] *MMP7*[*] *MMP8*[#] *MMP9*[*¤»] *MMP10*[#*¤] *MMP12*[*] *MMP13*[*] *MMP14*[*] *MMP15*[¤] *MMP16*[#*] *NCAM1*[*¤] *PECAM1*[#¤] *SELE*[#¤] *SELL*[#*] *SELP*[#¤] *SGCE*[*¤] *SPP1*[*] *TGFBI*[#*¤] *THBS1*[*] *THBS2*[¤] *THBS3*[*¤] *TIMP1*[#*] *TIMP2*[*¤] *TIMP3*[*¤] *TNC*[¤] *VCAM1*[*] *VCAN*[*¤] *VTN*[#¤]	*ACTA2*[#] *ADAMTS1*[¤] *ANGPT1*[#] *CD44*[¤] *CDH1*[#] *COL1A1*[#] *COL1A2*[#] *COL3A1*[#] *COL5A1*[#¤] *COL5A2*[#] *COL6A1*[¤] *COL6A2*[¤] *COL7A1*[¤] *COL12A1*[¤] *COL14A1* *COL16A1*[¤] *CTGF*[#*] *CTNNA1*[#] *CTNNB1*[¤] *CTNND1*[¤] *CTNND2*[#*] *CTSK*[#] *CXCL1*[#] *CXCL5*[#] *ECM1*[#*] *FGF2*[#] *FN1*[¤] *HAS1*[¤] *ICAM1*[¤] *IL1B*[#] *IL6ST*[#] *ITGA1*[¤] *ITGA2*[#¤]	*KAL1*[¤] *LAMA1*[#¤] *LAMA2*[#] *LAMB3*[¤] *LAMC1*[¤] *MAPK1*[#] *MAPK3*[#] *MIF*[#] *MMP2*[#¤] *MMP3*[#*¯] *MMP7*[#] *MMP8*[¤] *MMP9*[#*¤ ¯] *MMP1*[#*¤] *MMP12*[#¤] *MMP13*[#] *MMP14*[¤] *MMP16*[¤] *PLAU*[#] *PLAUR*[#] *PTEN*[#] *PTGS2*[#] *RHOA*[#] *SERPINE1*[#] *SGCE*[#*] *SPARC*[¤] *SPG7*[¤] *SPP1*[#¤] *TAGLN*[#] *TGFB1*[#] *THBS1*[¤] *THBS3*[#¤] *TIMP1*[#] *TIMP3*[#] *TNC*[#*]	[64, 67-70, 75]

	CXCL2[#]	*SGCE*[¤]	*ITGAM*[#¤]		*ITGA3*[¤]	*VCAM1*[#]	
	CXCL11[#]	*SPARC*[#*]	*ITGAV*[#*]		*ITGA5*[#¤]	*VCAN*[#*]	
	EGF[#]	*SPG7*[#*]	*ITGB1*[#*]		*ITGA6*[¤]	*VTN**	
	EGFR[#]	*STAT3*[#]	*ITGB2*[#*¤]		*ITGAV*[¤]	*WNT5A*[#]	
	F3[#]	*TGFA*[#]	*ITGB3*[#]		*ITGB1*[#¤]		
	F13A1[#]	*TGFBR3*[#]	*ITGB4*[#*¤]		*ITGB3*[¤]		
	FGA[#]	*THBS1*[#*]			*ITGB5*[#*]		
	FGF10[#]	*THBS2*[#*]					
	FN1[#*]	*THBS3**					
	*HAS1**	*TIMP1*[#*¤ ¯»]					
	HGF[#]	*TIMP2*[#]					
	*ICAM1**	*TNF*[#]					
	IFNG[#]	*VCAM1*[¤]					
	IGF1[#]	*VCAN*[¤]					
	IL10[#]	*VTN*[#*]					
	IL2[#]	*WISP1*[#]					
	IL4[#]						
	ITGA1[#*]						
	*ITGA2**						

Table 2: *A summary of the known gene expression in human dermal fibroblasts by PBM.* Indicates wounded cells in standard culture conditions, while ¤ indicates wounded cells in high glucose conditions. ° Indicates normal cells in high glucose cell culture conditions, while ^ indicates ischemic cell culture conditions. ¯ Indicates hypoxic wounded cells and » indicates hypoxic wounded cells in high glucose conditions. ¨ indicates that cells were taken from diabetic donors, and cultured under standard cell culture conditions. All genes demarcated # were found in standard cell culture conditions.*

5. Discussion

While a growing body of research suggests that PBM appears to be an effective intervention for accelerating wound healing, many aspects of the exact molecular and cellular

mechanisms underpinning these effects are still to be explored [8, 18]. While plausible mechanisms of PBM have been proposed, there is no current evidence describing the complete molecular and cellular effects [7]. The fundamental aim of this review was to synthesize the current evidence describing the effects of PBM on human dermal fibroblasts, particularly within five specific domains: cellular viability and proliferation; cellular migration; ATP production and mitochondrial membrane potential; protein expression and synthesis; and gene expression. Overall, it was found that in addition to a lack of clarity surrounding established physiological mechanisms, there are no evidence-based guidelines or investigational consistency regarding the optimal light parameters for investigating the biological effects of PBM *in vitro*. As the body of evidence for PBM continues to expand and evolve, several obstacles will need to be overcome to improve the consistency of research within the field. As the cost and prevalence of chronic wounds likely to increase in the future [4], the shortage of translational research with consistent methodology presents an explicit requirement for new research, as currently, animal models are left to bridge the gap from research to clinical practice.

PBM appears to be able to have dose-dependent biological effects, with stimulatory changes with lower to moderate doses, and inhibitory effects at high dose [77, 78]. It appears likely that total fluence and exposure time are associated with the efficacy of PBM [79]. On this basis, PBM appears to mostly accelerate physiological processes of fibroblasts *in vitro* that contribute to wound healing when applied at a fluence of 0.5-5 J/cm^2 [28, 29, 35, 46, 56]. Within these ranges, cellular proliferation, viability and migration appear to improve when compared to non-irradiated controls. Importantly, some of the research in this subsection fails to consider the distinction between migration, viability and proliferation, which must be explored further to determine the effectiveness of PBM interventions [80]. Having said this, these findings are generally in agreement with investigations on other cell types, suggesting a common underlying mechanism [81-87]. Furthermore, our review demonstrates that there appears to be a more defined dose-response relationship when testing PBM's effect on cellular proliferation, as opposed to viability *in vitro,* as illustrated by inconsistent cellular viability findings. Frequent intra- and inter-study inconsistencies regarding the optimum irradiation parameters required to produce a positive physiological effect may be in part responsible for these findings. Despite many experimental variables contributing to these inconsistencies in viability measurements, our findings do indicate that true proliferation assays may be better placed than viability assays to optimize *in vitro* PBM experiments before proceeding to more expansive experiments. Hence, the use of modern, sensitive and

accurate proliferation assays, such as the EdU assay, should be utilized more frequently at this phase of PBM research.

While the majority of investigations found benefit within these lower fluence ranges, there is some inconsistency in results. Some studies report a positive physiological change *in vitro* when exposed to doses as high as 180 J/cm^2 [41] casting uncertainty on the suggested inhibitory threshold of 9-10 J/cm^2 [77]. This is possibly due to the many differences in cell culture conditions, methods of assessment and light parameters, leaving the optimal fluency for positive effects unclear, and necessitates further focused research on dose-responses. In the field more broadly, detailed parameters are sometimes misreported or completely lacking. Variables including radiation spot size, target well configuration, distance from target sight, continual measurement of power output, and controlling for light scattering, amongst others, are often lacking [9]. Until all parameters are consistently reported, discrepancies in the literature both *in vitro* and *in vivo*, are likely to hamper further progress.

Changes to mitochondrial function are central to the current mechanistic theory of PBM [25]. It mostly appears irradiation up to 5 J/cm^2 can have short-term positive effects on ATP production and mitochondrial function, in the form of complex enzyme regulation and mitochondrial membrane potential [44, 59-61]. In cellular models of disease (physical trauma, hyperglycemia, acidosis and hypoxia), the optimal level of irradiation required to impact the mitochondria can change significantly or disappear entirely [59]. This finding is important, as it infers that different disease states and applications of PBM may warrant tailored application. In a clinical context, this is critical knowledge to allow the full therapeutic exploitation of PBM. Furthermore, despite indirect evidence of interactions between PBM and COX in the form of enzymatic reactions [59, 88], no research has demonstrated direct, preferential photonic interaction with COX, or any other parts of the mitochondria. Understanding the minutia of light-biomolecule interactions will open a gateway to deep mechanistic understanding of the therapeutic use of light.

Multiple investigations have shown changes to both gene and subsequent protein expression downstream as indicators of a physiological effect induced by PBM. Modulation of protein and gene expression through PBM represents an important mechanism by which the treatment may influence inflammatory cytokine production, wound healing rates, and thereby promote cellular viability, proliferation and migration [35, 46, 62, 63, 68]. Several

studies have demonstrated mixed results in modulating ECM, collagen and cytokine expression. However, the literature investigating these processes at large, exhibits vast heterogeneity of light application, leading to conflicting results amongst researchers. A fluence of up to 5J/cm^2 appears to cause upregulation in the genes associated with healing processes [46, 62, 64, 68]. However, other studies have reported similar results at fluences as high as 180 J/cm^2 [41]. Again, this contradictory evidence casts confusion on the optimal parameters required for influencing gene and protein expression. While PBM seems able to have an impact on transcription, the findings appear to lack consistency in terms of expression of specific groups of genes, or transcription factors. Furthermore, the pathways that underpin these transcriptional effects remain under investigated. Future reviews should investigate gene regulation more rigorously to provide insight into the clinical effects of the treatment. Investigating the transcription factors and cellular signalling related to gene expression may also provide a better mechanistic understanding of how the genes responsive to PBM contribute to wound healing.

Our review focused on fluence as the main variable governing the physiological effects of PBM. As such, we did not explore the effect of wavelength on fibroblast physiology: namely those between red and infrared. There is much debate within the PBM field as to whether wavelength, specifically those between 600-1070nm exert different physiological effects [8, 88]. Using the *optical window* model, near-infrared light is said to penetrate deeper than red due to decreased absorption by melanin and hemoglobin [7]. Looking at this through the lens of *in vitro* research, this seems less important given the amount of light penetration needed for this application. Having said this, some of the *in vitro* work we reviewed has demonstrated different physiological effects when comparing red light to NIR wavelengths [30, 38, 58], with other research showing a synergistic effect using multiple red and NIR wavelengths [89]. However, there has been no clear evidence to show an optimal wavelength for *in vitro* research [90]. Given that current evidence points to importance of correct fluence, as oppose to wavelength, as a determinant of successful *in vitro* work [90], a universal consensus to a set of *in vitro* experimental standards tightly controlling fluence reaching the tissue, should be developed first. Although other light variables such as wavelength, pulse structure and polarization orientation are important factors in *in vivo* research [22], improved *in vitro* experimental standards and guidelines will contribute to improved translation research, leading to advanced patient outcomes, and the wider uptake of PBM by the medical field more broadly.

6. Limitations

In an attempt to limit the impact of divergent technical variables, search criteria excluded wavelengths existing outside of the defined PBM parameters of 600nm-1070nm, possibly neglecting potentially relevant literature. The exclusion animal and non-dermal cell lines, may also have overlooked key literature, however, we felt that this would have made the review too broad.

7. Future Directions

Once a more consistent set of *in vitro* parameters has been established for PBM research more broadly, there are many innovative methods that could be used to develop more valid scientific data. Three-dimensional (3D) cell culture is an emerging method used in biomedical research that better resembles the *in vivo* environment and represents a more efficacious way of translating *in vitro* research to animal and human studies [91]. Initial studies investigating the effects of PBM on fibroblasts using a 3D collagen matrix model have demonstrated promising results. This study found increased cellular viability and upregulation in gene expression responses when exposed to a 780nm laser [92]. Furthermore, the advent of 3D bioprinting is an exciting technology that the field of phototherapy could take advantage of. This technology can allow research to produce detailed, 3D, multicellular models that may eventually supersede animal models [93]. Researchers should also consider next-generation sequencing (NGS) technology to create a more rapid and accurate representation of the genome, revealing potential modulatory effects of PBM. A significant shortcoming of NGS and its' widespread application is the significant cost associated with the technology. However, continued technological innovation is likely to make it more affordable and competitive [94]. Future research on PBM and wound healing should not only try to establish more concrete biological mechanisms behind the therapy, but also aim at developing more detailed models of photonic interactions with target cellular components.

8. Conclusion

This review found that PBM has the capacity for therapeutically significant effects on human dermal fibroblasts *in vitro*, particularly in the domains of cellular viability, proliferation and migration, ATP and mitochondrial function, as well as changes in protein and gene expression. With the research and application of PBM growing at an unprecedented rate, the development of an optimal, widely-accepted *in vitro* framework is vital to improve the validity

and consistency of the research in this field. While existing research demonstrates varied benefits, the variance in light parameters, methodological assessment methods leads to challenges in interpretation of results. The experimental standards will help to improve understanding of the precise cellular and molecular mechanisms of PBM. This therapy may offer a safe, non-invasive intervention for a variety of chronic and debilitating conditions; making it an exciting area for future research. Its clinical exploitation has the potential to relieve healthcare systems globally of huge costs relating to slow, or non-healing chronic wounds and their complications.

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Chapter 4: The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: A systematic review and meta-analysis of randomized control trials

Chapter context and preface

The effect the COVID-19 pandemic was, and remains a large burden across all aspects of society, with fundamental science research being no exception. Despite not been in the original research plan, given the uncertainty around the pandemic a decision was made to include a systematic review and meta-analysis in this thesis as a contingency for lost time in the laboratory. Given the aims of this thesis overall, the original meta-analysis was set to profile the effects of PBM on wound healing clinically, however, a study on this exact topic had just been published at the time, so the research topic changed to tendinopathy. Although not strictly related, the tenocyte, which makes up tendons, has a fibroblast lineage and hence shares many common traits, making comparisons applicable. Beyond the actual research topic, having completed and published a full systematic review and meta-analysis is a highly transferable skill which I can carry throughout the remainder of my academic career.

Chapter 4: Declaration of Co-Authorship and Co-Contribution

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted] 2/12/22

Signature Date

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

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The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: A systematic review and meta-analysis of randomized control trials

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ABSTRACT

Background: Tendinopathy is a common clinical condition that can significantly affect a person's physical function and quality of life. Despite exercise therapy being the mainstay of tendinopathy management, there are many potential adjunct therapies that remain under investigated, one of which is photobiomodulation (PBM). PBM uses varied wavelengths of light to create a biological effect. While PBM is used frequently in the management of tendinopathy, high quality evidence supporting its utility is lacking.

Methods: A systematic search of the Pubmed, CINAHL, SCOPUS, Cochrane Database, Web of Science and SPORTSDICUS databases was performed for eligible articles in August 2020. Randomized Control Trials that used red or near-infrared PBM to treat tendinopathy disorders that made comparisons with a sham or 'other' intervention were included. Pain and function data were extracted from the included studies. The data were synthesized using a random effects model. The meta-analysis was performed using the mean difference (MD) and standardized mean difference (SMD) statistics.

Results: A total of 17 trials were included (n=835). When compared solely to other interventions PBM resulted in similar decreases in pain (MD -0.09; 95% CI -0.79 to 0.61) and a smaller improvement in function (SMD -0.52; 95% CI -0.81 to -0.23). When PBM plus exercise was compared to sham treatment plus exercise, PBM demonstrated greater decreases in pain (MD 1.06; 95% CI 0.57 to 1.55) and improved function (MD 5.65; 95% CI 0.25 to 11.04). When PBM plus exercise was compared to other interventions plus exercise, no differences were noted in pain levels (MD 0.31; 95% CI -0.07 to 0.70). Most studies were judged as low-risk of bias. The outcome measures were classified as very low to moderate evidence quality according to the Grading of Recommendation, Development and Evaluation tool.

Conclusion: There is very-low-to-moderate quality evidence demonstrating that PBM has utility as a standalone and/or adjunctive therapy for tendinopathy disorders.

PROPERO registration number: CRD42020202508

KEYWORDS: Tendinopathy; Photobiomodulation; Pain; Low-level laser therapy; Meta-analysis; Systematic review

BACKGROUND

Tendinopathies represent a common presentation to clinical practice, particularly in active persons [1]. For instance, Achilles tendinopathy has been reported to occur at a rate of 2.35 per 1000 patients [2], whilst occurring between 6.2-9.5% in athletic populations [3]. Regardless of cohort, tendinopathy can profoundly affect a person's quality of life and ability to perform activities of daily living, and cause considerable economic impact [4]. Traditionally, tendon pain was known as tendinitis, referring to the pain and inflammation thought to be associated with this condition.[4] However, as research in this area advanced, it was noted that most painful tendon disorders are chronic disorders, lacking a primary inflammatory driver [5-7]. Hence, the next term that evolved to describe this disorder was tendinosis, referring to the deleterious histopathological changes that can occur within a painful tendon [5]. More contemporary research now advocates for the term tendinopathy when describing any painful tendon disorder [7, 8]. Despite the original definition being grounded in the histopathological and clinical findings [7], tendinopathy is now defined as persistent tendon pain and loss of function related to mechanical loading [8], which may be associated with radiological changes [9].

Despite extensive research efforts in recent years, the complete pathophysiological picture of tendinopathy remains poorly understood [1]. However, it is known that four key cellular changes typify tendon pathology: 1. Increased number and metabolism of tenocytes; 2. Large proteoglycan presence, causing increased water content; 3. Abnormal collagen alignment and 4. New blood vessel and nerve growth within the tendon [10]. Regardless of the exact pathophysiological mechanisms, diagnosis of tendinopathy is primarily clinical, rather than radiological [1]. Tendinopathy presents as localized tendon pain that is correlated to mechanical load, that is beyond the tendon's current capacity [8]. A clinician must pay close attention to changes in activity load and other rheumatological, metabolic and endocrine risk factors, with pain being produced during specific provocative movements, or by activities of daily living [1]. Furthermore, given the poor correlation between pain, function and histopathological radiological findings [10], and the absence of a defined nociceptive tendinopathic pathway [1], it is also important to consider the psychosocial influences of tendinopathy [1, 4, 11].

Due to the common prevalence of tendinopathy there is a large variety of treatment methodologies that have been employed, of which, exercise rehabilitation is the most well supported [1, 12, 13]. There are also a number of adjunct therapies used in the management of tendinopathy, including: Extracorporeal shock wave therapy (ESWT), Non-steroidal anti-inflammatory drugs (NSAIDs), injection therapies such as platelet rich plasma (PRP), corticosteroids (CS), and prolotherapy, transdermal application of CS through the method of

Iontophoresis, and also passive interventions such as stretching and deep friction massage [1, 13]. While some of these treatments show promise, most have been shown to be no better, or worse that exercise rehabilitation [1].

An emerging and underexplored treatment in the management of tendinopathy is photobiomodulation (PBM) [14]. While the exact physiological mechanisms underpinning PBM are yet to be fully described, the prevailing theory is based on the interplay between adenosine triphosphate (ATP), nitric oxide (NO) and cytochrome c oxidase (complex IV of the mitochondria) [15]. It is thought that both red and near-infrared (NIR) light have a high affinity for CCO [15]. During routine metabolism, or in instances of cellular stress, NO may competitively bind to CCO, displacing oxygen, slowing or limiting ATP production. PBM has been suggested to displace the NO from CCO, allowing oxygen to more freely interact with CCO, thus enhancing ATP production [15]. Despite this mechanism being widely accepted, there is no evidence to date that shows a direct photo-biological interaction with CCO [14, 16]. Additionally, there are many other secondary mechanisms by which PBM may exert its effects. These include an increased production of reactive oxygen species (ROS), which can lead to upregulations in gene transcription and downstream protein expression [14, 17], and additionally may modulate key immune cells leading to improved tissue healing and neural fibre inhibition [14, 18, 19].

At a more fundamental level, how PBM affects tendon tissue *in vitro*, and in animal models has been investigated. *In vitro* PBM appears to influence multiple mechanisms related to growth and proliferation. Specifically, PBM can increase the expression of genes related to proliferating cell nuclear antigen (PCNA) and transforming growth factor-β1 (TGF-β1) [20, 21]; Cyclins E, A, and B1 [21]; expression of genes related to type I collagen, decorin [22] and dynamin II [23], all of which are key regulators of the healing response. Interestingly, PBM has also been shown to decrease the expression of genes related to inflammation such as TNF-α [24] and IL-6 in tenocytes [25]. The positive effects of PBM have also been observed in animal models of tendinopathy, showing mild improvements in functional healing compared to non-irradiated controls [26]. However, as with many areas of study within the field of PBM, a recent review article reported that the lack of a standardized process for treating animal tendons with PBM makes comparison difficult, and its further development and standardization should be given priority [27].

The impact of PBM on tendinopathy has been appraised with reviews on specific tendinopathies such as: lateral elbow tendinopathy [28]; Achilles tendinopathy [29]; and shoulder tendinopathy [30]; all of which demonstrated mixed effects, possibly due to a lack of consistent PBM application variables between studies. There has also been a systematic

review and meta-analysis of the effects of PBM on all human tendinopathies, however it was reported in 2010, and included both randomized controlled trials (RCTs) and controlled clinical trials (CCTs) [31], and again mixed results were reported. Building on these previous works, and given the proposed universal effects of PBM, the aim of this work was to synthesize the current evidence describing the impact of low-intensity red and NIR PBM on pain and function in all tendinopathy disorders in human patients. Specifically, appraising only RCTs, we analyzed the effects of PBM on tendinopathy in three domains: Pain, PROMS and Strength.

METHODS

Protocol and Registration

This review was prospectively registered in the PROSPERO database (registration number: CRD42020202508). It was also completed and structured according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [32].

Eligibility Criteria

Studies included in this review were any randomized controlled trials that used up to a class 3B power laser, or equivalent light sources within the 600 nm – 1100 nm spectrum, to treat any diagnosed tendinopathy or tendinopathy-related disorders. Given the proposed universal effects of PBM, and the wide-ranging appraisal aim of this review, all tendinopathy and tendinopathy-related disorders were pooled. Comparisons had to be made to placebo or other clinical interventions in human adults. Further, the trials needed to report Visual Analogue Scale (VAS), validated Patient Reported Outcome Measure (PROM) data and/or changes in muscle strength. Studies were excluded if they were produced before the year 2000 given the change in both the diagnosis and understanding of tendinopathy [7] and the changes in PBM application [33] in that time. Articles unavailable in English were excluded.

Information Sources and Search Strategy

The search terms used in this review were: (Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial). The databases that were searched were: Pubmed, CINAHL, SCOPUS, Cochrane Database, Web of Science, SPORTSDiscus. This search was completed by 1st August, 2020. An updated search was performed in April 2021 and yielded no additional results. Reference lists of relevant PBM reviews were also searched. A detailed description of the search can be found in table 1 of Additional file 1.

Study Selection

The titles and abstract of all the studies yielded in the initial search were screened by two of the authors (NT and JF) for eligibility using the Covidence (Melbourne, Australia) platform. Any disagreements were resolved by a third author (MH). From here, full-text analysis was completed by the two of the authors (NT and JF) and again resolved by a third (MH). The authors of studies which reported insufficient data for the meta-analysis were contacted by email, however, were excluded if no response was given.

Risk of Bias

Two of the authors (NT and JF) assessed the included studies for bias using the Cochrane Collaboration's risk-of-bias tool [34]. Publication bias was assessed by funnel plot analysis generated by Review Manager Version 4.5 (The Cochrane Collaboration, Denmark), where there were more than 10 studies to analyze.

Data Collection Process

Data of interest was extracted individually by two of the authors (NT and JF), with any disputes or inconsistencies resolved by the addition of a third author (MH), and then reaching a consensus decision.

Data Items

The primary outcomes taken for this study were pain intensity, in the form of the VAS, validated PROMS and changes in muscle strength. Range of motion measurements were excluded as they are not considered to be a core domain of tendinopathy [35]. The secondary outcome taken was reporting of adverse effects.

Summary Measures

As the primary measurements were all reported as continuous data, VAS and PROM data were combined using the mean difference (MD) statistic, while change in muscle strength data was analyzed using the standardized mean difference (SMD) statistic (given the heterogeneity in measuring muscle strength) , using the change scores between time points. As only three of the included studies reported the SD change score [36-38], the correlation coefficient was calculated to be 0.8 based on these studies [39]. The data then underwent a sensitivity analysis comparing the meta-analysis results using a correlation coefficient of 0.2 and 0.8. As no change in the results were detected with either coefficient, the correlation coefficient of 0.8 was used for the final analysis VAS data was reported on a scale of 0-10, with data reported on a scale of 0-100 transformed to the 0-10 scale. PROM data was reported on a scale of 0-100. Studies that reported multiple VAS sub-scales (i.e. VAS rest,

VAS night, etc.) and strength testing measurements means were averaged, and their standard deviation pooled according to previously described measures [39]. Studies that reported a 95% confidence interval (CI), and not the SD, were converted to SD [39].

Synthesis of Results

Two authors (NT and JF) completed the analysis using both Microsoft Excel (Microsoft, USA) and Review Manager Version 4.5 (The Cochrane Collaboration, Denmark). A random effects meta-analysis was used to analyze the results, with the I^2 statistic being used to assess study heterogeneity. The trials were grouped according to VAS, specific PROM and strength measurements. Given the variability in design amongst the included studies, multiple subgroupings were made according to time points analyzed and comparison treatments and controls. 'End of treatment' was defined as end of a 2-4 week course of the treatment intervention, while 'Follow Up' was defined as 3 months post-treatment.

The evidence quality of each outcome was subjectively assessed using the Grading of Recommendation, Development and Evaluation (GRADE) tool [40]. Using the criteria from Tomazoni, Almeida [41], five factors and threshold criteria were used to assess the evidence quality: Risk of Bias: >25% of trials classified at high risk of bias; Inconsistency: $I^2 > 50\%$; Indirectness: > 50% of participants not related to trial's target audience; Imprecision: < 400 participants in the comparison for continuous outcomes; and Publication Bias: funnel plot if > 10 trials in same comparison [41]. The evidence quality could be categorized according to four ratings: High; Moderate; Low; and Very Low. Each time an outcome did not meet each of the threshold criteria it was downgraded one level per criteria. For example, if one measure did not meet the thresholds for risk of bias and Inconsistency it was classified as low-quality evidence, downgraded from high-quality evidence.

RESULTS

Search Summary

The detailed search strategy is shown in Table 1 of Additional file 1. The initial search strategy yielded 1230 results, after title and abstract screening of these results, 104 studies remained. When these were subjected to full-text screening 22 studies were eligible, of which 17 were included in the meta-analysis [36-38, 42-55] (Fig. 1). The five eligible, but excluded studies, were omitted due to insufficient data, which could not be obtained by contacting the authors [56-60]/ The pooled studies equated to a total of (n=835) participants.

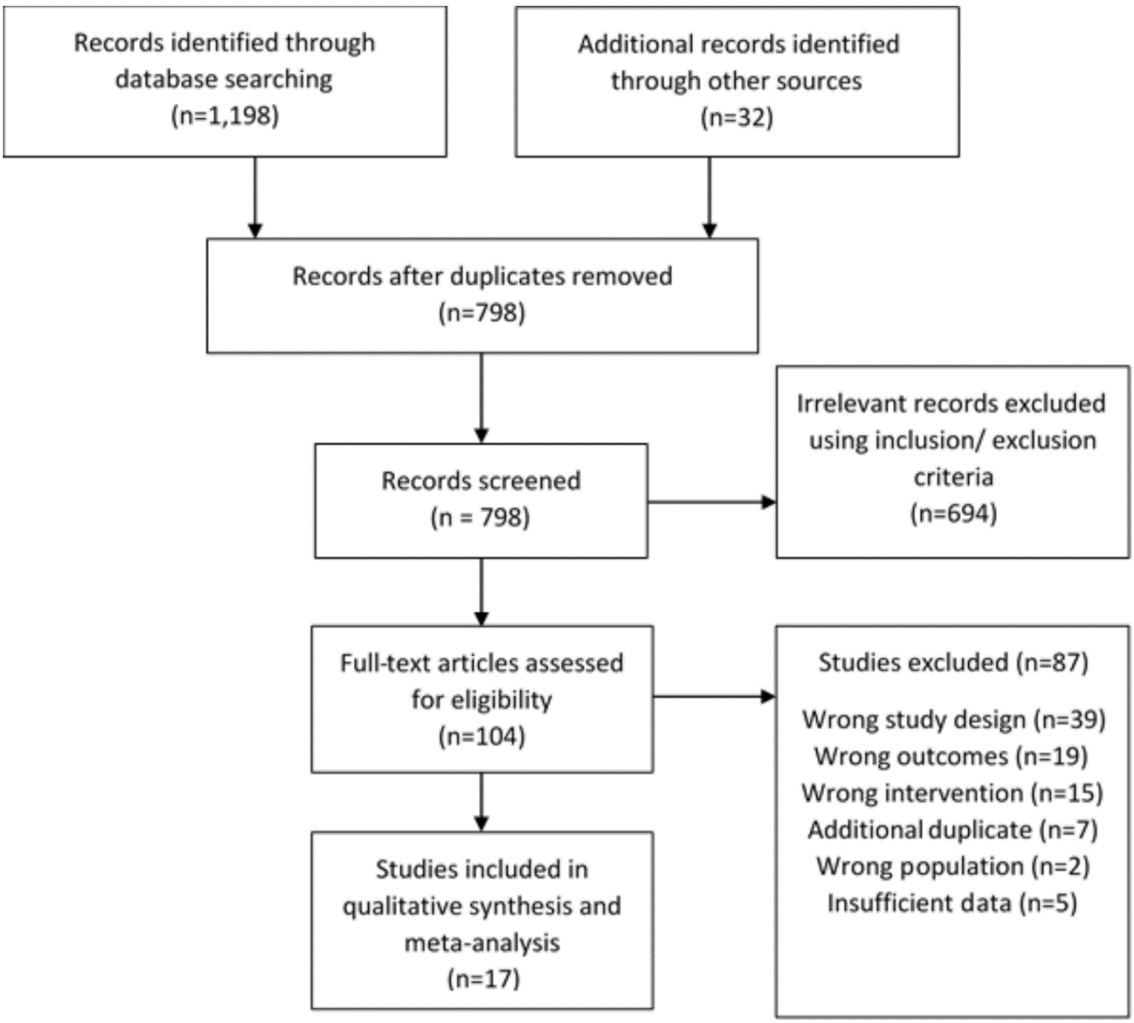


Fig. 1: *Literature search process according to the PRISMA guidelines*

Included Study Characteristics

Participant Diagnosis

Of the included studies, one investigated (n=1) Achilles Tendinopathy (AT) [53]; one investigated De Quervain's Tenosynovitis (DQT) (n=1) [51]; seven (n=7) investigated Lateral Elbow Tendinopathy (LET) [36, 43, 45, 46, 48, 50, 52]; one (n=1) investigated Patella Tendinopathy (PT) [38]; and seven (n=7) investigated Sub-acromial Syndrome/Rotator Cuff Tendinopathy (SAS/RT) [37, 42, 44, 47, 49, 54, 55] (Table 1).

Interventions

There were a wide array of PBM application variables used within the included studies. All the studies used NIR light, ranging from 0.5-5J/cm^2, and all studies irradiated multiple sites.

Additionally, there were a number of studies that did not report all necessary light application variables [36, 42, 46, 47, 49, 51, 54, 55] (Tables 1 and 2). Other comparative interventions ("other interventions") included: Phonophoresis and Iontophoresis [43]; ESWT [46]; High-Intensity Laser Therapy (HILT) [48]; Passive Physiotherapy [37]; and US [51]; with the remaining studies using exercise alone [36, 42, 50, 52, 53, 55], or exercise plus another intervention [45, 54]. Only four studies used the WALT guidelines [33] to inform their treatment protocols [36, 51, 53, 54] (Tables 1 and 2).

Outcome Measures

All the included studies used VAS as an outcome measure. Of the studies that used PROMS in their measures, four (n=4) studies used the Disabilities of the Arm, Shoulder and Hand (DASH) measure [36, 45, 50, 55]; with one (n=1) using the Quick DASH (Q-DASH) [48]; two (n=2) used the Patient Reported Tennis Elbow Evaluation (PRTEE) [36, 43]; two (n=2) used the Shoulder Disability Questionnaire (SDQ) [37, 55]; three (n=3) used the Shoulder Pain and Disability Index (SPADI) [44, 47, 49]; and one (n=1) study used the Victoria Institute of Sport Assessment-Patella Tendon (VISA-P) [38]. Due to the heterogeneous nature, and limited numbers of study interventions, only the DASH scores could be subject to meta-analysis. Additionally, there were 10 (n=10) studies that used muscle strength scores and an outcome measure [36, 38, 43, 45, 46, 48, 50-52, 55] (Table 1). Only five studies reported if any adverse effects occurred in the trial, of which there were none [42, 44, 47, 48, 55].

Risk of Bias

When pooled together the included studies were judged to a low risk of bias 68.1% of the time, an unclear risk of bias 23.5% of the time, and high risk of bias 8.4% of the time. Largely, the included studies tended to under report the randomization and blinding protocols, with some studies also failing to report all the required light parameters, hence being judged as being subject to 'other bias' (Fig. 2). Publication bias via funnel plot analysis was not completed as none of the individual forest plots contained >10 studies [34].

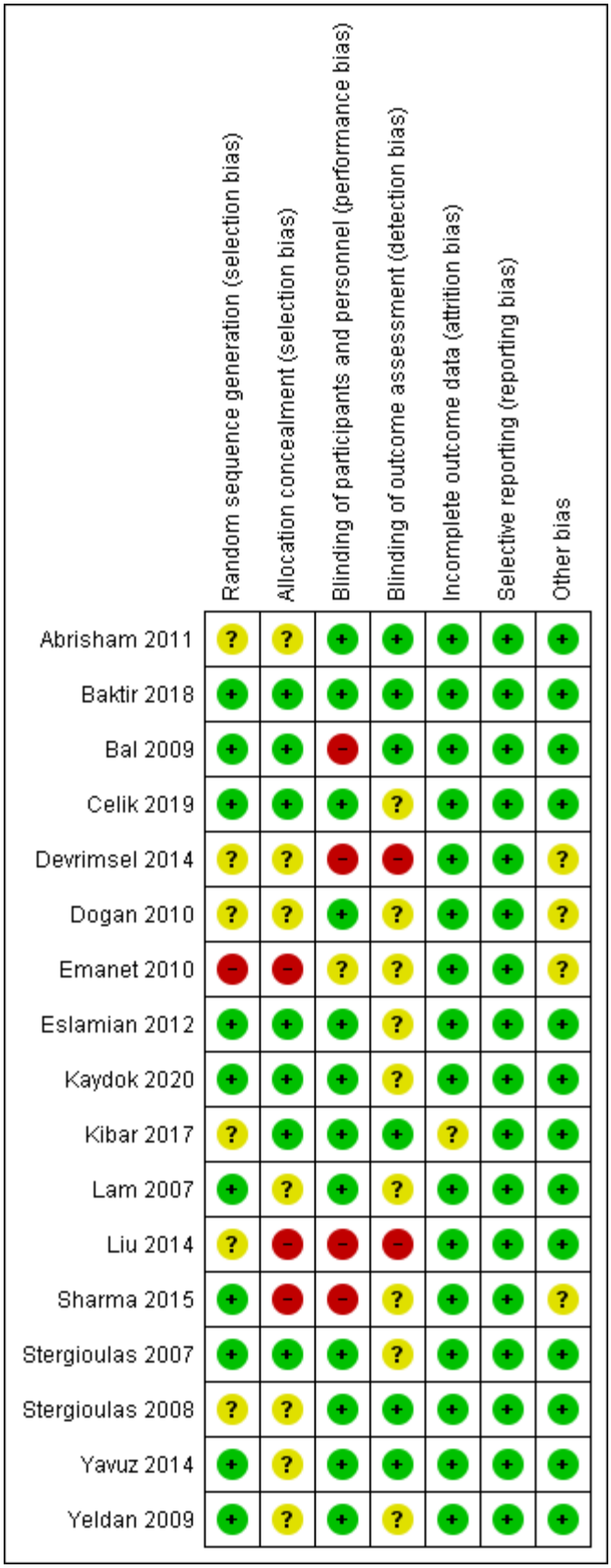


Fig 2: Risk of bias summary - review authors' judgements about each risk of bias item for each included study

VAS Measures

PBM Only versus Other Interventions Only

When compared to other interventions only (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), PBM only, demonstrated similar effects from baseline-end of treatment (MD -0.09; 95% CI --0.79 to 0.61; I^2=78%; n=105). The studies in this outcome were downgraded to very low-quality evidence due to risk of bias, inconsistency, and imprecision (Fig. 3a).

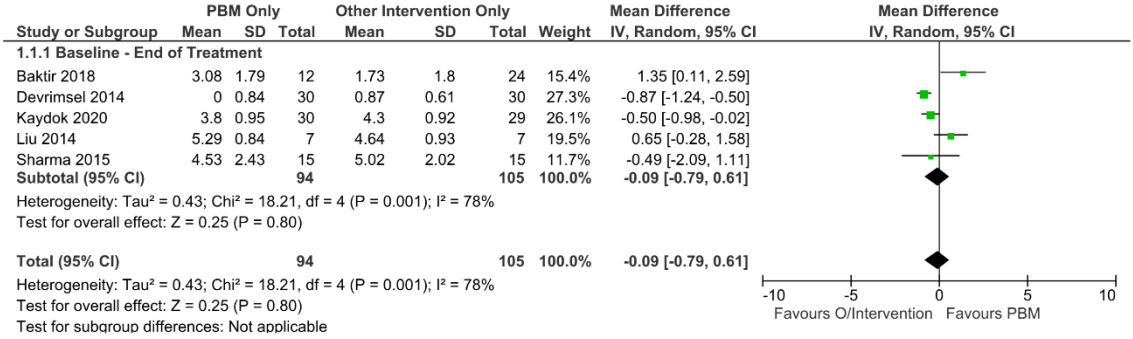
PBM plus Exercise versus Sham plus Exercise

Overall, PBM plus exercise demonstrated significant reductions in pain levels compared to sham plus exercise (MD 1.06; 95% CI 0.57 to 1.55; I^2=82%; n=224). The time period subgroup analysis showed similar results with, PBM plus exercise creating a more substantial decrease in pain at baseline-end of treatment (MD 0.96; 95% CI 0.27 to 1.64; I^2=89%; n=154), and baseline-follow up (MD 1.22; 95% CI 0.68 to 1.76; I^2=35%; n=70). There were no significant between-subgroup differences found (p=0.55). The studies in this outcome were downgraded to low-quality evidence due to inconsistency and Imprecision (Fig. 3b).

PBM plus Exercise versus Other Intervention plus Exercise

No significant difference was found between PBM plus exercise and other interventions (ESWT and US) plus exercise (MD 0.31; 95% CI -0.07 to 0.70; I^2=0%; n=70). The time period subgroup analysis demonstrated similar effects on pain within the baseline-end of treatment (MD 0.20; 95% CI -0.34 to 0.74; I^2=0%; n=35), and baseline-follow up (MD 0.43; 95% CI -0.12 to 0.97; I^2=0%; n=35) periods. There were no significant between-subgroup differences found (p=0.57). The studies in this outcome were downgraded to moderate-quality evidence due to imprecision (Fig 3c).

3a.



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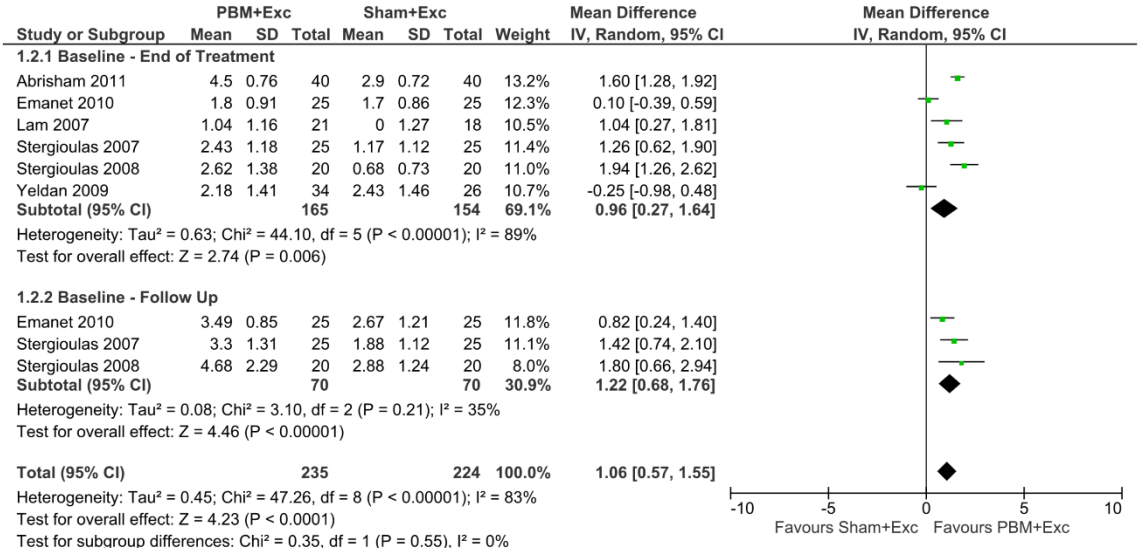

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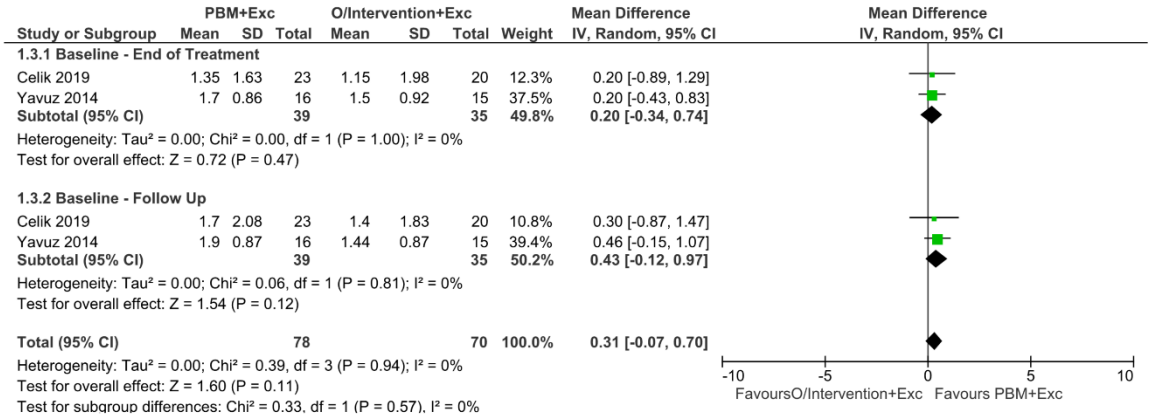

Fig. 3 - VAS: 3a: Forest plot of comparing PBM only and other interventions (O/Intervention) only; 3b: Forest plot of the effects of PBM plus exercise (Exc) versus sham treatment plus exercise; 3c: Forest plot of the effects of PBM plus exercise versus other interventions plus exercise.

PROMS

DASH: PBM plus Exercise versus Sham plus Exercise

PBM plus exercise demonstrated a significant improvement in the DASH PROM score compared to sham plus exercise (MD 5.65; 95% CI 0.25 to 11.04; I^2=78% n=112). The time period subgroup analysis showed no significant effect of PBM at baseline-end of treatment (MD 2.83; 95% CI -4.56 to 0.70; I^2=80%; n=69), while PBM plus exercise demonstrated a significant positive effect at the baseline-follow up period (MD 9.47; 95% CI 5.63 to 13.31; I^2=0%; n=43). There were no significant between-subgroup differences found (p=0.12). The studies in this outcome were downgraded to very low-quality evidence due to risk of bias, inconsistency and imprecision (Fig. 4).

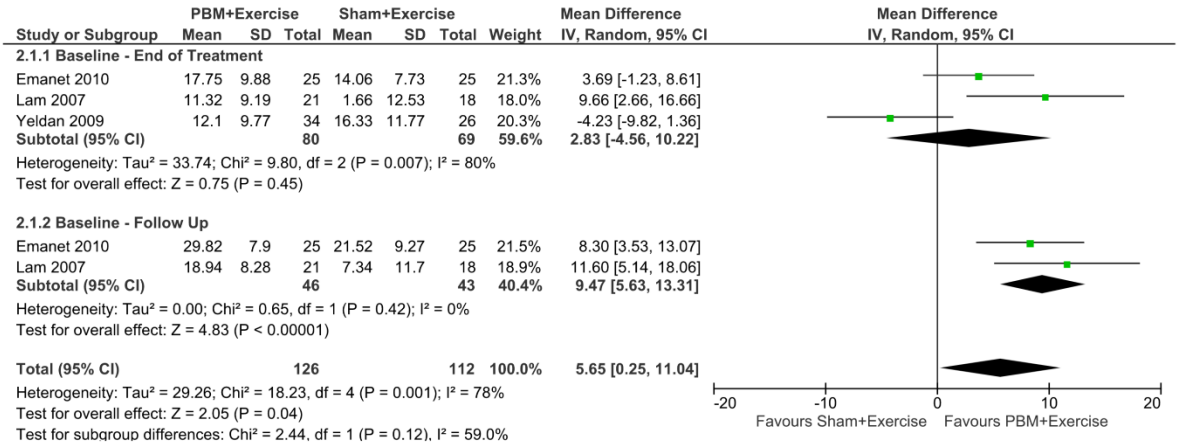

Fig. 4 - PROMS: Forest plot of comparing PBM plus exercise versus sham + exercise

Strength Measures

PBM Only versus Other Interventions Only

When compared to other interventions only (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), PBM only, demonstrated a significantly decreased effect from baseline-end of treatment (SMD -0.52; 95% CI -0.81 to -0.23; I^2=0%; n=105) (Fig. 5a). The studies in this outcome were downgraded to low-quality evidence due to risk of bias and imprecision.

PBM plus Exercise versus Sham plus Exercise

Overall, the results demonstrated that PBM plus exercise caused significant increase in strength compared to sham plus exercise (SMD 0.66; 95% CI 0.11 to 1.21; I^2=81%; n=144). The time period subgroup analysis however, demonstrated no significant effect for PBM plus

exercise on functional strength measures within both the baseline-end of treatment (SMD 0.59; 95% CI -0.13 to -1.31; I^2=83%; n=94) and baseline-follow up period (SMD 0.82; 95% CI -0.33 to 1.96; I^2=87%; n=50). There were no significant between-subgroup differences found (p=0.74). The studies in this outcome were downgraded to low-quality evidence due to Inconsistency and Imprecision (Fig. 5b).

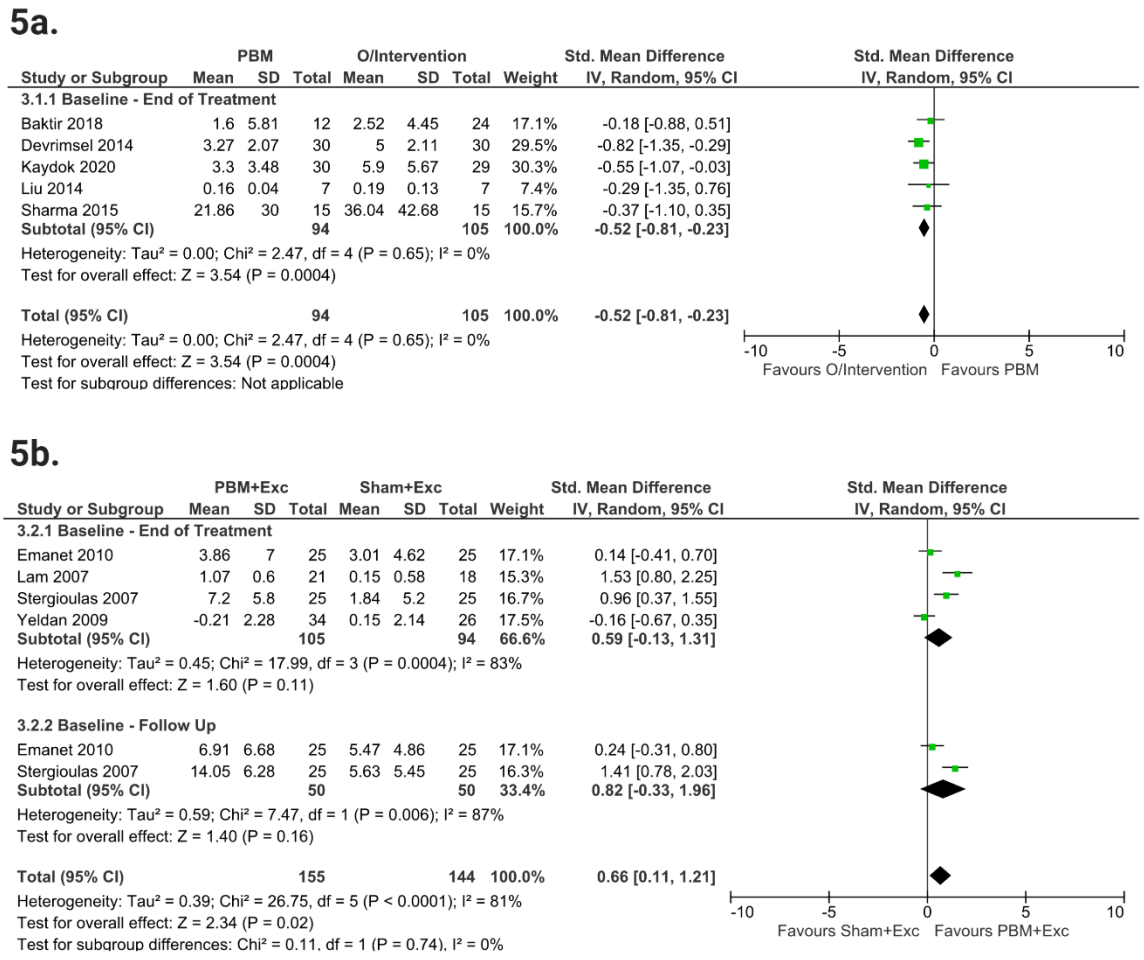

Fig. 5 - Strength Measures: 5a: Forest plot of comparing PBM only and other interventions (O/Intervention) only; 5b: Forest plot of the effects of PBM plus exercise (Exc) versus sham treatment plus exercise

GRADE Classifications

The quality of evidence classification for each outcome is located in Table 2 in Additional file 1.

DISCUSSION

The overarching aim of this review was to investigate the effect of low-intensity red and NIR PBM on pain and function in patients with tendinopathy and tendinopathy-related disorders. It was found that when compared to other interventions, with or without exercise added (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), that there is very low-moderate quality evidence to show that PBM with or without exercise were equally effective at reducing pain. This review also found very low-quality evidence demonstrating that when PBM is combined with exercise, it results in a significant improvement in PROMS compared to sham treatment plus exercise. There was also low-quality evidence demonstrating that other interventions (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US) were significantly better at improving functional strength measures compared to PBM, while when exercise was added to PBM therapy, it was significantly better at restoring functional muscle strength compared to sham treatment plus exercise.

Despite the small body of somewhat favorable evidence for PBM, as a whole, there were multiple limitations with the studies included in this review. Firstly, according to the GRADE classification system, all outcome measure assessed were classified as very low, low, or moderate quality of evidence. This was largely due to many of studies been classified as inconsistent (I^2>50%) and imprecise (< 400 participants per outcome measure) and judged to be at high risk of bias (> 25% trials are classified as high risk). Although the imprecision could be addressed with the inclusion of more studies, the fact that we were not able to assess for publication bias, as no outcomes had more the ten included trials, is something that will have to be addressed in future trials and reviews. Furthermore, 31.9% of the risk of bias variables assessed were judged to be of unknown or high-risk of bias, which should be taken into account when interpreting the results of this review.

It is well documented throughout the literature that the inconsistent nature of PBM experiments, both clinical [41, 61] and *in vitro* [14], are a significant hurdle in establishing both a concrete physiological mechanism, and a widely used and accepted set of clinical implementation guidelines. Appraising the studies included in this review, we see many differing forms of PBM application, including total number of treatments, treatment sites, and irradiation per site. This is understandable given they are treating different areas of tendon pathology, however, there were some studies that did not report all the required treatment variables [36, 42, 46, 47, 49, 51, 54, 55], making exact replication challenging, in the process affecting the quality of evidence. The WALT (World Association for Laser Therapy) recommendations are a set of therapeutic recommendations for clinical and scientific application of red and NIR spectrum PBM [33]. Only four of the trials in this review

referenced the WALT recommendations in their study design [36, 51, 53, 54], further underlining the need for higher levels of inter-study consistency.

Heavy strength and plyometric training, in addition to training load management, appear to be the most efficacious exercise modalities to employ during tendinopathy management [1]. This review demonstrated very low-quality evidence that PBM could be used as an adjunct therapy to enhance the effects of exercise rehabilitation. That said, a limitation of this analysis was that all the exercise modalities from each study were pooled in each outcome measure, hence different exercise prescriptions may have affected the results. Future research in this area should more stringently control the exercise prescription groups in line with tendinopathy best practice. Interestingly, this review also found that when compared to other interventions, PBM was equally as effective at decreasing pain, however, this was again limited by the pooling of all other interventions. Many of the other interventions that used a pharmacological anti-inflammatory agent, such as Phonophoresis, Iontophoresis and CS Injection, can cause unwanted patient side effects [62]. In fact, it is now recommended that practitioners move away from these methods, CS injections in particular, due to the long-term deleterious tissue effects they can have [62]. In light of this, PBM may represent a non-invasive, cost effective and safe alternative to the more traditional injection and anti-inflammatory based therapies used in tendinopathy management. However, more robust trials are needed to elucidate this effect.

To our knowledge only one other systematic review and meta-analysis has been performed on the effect of PBM on all tendinopathies previously [31]. This review demonstrated similar mixed results concerning the effects of PBM on pain and function in tendinopathy and similar issues with evidence quality to the present review, despite having fewer studies available for analysis. Tendinopathy specific systematic review and meta-analyses have been conducted for shoulder[30] and Achilles tendinopathy [29] and similarly to this review, found a mixed efficacy of PBM underpinned by trials of moderate-very low evidence. Taking these findings together, it is clear that more widespread and robust RCTs are needed to better inform the use of PBM in tendinopathy management.

The strengths of this review include a detailed search of multiple databases, as well as additional searches of paper reference lists. Further, two of the authors performed the entire search process and the risk of bias and GRADE categorization, with a third author resolving any disputes. Another limitation of this study was the fact that all tendinopathies were pooled together as a single diagnostic entity. Hence, the analysis may not have accounted for the heterogeneity of tendinopathy disorders. However, the analysis appeared to indicate similar effects of PBM, regardless of specific diagnosis. More specific-tendinopathy RCTs are

needed to underpin more robust single-tendinopathy systematic reviews and meta-analyses. Additionally, the exclusion of multiple studies whose required statistics were unobtainable from either the paper, or the contact authors may have changed the study results. As previously stated, the future research focus of PBM for the management of tendinopathy should be set on performing repeated robust RCTs that adequately report and justify all treatment parameters and follow the Consolidated Standard of Reporting Trials (CONSORT) guidelines. This will firstly better elucidate if PBM is an effective standalone and/or adjunct therapy for PBM, and secondly if high-quality evidence is found for this effect, it will underpin improved treatment guidelines, potentially translating to improved patient health outcomes.

CONCLUSION

PBM is an increasingly used treatment modality for a range of musculoskeletal disorders, however, there are many questions regarding its mechanisms and true effectiveness that remain under-investigated and unanswered. Currently, there is very-low-to-moderate quality evidence that low-intensity red and NIR PBM is an effective standalone and exercise-adjunctive treatment for tendinopathy disorders in humans. Further, a similar quality of evidence demonstrates that it may have utility as a less-invasive and more risk-averse adjunctive treatment to more traditional passive interventions. More robust RCTs that adhere to the CONSORT guidelines need to be performed to further elucidate its effectiveness.

TABLES

Table 1: Characteristics of included studies

Study First Author, Year	Diagnosis	Total Participants; Participants per group	Intervention Groups	Outcomes Extracted	Treatment Time	Measurement Time Points
Abrisham 2011 [42]	SAS	80; 40/40	PBM + Exercise, Sham; Laser + Exercise	VAS	Two weeks	1. Baseline; 2. Two weeks
Baktir 2018 [43]	LET	37; 12/13/13	PBM; Phonophoresis; Iontophoresis	VAS; PRTEE-t	Three weeks	1. Baseline; 2. Two Weeks
Bal 2009 [44]	SAS	44; 22/22	PBM + Exercise; Exercise Only	VAS; SPADI-t	Two weeks	1. Baseline; 2. Two weeks; 3. Three month follow up
Celik 2019 [45]	LET	43; 23/22	PBM + Exercise; ESWT + Exercise	VAS; DASH	Four weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Devrimsel 2014 [46]	LET	60; 30/30	PBM; ESWT	VAS	Four weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Dogan 2010 [47]	SAS	52; 30/22	PBM + Exercise; Sham PBM + Exercise	VAS; SAPDI-t	Three weeks	1. Baseline; 2. Three weeks
Emanet 2010 [36]	LET	50; 25/25	PBM + Exercise; Sham PBM + Exercise	VAS; DASH; PRETEE-t	Three weeks	1. Baseline; 2. Three weeks; 3. Three month follow up
Eslamian 2012 [37]	RT	50; 25/25	PBM + Passive Physiotherapy; Sham PBM + Passive Physiotherapy	VAS; SDQ	Three weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Kaydok 2020 [48]	LET	59; 30/29	PBM + HILT	VAS; QDASH	Three weeks	1. Baseline; 2. Three weeks
Kibar 2017 [49]	SAS	62; 30/32	PBM; Sham PBM	VAS; SAPDI-t	Three weeks	1. Baseline; 2. Three weeks
Lam 2007 [50]	LET	39; 21/18	PBM + Exercise; Sham + Exercise Only	VAS; DASH	Three weeks	1. Baseline; 2. Three weeks
Liu 2014 [38]	PT	21; 7/7/7	PBM; Exercise Only; PBM + Exercise	VAS; VISA-P	Four Weeks	1. Baseline; 2. Four weeks
Sharma 2015 [51]	DQT	30; 15/15	PBM; US	VAS	Two Weeks	1. Baseline; 2. Two weeks
Stergioulas 2007 [52]	LET	50; 20/20	PBM + Exercise; Sham + Exercise	VAS	Four and Eight Weeks	1. Baseline; 2. Eight weeks; 3. Two month follow up

Stergioulas 2008 [53]	AT	40; 20/20	PBM + Exercise; Sham + Exercise	VAS	Four and Eight Weeks	1. Baseline; 2. Four weeks; 3. Eight Weeks; 4. Three month follow up
Yavuz 2014 [54]	SAS	31; 16/15	PBM + Exercise; US + Exercise	VAS; SPADI-D	Four Weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Yeldan, 2009 [55]	SAS	60; 34/26	PBM + Exercise; Sham PBM + Exercise	VAS; DASH; SDQ	Three Weeks	1. Baseline; 2. Three weeks

Table 2: PBM variables of included studies

Study First Author, Year	PBM light source; Wavelength	Light source power output during treatment (mW)	Fluence per spot (J/cm^2)	Treatment spots	PBM sessions per week; Total PBM sessions	WALT recommendations informed trial?
Abrisham 2011 [42]	'Laser Device;' 890nm	Not Reported	2-4	3	5; 10	No
Baktir 2018 [43]	GaAs Laser Diode; 904nm	0.12	Not Reported	5	5; 15	No
Bal 2009 [44]	GaAs Laser Diode; 904nm	13.2	2	4	5;10	No
Celik 2019 [45]	GaAs Laser Diode; 904nm	40	2.4	6	3;12	No
Devrimsel 2014 [46]	'Laser;' 850nm	Not Reported	Not Reported	Not Reported	2; 10	No
Dogan 2010[47]	GaAlAs; 850nm	Not Reported	5	5-6	4-5; 14	No
Emanet 2010 [36]	GaAs Laser; 905nm	Not Reported	1	2	5; 15	Yes
Eslamian 2012 [37]	Ga-Al-As Laser Diode; 850nm	100	4	Up to 10	3; 9	No
Kaydok 2020 [48]	Ga-Al-As Laser Diode; 904nm	240	2-4	6	3; 9	No
Kibar 2017 [49]	Ga-Al-As Laser Diode; 850nm	Not Reported	4	11	3; 9	No
Lam 2007 [50]	Ga-Al-As Laser Diode; 904nm	25	2.4	Average 2.4	3; 9	No
Liu 2014 [38]	Ga-Al-As Laser Diode; 810nm	200	Not Reported	3	6; 24	No
Sharma 2015 [51]	Ga-Al-As Laser Diode; 830nm	30-40	3	Not Reported	3-4; 7	Yes
Stergioulas 2007 [52]	Ga-As; 904 nm	40	2.4	6	1-2; 12	No
Stergioulas 2008 [53]	Ga-Al-As Laser Diode; 820nm	30	0.5	6	1-2; 12	Yes
Yavuz 2014 [54]	Ga-Al-As Laser Diode; 850nm	Not Reported	3	5 maximum	2-3; 10	Yes
Yeldan, 2009 [55]	GaAs; 904nm	Not Reported	Not Reported	5 Maximum	Not Reported	No

Additional File, Table 1: Review Search Strategy and Results

Database	Search Strategy	Number of Results
Pubmed	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	203
CINAHL	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	97
SCOPUS	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	482
Cochrane Database	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	5
Web of Science	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	353
SPORTSDiscus	(Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial)	58
Other Sources: Searching relevant PBM review references lists	N/A	32
Total		**1,230**

Additional File, Table 2: GRADE Classifications

Grade Criteria

- Risk of Bias : Yes if >25% trials are classified as high risk
- Inconsistency: Yes if I^2 >50%
- Indirectness Yes if >50% of participants not related to trial's target audience
- Imprecision: Yes if <400 participants in the comparison for continuous outcomes
- Publication Bias: Yes if funnel plot if >10 trials in same comparison

Overall Quality Criteria

- High: 0 Yes responses
- Moderate 1 Yes response
- Low: 2 Yes responses
- Very Low: 3 or more Yes responses

Outcome	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication Bias	Overall Quality
VAS: PBM vs. Other Intervention	Yes	Yes	No	Yes	No	Very Low
VAS: PBM + Exercise vs. Sham + Exercise	No	Yes	No	Yes	No	Low
VAS: PBM + Exercise vs. Other Intervention + Exercise	No	No	No	Yes	No	Moderate
PROM – DASH: PBM + Exercise vs. Sham + Exercise	Yes	Yes	No	Yes	No	Very Low
Strength: PBM vs. Other Intervention	Yes	Yes	No	No	No	Low
Strength: PBM + Exercise vs. Sham + Exercise	No	Yes	No	Yes	No	Low

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Chapter 5: Light irradiation parameter set up and characterisation

Chapter Context and Preface

Although there is a growing body of *in vitro* PBM research, there remains a large amount of heterogeneity within the experimental and reporting parameters within the field. This chapter is a non-publication chapter that details the light source characterisation and light rig set up that contributed to the light irradiation protocols used in the subsequent chapters of this thesis. Although the light parameters used are reported briefly in each chapter, they were limited by the constraints of their publication format. Hence, this chapter's purpose is to report these parameters in greater detail to allow for greater protocol transparency and ease of reproducibility for future research.

Introduction

All translational and clinical research begins with basic science experiments. Cellular viability and proliferation assays are a common way within the Photobiomodulation (PBM) field to determine the most efficacious fluence(s) from a given light source to carry out subsequent experiments with [1]. However, before these can commence, it is important that researchers conduct adequate light characterization tests to check that power measurements are in line with the manufacturers claims, and also to improve the light application reliability between experiments [2]. The most widely accepted measure of PBM dosimetry is fluence. Reported in Joules per centimeter squared (J/cm^2), fluence is a function of power (W), time (sec) and beam area (cm^2) (Figure 1) [3].

$$\frac{\text{Power (W) x Time (s)}}{\text{Beam Area (cm}^2\text{)}} = \text{Fluence (J/cm}^2\text{)}$$

Figure 1: Fluence calculation commonly used in PBM applications. Created with biorender.com

Despite the strong consensus on fluence reporting, the basic science and clinical literature within the PBM field contains many experimental inconsistencies. Compounding this, there is yet to be a widely-adopted consensus regarding the reporting of light parameters in PBM research [2, 4, 5]. Specifically, key parameters such as power, irradiation time, treatment cycles, distance from target/anatomical area are often under-reported or missing [2]. As the field moves towards producing more meaningful and impactful clinical research, the implementation of consistent fundamental science protocols should be considered in the effort to better elucidate the fundamental biological mechanisms of PBM. To this end, the aim of this chapter is to report the detailed set-up and characterization of the light sources used in this project to improve the re-test reliability and reproducibility.

Methods

Light Source and Stage

There were two main light sources used in this project. The first was a 660nm InGaAsP laser diode (LD) (Thor Photomedicine, Chesham, Buckinghamshire, UK), which was used in the 96-well experiments. The second was a 670nm laser diode (LD) (B&W Tek Inc., Newark, Delaware), which was used in the 24-well experiments. The key specifications of each laser

diode are reported in Table 1 below. These two devices were used as the wavelengths and specifications are equal or similar to what had been used in previous analogous experiments [5, 6].

Manufacturer and Model	Thor Photomedicine, Visible Red Single Laser Probe	B&W Tek, BWF1
Emitter Type	Laser Diode	Laser Diode
Wavelength	660nm	670nm
Bandwidth	<3 nm	<3 nm
Class	III B	III B
Pulse Mode	Continuous wave	Continuous wave
Distance from target	15mm	80mm
Target spot size	0.32cm^2 (area of a 96well)	1.9cm^2 (area of a 24 well)
Power at target site (mW)	Various	Various
Exposure Duration (sec)	Various	Various
Total Fluence per site (J/cm^2)	Various	Various

Table 1: Light source specifications

The 660nm Thor InGaAsP LD is a commercially available PBM product designed for dental and dermal PBM applications. It takes advantage of a multiple quantum well heterostructure LD, whose power output can be varied with the aid of a variable voltage source. The LD is housed within a specially designed probe allowing for hand held operation (Figure 2). This probe tip is terminated with an integrated collimator, ensuring a circular beam spot at the tip's plane with a spot size in the order of 0.575 cm.

The B&W Tek, BWF1 LD, is a high brightness fiber coupled laser system designed typically for Raman spectroscopy and laser pulsing applications. The power output of this device was controlled via a variable current source. It is integrated within a compact thermoelectric cooler (TEC) housing with forced air cooling, to protect against overheating, particularly with long and/or high-powered irradiation. It terminates with a non-removable fiber pigtail (FC connection). A fiber collimator as a result, is used to control the size of the beam spot at the well plane.

Each of these light sources were mounted to a custom-built light stage offering macro and micro adjustments in x, y, z planes of the well plate holder with the aid of two horizontal translational stages (Onset, SuZhou, China) that could be controlled with the aid of integrated micrometer adjustment knobs. The well plate holder's vertical position was fine-tuned using a vertical translational mount enabling similar micrometer movement. Coarse translation of the LD holders was ensured with the aid of mechanical posts and right-angle post clamps. The Thor LD utilized a ring clamp to fix the shaft of the laser (Figure 2), while the B&W Tek LD was secured using a screw mount given the fiber pig tail at the laser interface (Figure 3). Both were mounted with respect to the position of the well plate, which was fixed with a custom 3D printed plate holder atop the vertical translational mount (Creality, Shenzhen, China) (Figure 2 and 3). Special emphasis was placed on ensuring consistent illumination conditions and target spot sizes at the well interface. To achieve that the following calibration protocol was devised.

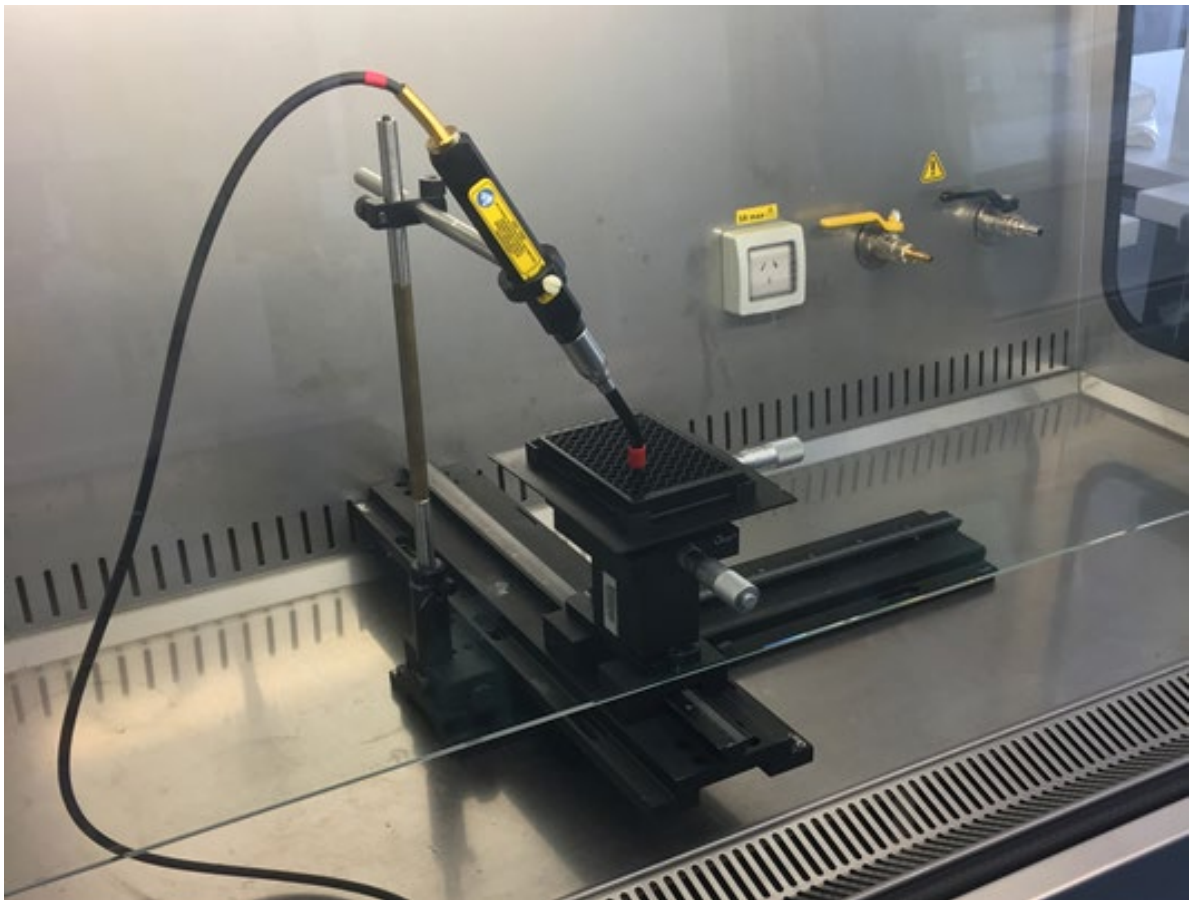

Figure 2: Custom stage built for light irradiation with the Thor LD set up

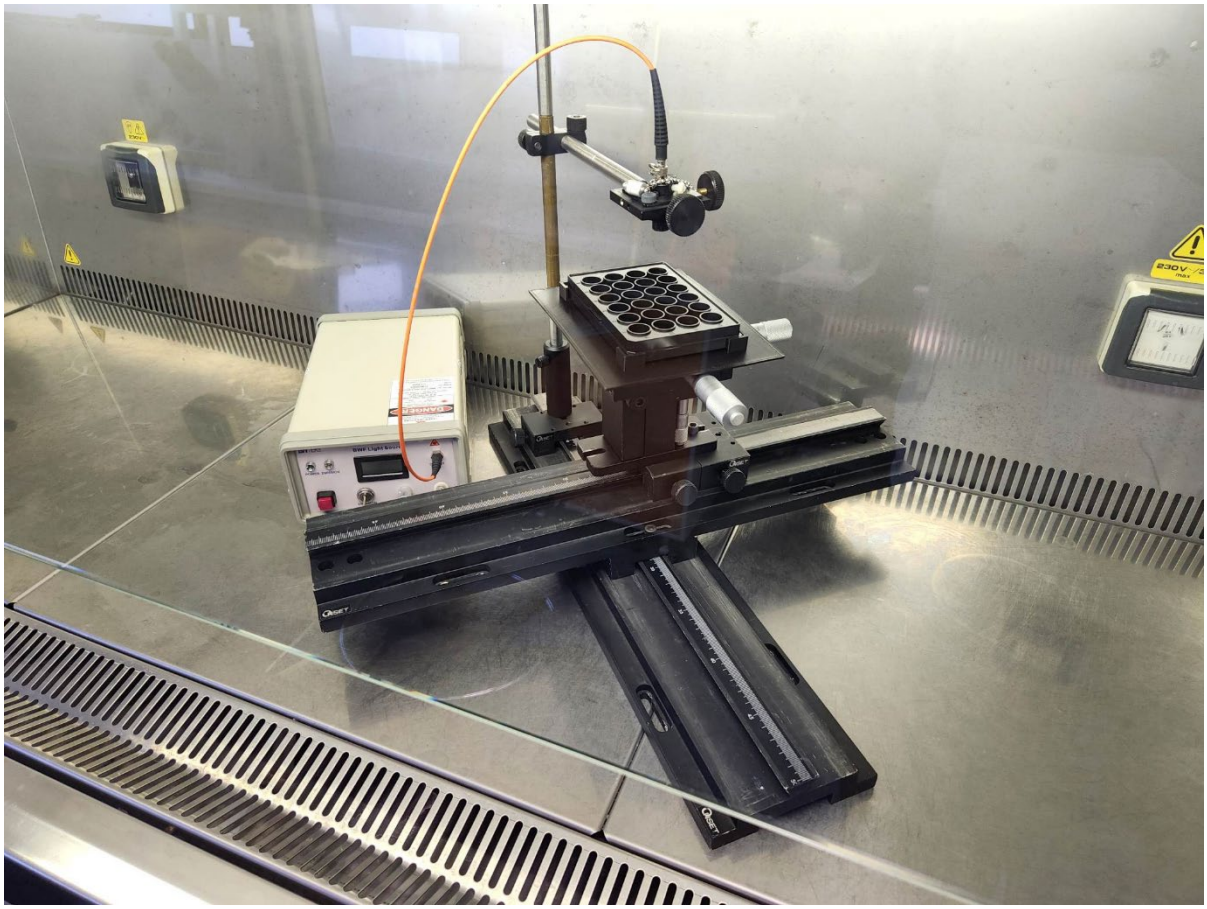

Figure 3: Custom stage built for light irradiation with the B&W Tek LD set up

Optical Power Measurement Procedures

Due to each light source being used having a different size accompanying well plate, each was tested with a matching optical power meter head, with the power meter head size corresponding to either the 96-well area, or the 24-well area. The power meter used for the 96-well application was a Coherent Fieldmaster optical power meter (Coherent, Santa Clara, CA, USA), and the power meter used for the 24-well application was a Spiricon MPE-2500 power meter (Ophir-Spiricon, Utah, USA). To ensure consistency in the readings between the two sources, each light source was also tested using either power meter.

Multiple measurements (n≥5) for a range of light intensities were then acquired as a function of distance from the fibre. The mean power reading (mW) was taken from each set of five measurements and this was the power figure used to calculate the fluence for the subsequent experiments in the project. The 96-well light source was placed 15mm above the power meter interface, while the 24-well light source was placed 80mm away from the power

meter interface. This was done so that the beam spot size was exactly equal to that of either the 96- or 24-well area.

Data Reporting

Power data was entered manually into Microsoft excel (Washington, USA) where it was plotted as means and reported descriptively.

Results

96-well light source characteristics

The acquired measurements indicated a generally linear increase in optical power output (mW) as the input voltage was varied at the laser diode power unit (Figure 4).

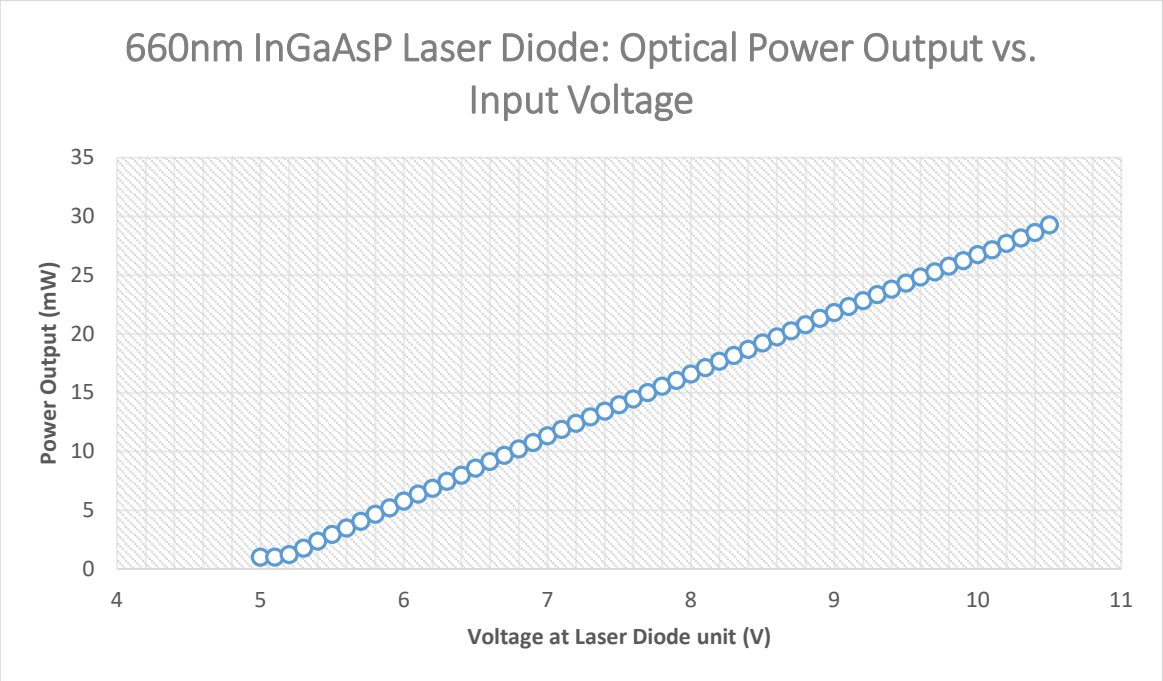

Figure 4: 96-well light source characteristics – power output of the Thor LD as a function of input voltage (mW: Milliwatt; V: Voltage; XY Scatter).

24-well light source characteristics

Similarly to the 96-well light source, the results indicate a generally linear increase in optical power (mW) as the input current was increased at the laser diode power unit (Figure 5).

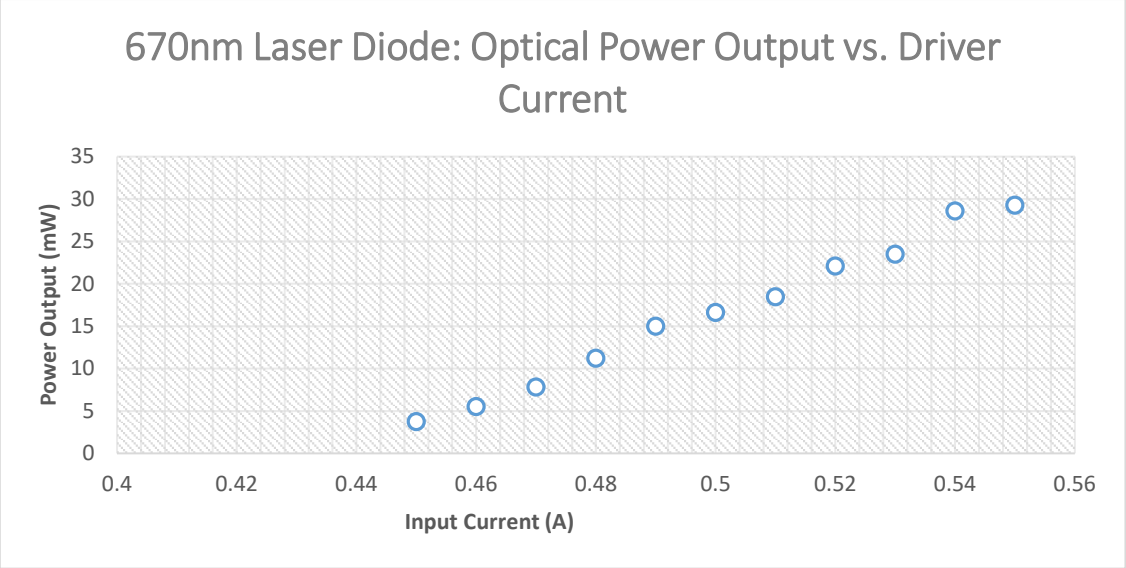

Figure 5: 24-well light source characteristics – power output of the B&W Tek LD as a function of input voltage (mW: Milliwatt; A: AMP; XY Scatter).

Conclusion

Despite the breadth of clinical and basic science research in the PBM field, there remains a large amount of intra-experimental variability, and a lack of consistent parameter reporting [2, 6]. Hence, the aim of this chapter was to take detailed measurements of each light source used across the project so that there was confidence in the re-test consistency across the various assays proposed in this thesis. Once the light stage and source were finalised, accurate power measurements (mW) for a given voltage/current output and set target distance were determined, and hence, allowed for a consistent fluence to be used in future experiments. The next step in the project was to perform cellular viability and proliferation pilot studies to assist in determining which fluence, and which variables that make up fluence, in particular output power and exposure time, would be the most effective for the future experiments. This is explored in the following chapter.

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Chapter 6: Cellular Viability, Proliferation and Apoptosis Pilot Studies

Chapter Context and Preface

Within the underreporting of many light parameters within *in vitro* PBM research, the rationale for using the specified light parameters, especially fluence dose (J/cm^2), is also often reported. The following non-publication chapter details the pilot experiments that were performed to optimise and determine the ideal fluence for the subsequent experiments of this thesis. Specifically, cellular proliferation and apoptosis were used to determine the optimal fluence, underpinned by the light characterisation work performed in the previous chapter. As per the last chapter, this section not only contributed to the important and consistent findings reported in subsequent chapters, but again facilitated greater protocol transparency and ease of reproducibility for future research. It is also important to note that this chapter is not meant to be viewed as a key thesis result and/or outcome, rather it describes the early work done during this thesis, in an attempt to document the experimental refinements made to achieve the published results presented later in the thesis.

Introduction

In a large portion of basic science PBM research, cellular viability assays are performed at multiple fluences in the first instance [1]. Once completed, the highest proliferation/viability reading determines the fluence that the subsequent, and often more time consuming and expensive experiments will be performed (i.e. gene arrays, western blots, FACS, etc.). Common viability and proliferation assays used in PBM research include MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Trypan Blue, Crystal Violet, ATP luminescent, WST-8 (2-(2-methoxy- 4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt), EdU (5-ethynyl-2'-deoxyuridine) and BrdU (5-Bromo-2´-Deoxyuridine) [1]. Although the MTT assay is probably the most commonly used assay traditionally, this chapter will focus on the WST-8—similar to MTT, but more sensitive, EdU assays, and will also examine the potential cytoprotective effect of PBM via the Annexin V/Propidium Iodine (PI) cellular apoptosis assay.

The WST-8 colorimetric assay is used to determine viable cell number [2]. WST-8 is reduced to formazan by NADH extracellularly resulting in a dye reduction that is proportional to the number of viable cells and is quantified by absorption measurement at 450-470nm [3]. Advantages of this method over more traditional measures such as MTT include being able to take multiple measurements on the same cells due to the low cytotoxicity of WST-8, and less methodological steps, making for a simpler procedure [2], leading to an increase in popularity amongst PBM researchers with it appearing in many of the newest studies [4, 5]. EdU a nucleoside analogue is used to directly measure cell proliferation and cell cycle progression [6]. EdU readily incorporates into the DNA of replicating cells and its ability to bind with an azide via a copper-catalysed reaction allows for efficient EdU detection using flow cytometry or fluorescence microscopy [7]. It is widely seen as an improvement on the existing Bromodeoxyuridine (BrdU) assay, a similar nucleoside analogue that incorporates itself into DNA. However, the BrdU assay requires the use fluorescent antibodies and requires cellular fixation, leading to variability in the staining intensity [7]. Although there are PBM studies which have used BrdU as a measure of proliferation and cell cycle progression [8, 9], there appears to be few that have used the EdU assay for similar measures.

A point of contention within the PBM field, and basic science research more broadly, is the delineation between cell viability and proliferation measures, and which assays actually measure each of these variables. Despite commonly being labelled cell proliferation assays, colorimetric assays such as MTT and WST-8 measure cellular metabolic activity [2] and therefore are in fact cell viability assays primarily, and secondly, an indirect measure of proliferation. Conversely, the BrdU and EdU assays directly measure the proliferating cells

via florescent incorporation with the proliferating cells' DNA [7]. Although seemingly trivial, the distinction between the two are crucial when planning PBM *in vitro* research.

In addition to the potential positive biological effects of cellular viability and proliferation that PBM can have, other research has found that PBM may induce a cytoprotective effect where tissue are exposed to cellular stress [10]. Specifically, studies have shown that PBM can decrease cyanide-induced apoptosis [11], hydrogen peroxide (H_2O_2)-induced apoptosis [12] and Alzheimer's-induced apoptosis [13]. There are a few proposed pathways by which PBM may exert its anti-apoptotic effects, namely its interactions with the Akt/GSK3β/β-catenin and Akt/YAPp73 signalling pathways [32], however, more research is needed to further elucidate these.

The field of PBM is hampered by a lack of experimental homogeneity, hence making detailed and transparent pilot studies imperative. Looking at fibroblast cell lines for example, studies have used many different protocols often without proper piloting, varied timeframes, and reagent concentrations for the same assays, making comparisons difficult, while some have even omitted entire parts of assay methodology making replication impossible [14]. Additionally, many studies have also not used media free of coloured pH indicators such as phenol red, which is known to interfere with the optical density measurements of colorimetric assays [15]. As more move towards producing more meaningful and impactful translation of research, we must strongly consider the use of consistent basic science protocols, to better elucidate the fundamental biological mechanisms of PBM. To this end, the aim of this chapter was to investigate the effects of different fluences on fibroblast cellular viability, proliferation and apoptosis to determine the optimum irradiation parameters for the subsequent chapters of this thesis.

Methods

Setting

All procedures were performed in the PC2 laboratory facilities at the Victoria University, Werribee, Australia, under standard laboratory conditions, with aseptic technique.

Cell Culture

All experiments were completed using the human Caucasian foetal foreskin fibroblast (HFFF2) cell line. These cells were sourced commercially from Cell Bank Australia (NSW, Australia). Cells were cultured in low glucose (1000 mg/L) Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate, without phenol red pH indicator dye (Thermo Fisher Scientific,

Waltham, MA, USA), 10% Fetal Bovine Serum (FBS) (Cell Sera, Rutherford, NSW, Australia), 1% 5000 U/mL Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 1% 200mM Glutamax (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in a humidified incubator at 5% CO_2 and 37˚C (manufacturer). Cells were sub-cultured at 80% confluency until sub-culture 4, at which point they were transferred to 2ml cryovials with 90% Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA) and 10% FBS. Cells were frozen at 1x10^6 per aliquot in a Mr. Frosty Freezing Container (MilliporeSigma, Burlington, MA, USA) at -80˚C for 1 week before being transferred to liquid nitrogen storage. When the cells were required for experiments, cells were thawed into the same growth media used for cell culture.

Light Sources

For this experiment two light sources were used, this due to experimental beam size requirement. Firstly, for the WST-8 and EdU experiments, Thor Photomedicine, Visible Red Single Laser Probe (Thor Photomedicine, Chesham, Buckinghamshire, UK). It was fixed at a 15mm distance above the monolayer resulting in a beam area of 0.32cm^2 (equal to the area of the well). All power measurements were measured by a Coherent Fieldmaster optical power meter (Coherent, Santa Clara, CA, USA), at the same distance from the power meter interface to mirror the expected power at the cell monolayer. For the Annexin V/PI experiments, a B&W Tek, BWF1 laser system (B&W Tek, Newark, Delaware, USA), was placed 80mm above the cell monolayer so the beam covered an area of 1.9cm^2, equating to the size of the well. Laser power was calculated using a Spiricon MPE-2500 power meter (Ophir-Spiricon, Utah, USA), by placing it at the same distance away from the laser tip, as the cell monolayer was situated (80mm). The specifications of both laser systems are located in Table 1. A custom-built stage with x, y, and z axis, macro and micro adjustment (Onset, SuZhou, China), and a 3D printed plate holder atop the stage (Creality, Shenzhen, China).

Manufacturer and Model	Thor Photomedicine, Visible Red Single Laser Probe	B&W Tek, BWF1
Emitter Type	Laser Diode	Laser Diode
Wavelength	660nm	670nm
Class	III B	III B
Pulse Mode	Continuous wave	Continuous wave

Distance from target	15mm	80mm
Target spot size	0.32cm^2 (area of a 96well)	1.9cm^2 (area of a 24 well)
Power at target site (mW)	Various	Various
Exposure Duration (sec)	Various	Various
Total Fluence per site (J/cm^2)	Various	Various

Table 1: Light source specifications

WST-8 Assay

The WST-8 colorimetric assay was used to measure cell viability. Cells were seeded in black-walled, 96 well plates (Greiner Bio-One, Kremsmünster, Austria), with a transparent bottom to minimize light scattering [16], at a density of 7x10^3, in 200µl of 10% FBS growth media (Figure 1). 24 hours post seeding, media was removed and replaced with 100µl of serum depleted media (0.5% FBS) to more closely simulate *in vivo*-like conditions [17]. Immediately after media change, cells were irritated once, with either a 0.5, 1, 2, 4, 8, 16 J/cm^2 fluence, and either 5, 10, 20, or 30mW of power (the 5mW group was not irradiated at 16 J/cm^2 as the cells would have an unacceptable time outside the incubator (1024 seconds) (Table 2). All plates had control wells that were not exposed to light. Cells were irradiated through an opaque mask, to ensure only one well was exposed to the light at any one time [18]. From here, 10µl of WST-8 solution was added to each well and placed back in the incubator for 4 hours. Any bubbles that formed during the addition of WST-8 were removed by centrifugation at 200xg for 2 minutes. Optical Density (OD) at 450nm was measured at 24 hours using a Bio-Red xMark microplate reader (Bio-Rad. Hercules, CA, USA). Each group was irradiated in quadruplicate.

Power (mW)	Fluence (J/cm^2)	Time (sec)
5	0.5	32
5	1	64
5	2	128
5	4	256
5	8	512
10	0.5	16
10	1	32
10	2	64
10	4	128

10	8	256
10	16	512
20	0.5	8
20	1	16
20	2	32
20	4	64
20	8	128
20	16	256
30	0.5	5.3
30	1	10.7
30	2	21.3
30	4	42.7
30	8	85.3
30	16	170.6

Table 2: PBM exposure details for the WST-8 assay

EdU Assay

The EdU assay was used to measure proliferation. The cells were seeded in black-walled, 96 well plates, with transparent bottoms, to minimize light scattering from the laser [16] (Greiner Bio-One, Kremsmünster, Austria) at a density of $7x10^3$, in 200µl of 10% FBS growth media per well). At 24 hours post seeding, the media was removed and replaced with 100µl of serum depleted media (0.5% FBS) to more closely simulate *in vivo*-like conditions [17]. Immediately after the media change each seeded well was irradiated at either 0.5, 1, 2, 4, 8, 16 J/cm^2 fluence at 10mW of power (Table 3). Cells were irradiated through a 'mask', to ensure only one well was exposed to the light at any one time [18]. All plates had control wells which were not exposed to light. 24 hours post-treatment the samples were prepared for flow cytometry analysis again using the manufacturer's instructions.

Power (mW)	**Fluence (J/cm2)**	**Time (sec)**
10	0.5	16
10	1	32
10	2	64
10	4	128
10	8	256
10	16	512

Table 3: PBM exposure details for the EdU assay

Annexin V/PI Assay:

Cells were plated at 4x10^4 cells per well in a black-walled, clear bottom 24-well plate (Eppendorf, Hamburg, Germany) in 500uL of growth media to reduce possible light scatter [25]. After 24 hours the cells were exposed to 0.5mM of H_2O_2 to induce oxidative stress [42], this concentration of H_2O_2 was determined in experiments reported in the following chapter. Immediately after the H_2O_2 exposure, each well irradiated at a fluence of 1J/cm^2 (Table 4). Cells were irradiated through a 'mask', to ensure only one well was exposed to the light at any one time [18]. All plates had control wells which were not exposed to light. There were three experimental groups, with six replicates: 1: PBM + H_2O_2; 2: No- Light + H_2O_2 (Positive control); and 3. No light and no H_2O_2 (Negative control). 24 hours post treatment the experimental assays were commenced. 24 hours post treatment each sample was prepared for flow cytometry analysis according to the manufacturer's instructions. 15 minutes before analysis 5uL of Annexin V and 1uL of Propidium iodine (PI) was added into each sample, and was incubated at room temperature.

Power (mW)	Fluence (J/cm^2)	Time (sec)
11.2	1	169

Table 4: Fluence parameters for the Annexin V/PI assay

Flow Cytometry

All flow cytometry was performed on an Accuri C6 flow cytometer (BD Biosciences, NJ, USA), with a 488 nm laser, and 530/30, 585/40 and 670LP filters. Automated sampling was used with regular sample agitation. Acquisition was performed with the BD Accuri C6 Plus Software (v1.0.23.1, BD Biosciences, NJ, USA), with post processing analysis performed on the software FlowJo (v.10.8.1). A sequential gating process was used to identify events of interest, using unstained, negative, and positive fluorescent controls. Firstly, size and density parameters were used to eliminate cellular debris, and forward and side scatter pulse processing used for doublet discrimination, before finally fluorescence of interest was analysed.

Statistical Analysis

Raw data was exported into either SPSS Version 22 (IBM, New York, USA) or JASP (JASP, Amsterdam, The Netherlands) for statistical analysis. All data is expressed as mean and standard deviation. A one-way ANOVA followed by Tukey's post-hoc was used to analyse

the differences between group means. Results were considered statistically significant at $p<0.05$.

Results

WST-8 Assay

PBM treatment increased the viability of cells compared to their matched controls across many different fluence ranges (Figure 1a-g). The intensities and time point measurements that demonstrated a statistically significant change compared to either the controls or other fluence are listed in Table 5.

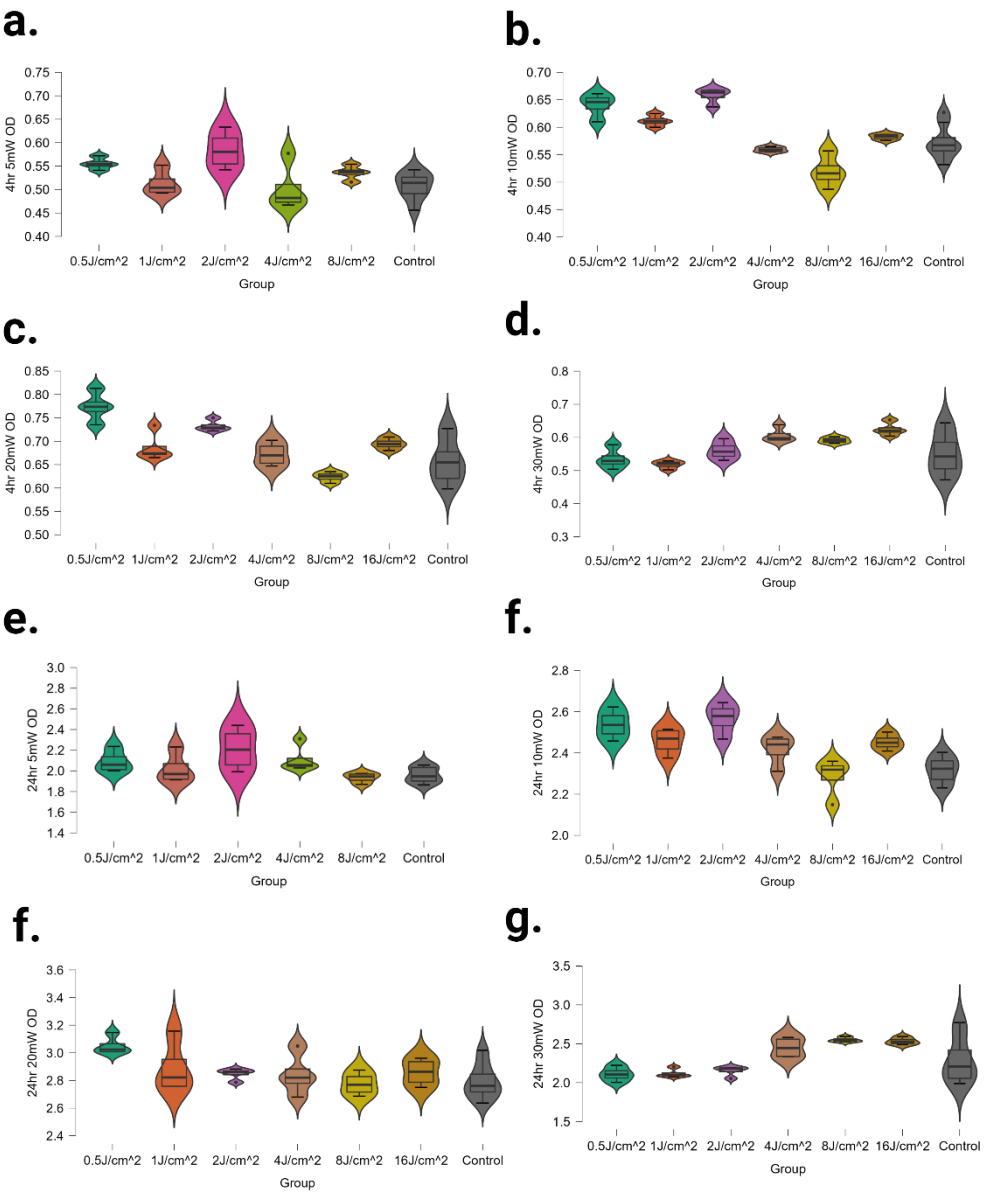

Figure 1: Cell viability expressed as optical density (OD) for 1a: 4-hour (hr), 5mW; 1b: 4hr 10mW; 1c: 4hr 20mW; 1d: 4hr 30mW; 1e: 24hr 5mW; 1f: 24hr 20mW; 1g: 24hr 30mW

Power (mW)	Time Point (hr)	Fluence (J/cm^2)	Comparison	Increased or decreased OD compared to Comparison	*p* Value
5	4	$1J/cm^2$	$2J/cm^2$	Decreased	0.044
5	4	$2J/cm^2$	$4J/cm^2$	Increased	0.015
5	4	$2J/cm^2$	Control	Increased	0.008
5	24	$2J/cm^2$	$8J/cm^2$	Increased	0.042
5	24	$1J/cm^2$	Control	Increased	0.031
10	4	$0.5J/cm^2$	$4J/cm^2$	Increased	< .001
10	4	$0.5J/cm^2$	$8J/cm^2$	Increased	< .001
10	4	$0.5/cm^2$	Control	Increased	< .001
10	4	$1J/cm^2$	$4J/cm^2$	Increased	0.033
10	4	$1J/cm^2$	$8J/cm^2$	Increased	< .001
10	4	$2J/cm^2$	$4J/cm^2$	Increased	< .001
10	4	$2J/cm^2$	$8J/cm^2$	Increased	< .001
10	4	$2J/cm^2$	$16J/cm^2$	Increased	< .001
10	4	$2J/cm^2$	Control	Increased	< .001
10	4	$8J/cm^2$	$16J/cm^2$	Decreased	0.006
10	4	$8J/cm^2$	Control	Decreased	0.007
10	24	$0.5J/cm^2$	$8J/cm^2$	Increased	< .001
10	24	$0.5J/cm^2$	Control	Increased	< .001
10	24	$1J/cm^2$	$8J/cm^2$	Increased	0.029
10	24	$1J/cm^2$	Control	Increased	0.043
10	24	$2J/cm^2$	$8J/cm^2$	Increased	< .001
10	24	$2J/cm^2$	Control	Increased	< .001
10	24	$8J/cm^2$	$16J/cm^2$	Decreased	0.036
20	4	$0.5J/cm^2$	$1J/cm^2$	Increased	0.009
20	4	$0.5J/cm^2$	$4J/cm^2$	Increased	0.002
20	4	$0.5J/cm^2$	$8J/cm^2$	Increased	< .001
20	4	$0.5J/cm^2$	$16J/cm^2$	Increased	0.020
20	4	$0.5J/cm^2$	Control	Increased	< .001
20	4	$2J/cm^2$	$8J/cm^2$	Increased	< .001
20	24	$0.5J/cm^2$	$8J/cm^2$	Increased	0.043
20	24	$1J/cm^2$	Control	Increased	0.022
20	24	$2J/cm^2$	$4J/cm^2$	Increased	0.030
30	4	$0.5J/cm^2$	$16J/cm^2$	Decreased	0.039
30	4	$1J/cm^2$	$4J/cm^2$	Decreased	0.042
30	4	$1J/cm^2$	$16J/cm^2$	Decreased	0.009
30	24	$16J/cm^2$	Control	Increased	0.047
30	24	$0.5J/cm^2$	$8J/cm^2$	Decreased	0.015
30	24	$0.5J/cm^2$	$16J/cm^2$	Decreased	0.021
30	24	$1J/cm^2$	$8J/cm^2$	Decreased	0.018
30	24	$1J/cm^2$	$16J/cm^2$	Decreased	0.025
30	24	$2J/cm^2$	$8J/cm^2$	Decreased	0.035
30	24	$2J/cm^2$	$16J/cm^2$	Decreased	0.049

Table 5: Summary of statistically significant change in cell viability across all groups

EdU Assay

When analysed using median fluorescent intensity (MFI) and normalising the data according to percentage change to matched control, PBM at a fluence of 1J/cm^2 (p=0.027) and 4J/cm^2 (p=0.007) created a significant increase in proliferation compared to their matched controls (Figure 2).

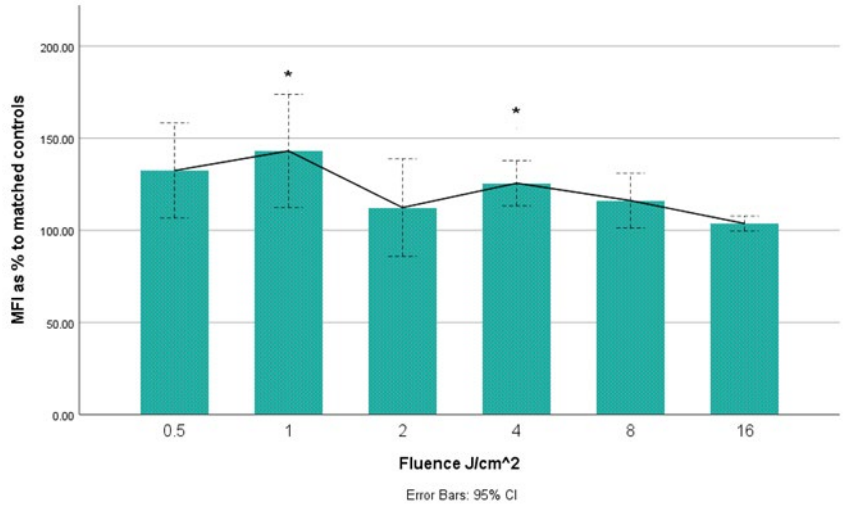

Figure 2: Normalized cell proliferation relative to control as a function of fluence; error bars represent the 95% confidence interval (demarcates Tukey adjusted p < 0.05).*

Annexin/PI Assay

There were no statistically significant differences between the PBM and PC groups when measuring the percentage of dead non-apoptotic cells, early apoptotic cells or non-apoptotic cells, although the mean was in favour of the PBM group across these three analyses. There was a statistically significant difference between the NC and both the PC and PBM groups when quantifying early apoptotic cells ($p < 0.001$) and non-apoptotic cells ($p = 0.002$). There was no statistically significant difference between all three groups when measuring dead non-apoptotic cells frequency (Figure 3).

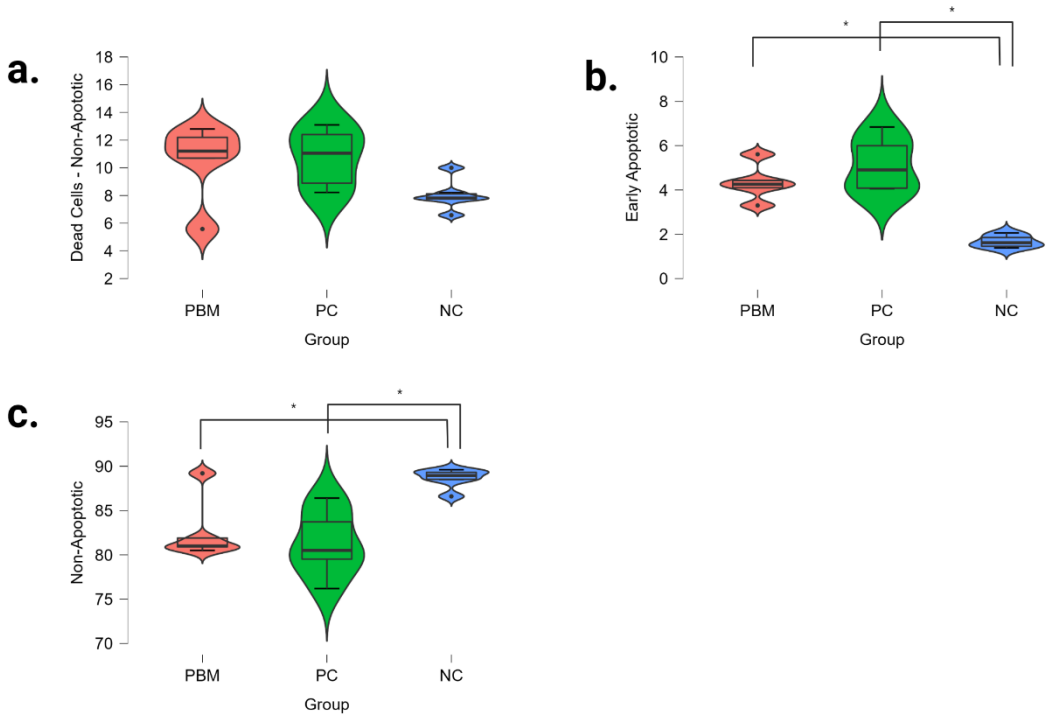

Figure 3a: Percentage of dead, non-apoptotic cells. Figure 3b: Percentage of early apoptotic cells. Figure 3c: Percentage of healthy, non-apoptotic cells (demarcates p <0.05).*

Discussion and Conclusion

PBM is a widely-used clinical therapy, that has been shown to be effective for a multitude of conditions [19]. This said, there remains conjecture about the exact physiological mechanisms that underpin its effects [14, 20]. Compounding this, in the attempts to uncover these mechanisms, there are large amounts of methodological heterogeneity across these experiments [20, 21]. Hence the aim of this chapter was to perform a number of pilot studies in an attempt to determine the optimum set of irradiation parameters for the subsequent chapters of this thesis via cellular viability, proliferation, and apoptotic assays.

Firstly, a number of WST-8 experiments were performed to determine the effect of PBM on fibroblast cellular viability. The quick, and cost-effective nature of the WST-8 assay afforded us to investigate a number of total fluence levels, and also to manipulate the variables that make fluence i.e. time and power. Overall, an increased viability across varied was seen across varied time points and fluence variables. However, these changes were inconsistent

with the dose-response principles of PBM [22, 23], and also had a broad standard variation, making statistical significance difficult to achieve. The inconsistent findings found with the cellular viability assay are common place in the literature—with many papers publishing conflicting results [14, 20]. WST-8 has been shown to be more sensitive to cellular changes than other analogous assays such as MTT [3], however, given the relatively small *in vitro* effects of PBM [14] and the findings reported here, it appears that WST-8 may not be sensitive or accurate enough to accurately determine the effects of PBM in the short time frames and small well sizes used. Therefore, it was decided a more sensitive measure was needed, in the form of a cellular proliferation EdU assay to better determine the optimum irradiation protocols for the remainder of the project.

As opposed to the results reported with the WST-8 assay, the EdU results illustrated a trend more closely adhering to the dose-response principles of PBM. Due to the cost of EdU, we performed the experiments on the full fluence range, however we kept the power level consistent and 10mW. We based this number predominately off the literature, and for experimental time efficiency [14]. Due to plate set up minimising light spill over in a 96-well setting, we needed to perform the experiment over two plates, each with their own controls—with statistical significance being achieved at 1 and 4 J/cm^2. These results mostly fit with other studies that have measured cell proliferation in human dermal fibroblasts, with a fluence from 0.45 to 10 J/cm^2 demonstrating a significant increase in proliferation compared to un-lit controls [5, 16, 22, 24-27]. However, the 16 J/cm^2 group was essentially similar to the control, in the context of the literature, we would have expected this figure to start to decrease and fall below the control [17, 26, 28]. Perhaps the downward trend may have continued at a fluence above 16 J/cm^2, however, given the previous literature we did not pursue this measurement. Despite the positive results, the output again demonstrated a broad standard deviation, raising question marks about the repeatability of the current protocol. From this, in consultation with the literature and other experts in the field, we decided to change some key experimental protocols. Two main changes were made: 1. Stimulate cellular stress through the addition of low-concentration [12, 27] H_2O_2 to the cells to potentially amplify the biological effect of PBM, and 2. The upscaling from a 96-well plate, to a 24-well plate with the thought that more cell samples would improve the consistency of the assay results, given the larger cell counts.

As expected, the addition of H_2O_2 caused a significant increase in apoptosis compared to the NC, however, although the mean was in favour of the PBM group, there was no

significant change in apoptosis between the PBM and PC group. This is mostly in disagreement with the limited available literature on PBMs effect on cellular apoptosis [10], with research demonstrating the anti-apoptotic effects of PBM with cells are exposed to cyanide-induced apoptosis [11], H_2O_2-induced apoptosis [12] and Alzheimer's-induced apoptosis [13]. This said, the study the study that looked H_2O_2-induced apoptosis, was done on cortical neurons, making comparisons to fibroblasts more challenging. Positively, there was an indication that the standard deviation was becoming less broad, notwithstanding the odd outlier. In the experiments in the next chapter and beyond, as the experiments were further refined, with the result mean and standard deviations became more consistent, making statistical significance easier to achieve. More importantly, the experimental protocol going forward was established, setting the stage for the comparison of not only PBM to the positive and negative control groups, but also for the comparisons to polarized PBM to all of these groups.

Overall, this chapter documented the pilot studies that informed the light and treatment parameters of the subsequent chapters of this thesis. Despite some inconsistencies in the early results, as the experiments and procedures were further refined, across this chapter and the proceeding, the results became more predictable and consistent, in the process better aligning to analogous results in other *in vitro* PBM settings. It also highlights both the importance and difficulty of both piloting *in vitro* experiments, and agreeing on a set of experimental protocols for a given project.

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Chapter 7: The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: potential applications to wound healing

Chapter context and preface

There are many light properties that can be manipulated to attenuate the delivery and subsequent effects of PBM, many which require further research. Of these, polarization was chosen as the primary variable to investigate within this thesis. This published chapter details specifically how polarization of PBM can affect cellular viability, proliferation, apoptosis, and mitochondrial membrane potential. This chapter also presented an *in vitro* model to produce cellular oxidative stress to model what can be seen in chronic wounds *in vivo*. The novel results detailed in this chapter represent a way in which the delivery of PBM may be improved further.

Post review note: A typographical error was pick up by one of the examiners. The 0.5uM H_2O_2 concentration stated in the methods section was in fact 0.5mM. The publishing journal has been made aware of this error.

Chapter 7: Declaration of Co-Authorship and Co-Contribution

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

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The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: potential applications to wound healing

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Abstract

Photobiomodulation (PBM) is a widely used therapeutic intervention used to treat several chronic conditions. Despite this, fundamental research underpinning its effectiveness is lacking, highlighted by the lack of a definitive mechanism of action. Additionally, there are many treatment variables which remain underexplored, one of those being the effect of polarization the property of light that specifies the direction of the oscillating electric field. When applied to PBM, using linearly polarized light, when compared to otherwise identical non-polarized light, may enhance its biological efficacy. As such, we investigated the potential biological effects of polarized PBM when compared to non-polarized and non-irradiated controls in the domains of cellular viability, proliferation, apoptosis and mitochondrial membrane potential ($\Delta\Psi$) within cells exposed to oxidative stress. It was noted that polarized PBM, when compared to non-polarized PBM and non-irradiated controls, demonstrated mostly increased levels of cellular proliferation and $\Delta\Psi$, whilst decreasing the amount of cellular apoptosis. These results indicate that polarization may have utility in the clinical application of PBM. Future research is needed to further elucidate the underpinning mechanisms of PBM and polarization.

Keywords: Photobiomodulation; polarized light; polarization; low level light therapy; cellular viability; cellular proliferation; cellular apoptosis; mitochondrial membrane potential; wound healing; oxidative stress

1. Introduction

Photobiomodulation (PBM) is an umbrella term given to any light source used to treat clinical conditions [1]. Although there is some debate about which wavelengths and intensities constitute PBM, the most common form used is red and/or infrared light with less than 1 Watt (W) in power. PBM has demonstrated clinical benefits across a wide spectrum of conditions affecting the population, including: musculoskeletal and neuropathic pain [2], dermatological conditions [3, 4], would healing [5, 6] and is currently being evaluated as a treatment in neurodegenerative conditions and traumatic brain injury [7]. Despite a large, and growing body of evidence demonstrating the positive effects of PBM, a full understanding of its molecular and cellular effects is lacking [8].

The leading proposed mechanism underpinning PBM, is that red and near-infrared light specifically interacts with the cytochrome c oxidase (CcO) enzyme in the mitochondria. It is thought that light displaces nitric oxide (NO), which competes with oxygen at the CcO substrate binding site, and ultimately increases ATP production [9] (Figure 1). While indirect evidence supports this, there is not yet any confirmatory evidence of a direct interaction between light and the aforementioned mitochondrial machinery [8]. Additionally, PBM is thought to promote the production of reactive oxygen species (ROS), presumably through the increase in oxygen metabolism [10]. This increase in ROS is thought to be a key driver in many of the observed changes in gene regulation and transcription factors [10]. Recently however, it has been shown that PBM can exert a proliferative effect on cells despite the absence of CcO, casting some doubt on the CcO/NO/ATP hypothesis of PBM [8]. This strongly underlines the need for more basic science research to gain further insights into the fundamental mechanism(s) of PBM.

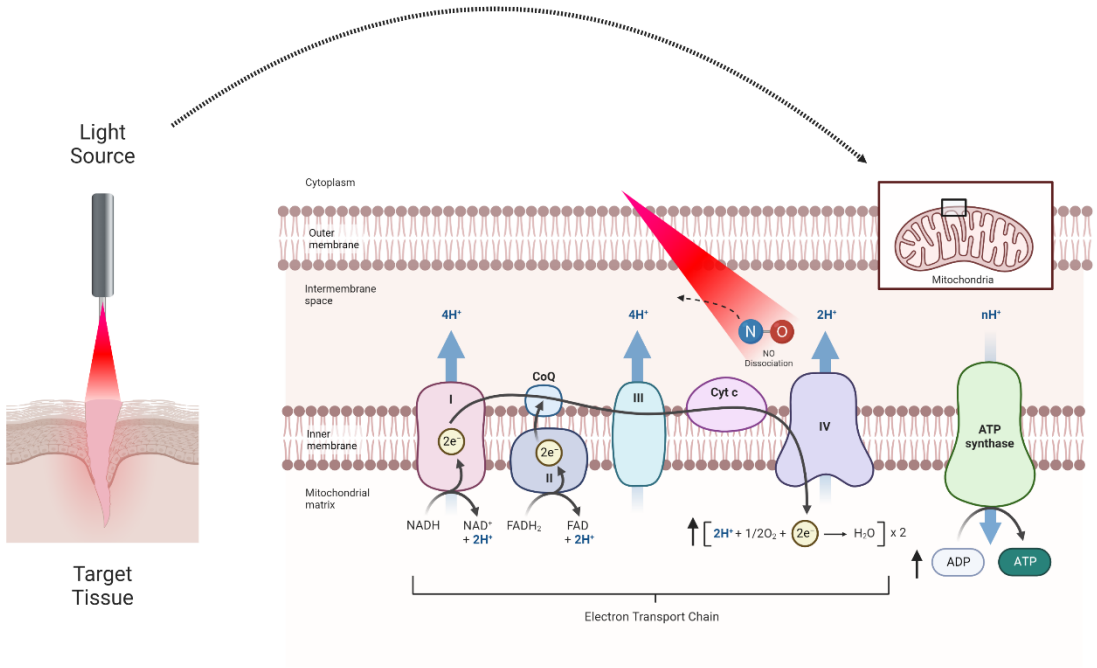

Figure 1. Proposed PBM biological mechanisms. ATP: adenosine triphosphate; ADP: adenosine diphosphate; NO: nitric oxide; NAD: Nicotinamide adenine dinucleotide; FAD: Flavin adenine dinucleotide; H: Hydrogen; e, electron; O_2: Oxygen; H_2O: Water; Cyt c: Cytochrome c; I-IV: Cytochrome I-IV. Created with biorender.com

Regardless of the precise photonic and molecular mechanisms of PBM, it has been shown to improve both cellular viability and proliferation across a wide range of *in vitro* settings [11]. However, there are mixed results on the effect of varying fluences (Joules (J)/cm^2) on cellular viability, with data showing increases, decreases and no effect across a range of light doses. Specifically, fluence between 0.5-5.5 J/cm^2 have shown to increase viability [12-15], whilst fluence of 1.5-25 J/cm^2 demonstrated no change in viability [16-21], and doses of both 0.5 and 10 J/cm^2 resulted in a decrease in cellular viability [22]. These inconsistencies are likely due to varying irradiation and treatment conditions, highlighting the need for a consistent set of experimental standards when it comes to *in vitro* PBM research [11, 23]. Direct measures of cellular proliferation have also been conducted widely in this field with more consistent results. Measures of cellular proliferation appear to show a consistent dose-response relationship. PBM induces an increase in proliferation proportional to the light intensity, until a point, at which it plateaus and decreases the biological effect as power keeps increasing [24]. Multiple *in vitro* studies describe that a fluence from 0.45-10 J/cm^2

increases cellular proliferation [22, 25-30], with fluences of 10-20 J/cm^2 shown to decrease proliferation [19, 28, 31]. These effects on cellular behaviour are thought to underpin the positive clinical effects shown by PBM, particularly in the context of wound healing [9, 32].

Another mode by which PBM is thought to exert its biological effect is through cell protection, specifically in decreasing cellular apoptosis in response to cellular stress [33]. The caspase enzymes are known to be a key player in cellular apoptosis [34], and are of particular interest in PBM studies given their relationship to the mitochondria, where free cytochrome c within the cytosol helps generate caspase cellular machinery [34]. Indeed, it has been shown that when cells are exposed to cellular stress in the form of H_2O_2 *in vitro*, PBM caused a decrease in CASP 3 and CASP 8 activity when compared to non-irradiated controls [29, 35]. There are also additional pathways by which PBM may induce an anti-apoptotic effect such as the Akt/GSK3β/β-catenin and Akt/YAPp73 signalling pathways and hepatocyte growth factor [32]. However, more research is required to elucidate the optimum dose and interaction between PBM and the apoptotic pathways. Given the known link between mitochondrial membrane potential (ΔΨ) and apoptosis [36], this has also been evaluated in the context of PBM [11]. In line with the effects of PBM on apoptosis, a mostly consistent dose-response curve, with a fluence of 5 J/cm^2 increasing ΔΨ has been noted, while a fluence of both 15 J/cm^2 and 45 J/cm^2 caused a decrease in ΔΨ [37]. However, there are some conflicting results, where a fluence of 3 J/cm^2 can cause a decrease in ΔΨ [20], further highlighting the need for more research.

In addition to the incomplete understanding of the fundamental photonic and physiological mechanisms underpinning its effects, and an absence of an accepted set of *in vitro* experimental standards, there are a number of technical properties of light that remain under investigated in PBM [23]. Variables such as wavelength, power, irradiation time, beam area, radiant energy, fluence, polarization, pulse parameters and treatment cycles, are all factors which can influence the outcome of PBM application [23]. Of these, polarization— the property of light that specifies the direction of the oscillating electric field —is an intriguing variable to investigate. Using linearly polarized light, when compared to otherwise identical non-polarized light, may increase its biological efficacy [38, 39]. Specifically, polarized PBM has been shown to increase fibroblast proliferation and procollagen mRNA expression [40], alter immune cell function [41], and in animal models, improve the recovery time of rats exposed to spinal cord injury [42]. Despite, the promising biological effects of polarized PBM when compared to non-polarized PBM, more research is needed to fully uncover any

potential benefits of polarized light in the field of phototherapy. Herein, we determined the biological effects of polarized PBM when compared to non-polarized and non-irradiated controls in the domains of cellular viability, proliferation, apoptosis and ΔΨ.

2. Methods

2.1. Setting

All procedures were performed in the PC2 laboratory facilities at Victoria University, Werribee campus, Australia, under standard laboratory conditions, with aseptic technique.

2.2. Cell culture and treatments

All experiments were completed using the human caucasian foetal foreskin fibroblast (HFFF2) cell line. These cells were sourced commercially from Cell Bank Australia (NSW, Australia). Cells were cultured in low glucose (1000 mg/L) dulbecco's modified eagle medium (DMEM) with sodium pyruvate, without phenol red pH indicator dye (Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS) (Cell Sera, Rutherford, NSW, Australia), 1% 5000 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 1% 200 mM glutamax (Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown in a humidified incubator at 5% CO_2 and 37 °C. Cells were sub-cultured at 80% confluency until sub-culture 4, at which point they were transferred to 2 ml cryovials with 90% Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA) and 10% FBS. Cells were frozen at $1x10^6$ per aliquot in a Mr. Frosty Freezing Container (MilliporeSigma, Burlington, MA, USA) at -80 °C for 1 week before being transferred to liquid nitrogen storage. When the cells were required for experiments, cells were thawed into the same growth media used for cell culture. Each individual assay was exposed to identical treatment conditions. Firstly, the cells were plated at $4x10^4$ cells per well in a black-walled, clear bottom 24-well plates (Eppendorf, Hamburg, Germany) in 500 µL of growth media to reduce possible light scatter and interference between adjacent wells [25]. After 24 hours the cells were exposed to 0.5 µM of H_2O_2 to induce oxidative stress [43]. Immediately after H_2O_2 exposure, each well irradiated at a fluence of 1 J/cm^2. The full irradiation parameters are shown in Table 1. There were four experimental groups, all conducted in quadruplicates, unless otherwise indicated: 1: linearly polarized light + H_2O_2 (P-PBM); 2: non-polarized light + H_2O_2 (NP-PBM); 3. no-light + H_2O_2 (positive control - PC); and 4. no-light and no H_2O_2 (negative control - NC). 24 hours post

treatment the experimental assays were commenced. All experimental groups were otherwise exposed to the same conditions.

2.3. Light Source

For each experiment a 670 ± 5 nm BWF laser diode fiber coupled laser system (B&W Tek, Newark, Delaware, USA) was used. The details of this laser system are in table 1. The experimental fluence was calculated by monitoring the optical power output using a Spiricon MPE-2500 power meter (Ophir-Spiricon, Utah, USA), at the same distance from the laser tip, as the cell monolayer. A 25 mm linear glass polarizing filter (Edmund Optics, New Jersey, USA) was used to produce linearly polarized light. The laser output power was recalibrated with the polarizer in place, to ensure matched fluence for both polarized and non-polarized treatments. The polarizer was oriented in the same position as calibrated for all treatments to ensure consistent light parameters [42].

Manufacturer and Model	B&W Tek, BWF1
Emitter Type	Laser Diode
Wavelength	670 nm
Class	III B
Pulse Mode	Continuous wave
Distance from target	80 mm
Target spot size	1.9 cm^2 (area of a 24 well)
Power at target site (mW)	11.2
Exposure Duration (sec)	169
Total Fluence per site (J/cm^2)	1

Table 1. Laser system and fluence parameters

2.4. H_2O_2 Dilution

The Annexin V/propidium iodide (PI) assay was used to determine the optimum concentration of H_2O_2 to stimulate cellular stress and apoptosis. Cells were exposed to the following concentrations of H_2O_2: 16 mM, 8 mM, 4 mM, 2 mM, 1 mM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.63 µM and 0 µM for 24 hours, before being stained with Annexin V-Alex Fluor 488 and PI according to manufacturer instructions to identify optimal conditions of cellular stress.

2.5. WST-8 Assay

To measure cellular viability, a WST-8 assay was performed. Briefly, 50 µl of WST-8 solution was added to each well immediately after irradiation and placed back in a humidified incubator set to 5% CO_2 and 37 °C for a period of 24 hours. Any bubbles that formed during the addition of WST-8 were removed by centrifugation at 200xg for 2 minutes. Optical Density (OD) at 450 nm was measured at 24 hours using a Bio-Red xMark microplate reader (Bio-Rad. Hercules, CA, USA).

2.6. EdU Assay

To measure cellular proliferation, the EdU assay was performed. Briefly, the cells were fixed and permeabilized, then 10 µL of EdU solution was added to each sample according to the manufacturer's instructions (BD Biosciences, San Jose, California) 24 hours post treatment. 15 minutes before flow cytometry analysis, each sample was incubated for 15 minutes in 1 µL of PI to determine total DNA content. 24 hours post-treatment the samples were prepared for flow cytometry analysis again using the manufacturer's instructions.

2.7. Annexin V/PI Assay

To measure healthy (non-apoptotic), dead and apoptotic cells, an Annexin V/PI assay was performed (BD Biosciences, USA). Briefly, 24 hours post treatment each sample was prepared for flow cytometry analysis according to the manufacturer's instructions. 15 minutes before analysis 5 µL of Annexin V and 1 µL of PI were added into each sample and incubated at room temperature before being analyzed by flow cytometry.

2.8. MitoProbe JC-1 Assay

To measure the $\Delta\Psi$ of the samples, a MitoProbe JC-1 assay was performed (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 24 hours post treatment 0.5 µM of JC-1 dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample. A CCCP (carbonyl cyanide m-chlorophenyl hydrazone) control (50 µM) was also used to confirm that the JC-1 response is sensitive to changes in membrane potential. After 24 hours, samples were analyzed by flow cytometry as described below.

2.9. Flow Cytometry

All flow cytometry was performed on an Accuri C6 flow cytometer (BD Biosciences, NJ, USA), with a 488 nm laser, and 530/30, 585/40 and 670LP filters. Automated sampling was used with regular sample agitation. Acquisition was performed with the BD Accuri C6 Plus Software (v1.0.23.1, BD Biosciences, NJ, USA), with post processing analysis performed on the software FlowJo (v.10.8.1). A sequential gating process was used to identify events of interest, using unstained, negative, and positive fluorescent controls. Firstly, size and density parameters were used to eliminate cellular debris, and forward and side scatter pulse processing used for doublet discrimination, before finally fluorescence of interest was analysed.

2.10. Statistical Analysis

All raw data were exported into JASP (JASP, Amsterdam, The Netherlands) for statistical analysis. All data is expressed as mean and standard deviation. A one-way ANOVA followed with Tukey's post-hoc testing was used to analyse the differences between group means. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Determination of the optimal H_2O_2 dilution

An almost linear decline in cell apoptosis was observed as the concentration of H_2O_2 decreased. The effect of H_2O_2 appeared to diminish at a concentration of 0.25 mM (Figure 2). Based on this an experimental concentration of 0.5 mM was selected for ongoing experiments, as previous work had used the lowest apoptosis-inducing concentration of H_2O_2 possible [43].

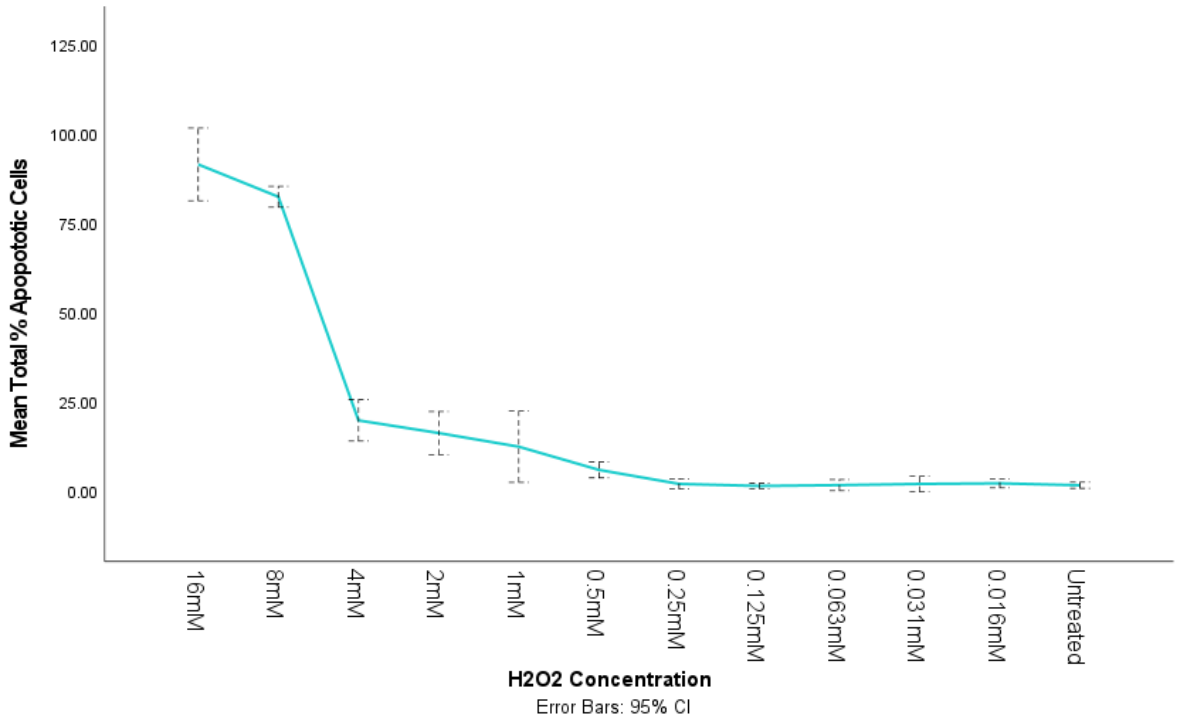

Figure 2. Mean apoptotic human caucasian foetal foreskin fibroblast (HFFF2) cells as a percentage of total cells plotted with H_2O_2 concentration

3.2. WST-8 Assay

There were no significant differences in cellular viability between any of the groups that were exposed to cellular stress ($p > 0.05$) using the WST-8 assay. As expected, the negative control group demonstrated a significantly higher cellular viability when compared to the P-PBM ($p = 0.001$), the NP-PBM ($p = 0.007$), and the PC ($p = 0.006$) groups (Figure 3).

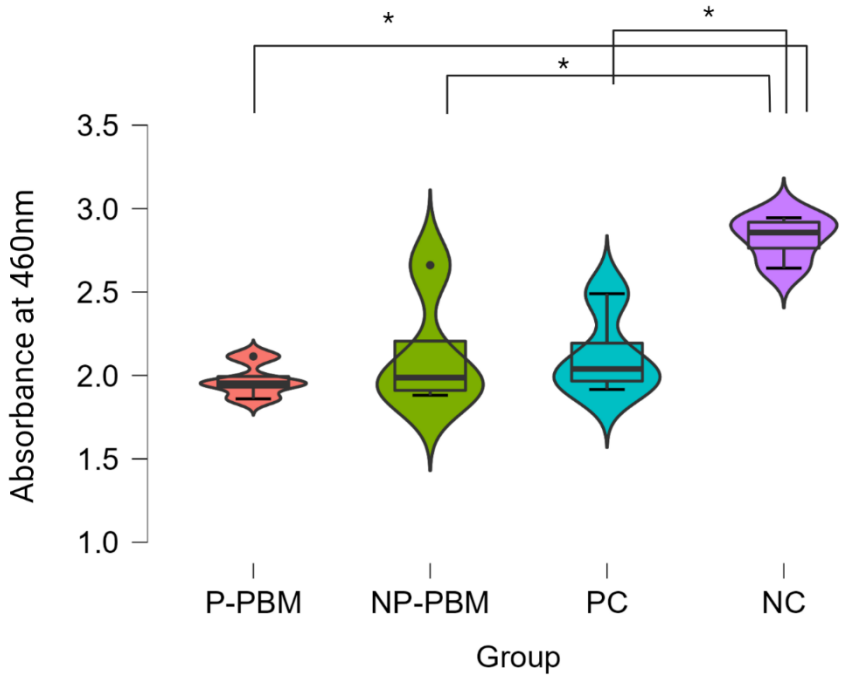

Figure 3. HFFF2 cell viability using the WST-8 assay. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

3.3. EdU Assay

The P-PBM group demonstrated a significant increase in total proliferating cells compared to the NP-PBM ($p = 0.029$) and the PC ($p = 0.006$) groups (Figure 4). The NC group demonstrated significantly increased proliferation compared to the P-PBM ($p < 0.001$), NP-PBM ($p < 0.001$), and PC ($p < 0.001$) groups.

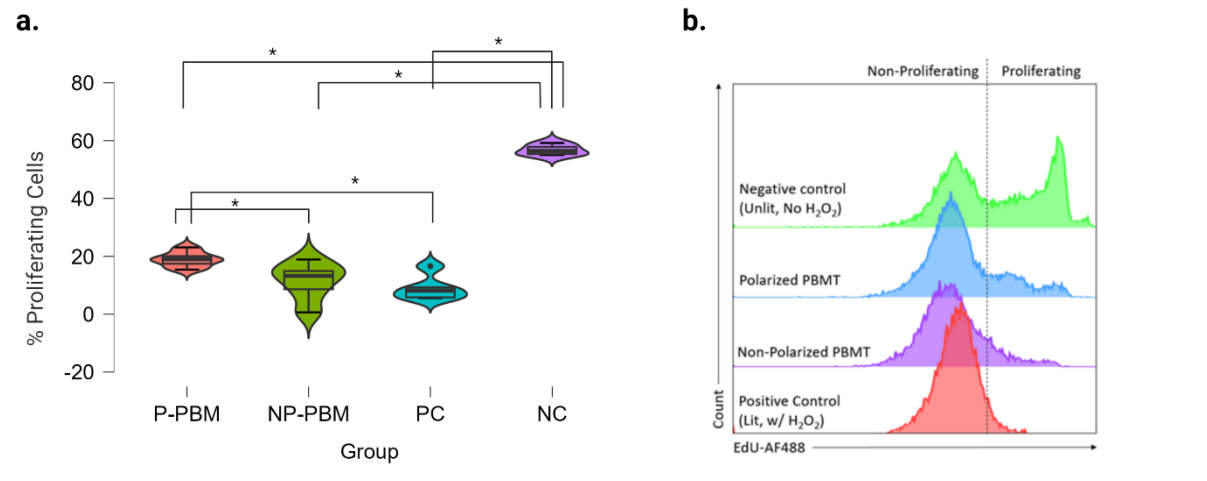

Figure 4. HFFF2 cell proliferation using EdU assays. (a) Percentage of proliferating cells, and (b) histogram of proliferating and non-proliferating cells. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates P < 0.05.

Upon assessing the different stages of the cell cycle (Figure 5) there were no significant differences between the groups with regards to percentage of cells in the G1 and Sub G1 phases. In the P-PBM group, there was a significant increase in the percentage of cells in the S-Phase compared to the NP-PBM (p = 0.034) and the PC (p = 0.014) groups (Figure 5a), while the NC group demonstrated a significant increase in the percentage of cells in the S-Phase cycle compared to all other groups (p < 0.001). Additionally, the percentage of cells in the G2-Phase was significantly decreased in the NC group, compared to all other groups (P < 0.001) (Figure 5b).

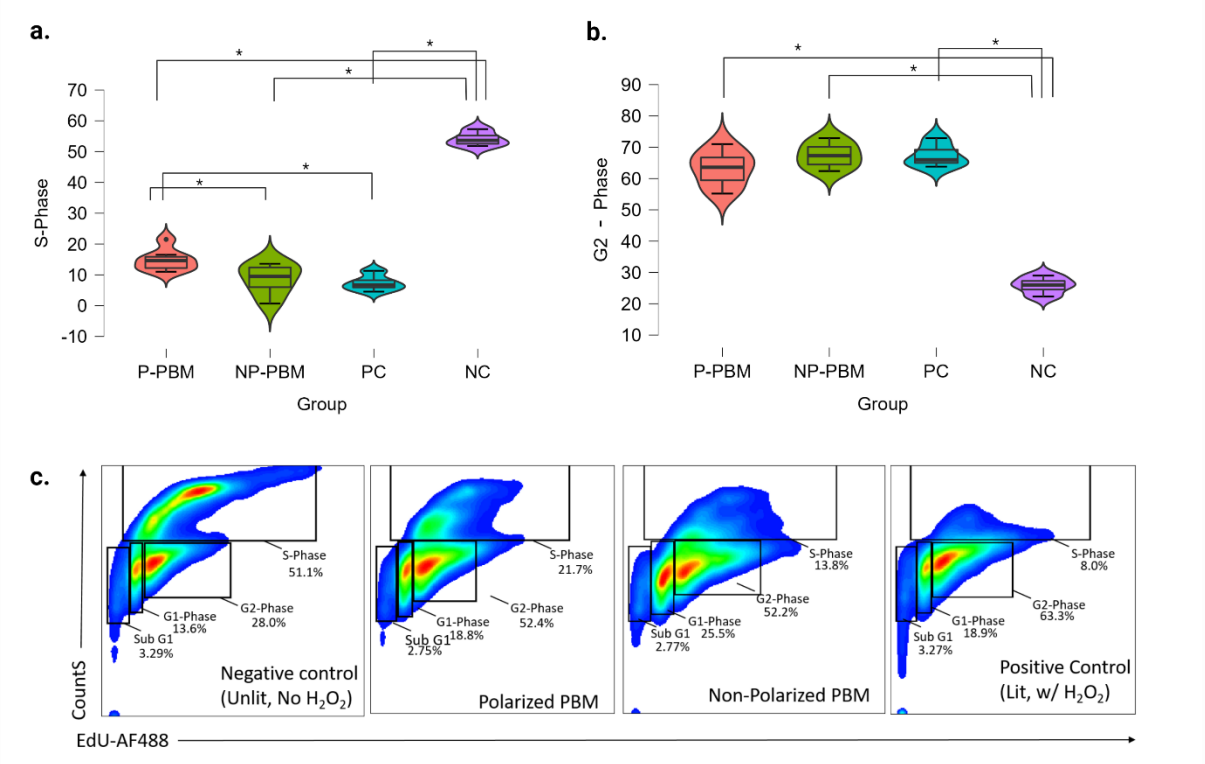

Figure 5. (a) Percentage of HFFF2 cells in the S cell cycle phase; (b) Percentage of HFFF2 cells in the G2 HFFF2 cell cycle phase, and (c) fluorescent dot plots of all HFFF2 cell cycles by percentage. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates P < 0.05.

3.4. Annexin V/PI Assay

Annexin V/PI assay was used to determine apoptosis of cells following PBM with or without polarization. There was a significantly higher proportion of healthy (non-apoptotic) cells in the NC group compared to the PC ($p = 0.003$) and NP-PBM groups ($p < 0.001$). There was also a significantly higher proportion of healthy cells in the P-PBM group compared to the NP-PBM ($p = 0.005$) group (Figure 6a). There were a significantly less proportion of early apoptotic cells in the NC group compared to the NP-PBM ($p < 0.001$) and PC ($p = 0.006$) groups (Figure 6b). Additionally, there was a significant decrease in late apoptotic cells in the NC group compared to the PC group ($p < 0.001$), and a significant decrease in late apoptotic cells in P-PBM group compared to the NP-PBM ($p = 0.019$) and PC ($p < 0.001$) groups (Figure 6c).

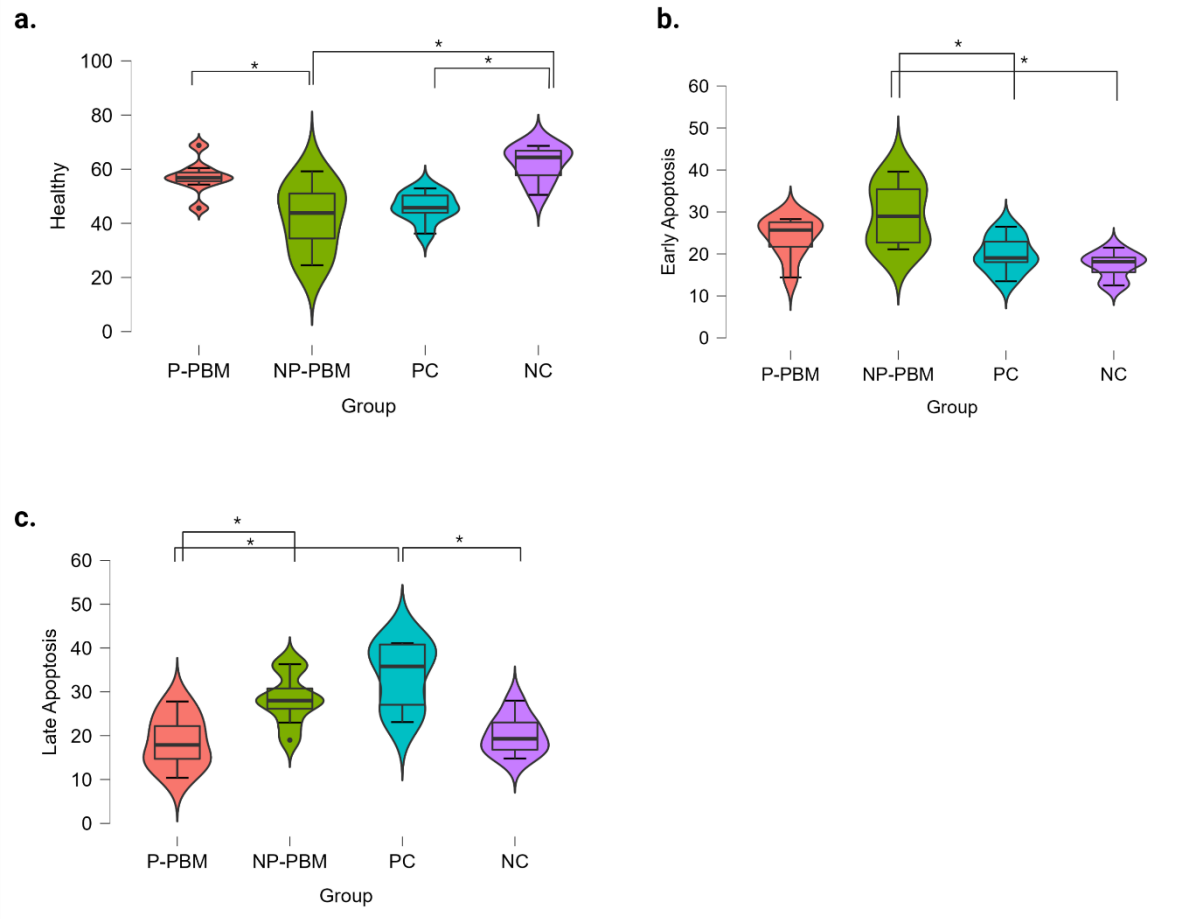

Figure 6. (a) Percentage of healthy (non-apoptotic) HFFF2 cells, (b) percentage of early apoptotic HFFF2 cells and (c) percentage of late apoptotic HFFF2 cells across all the groups. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

3.5. Mitoprobe Assay

Using the mitoprobe assay it was shown that P-PBM significantly increased ΔΨ when compared to the NP-PBM ($p = 0.003$) and the PC ($p < 0.001$) groups when analyzed as red/green fluorescence intensity ratio. The NC group was shown to have a significantly higher ΔΨ when compared to all other groups ($p < 0.001$) (Figure 7).

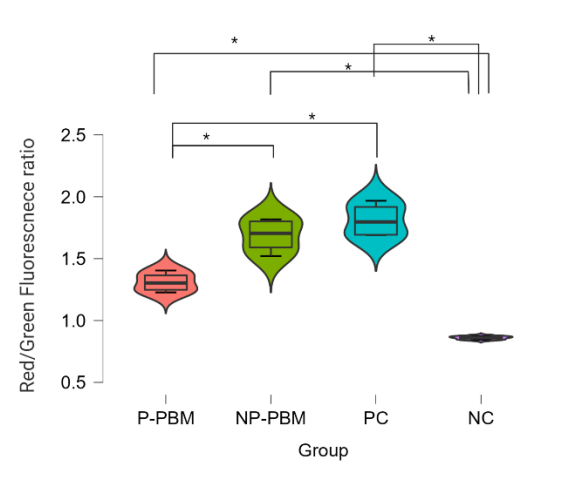

Figure 7. (a) Red/green fluorescence intensity ratio of HFFF2 cells. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

4. Discussion

PBM is a widely-used clinical therapy, and in particular, has been used extensively in the treatment of wounds and musculoskeletal injury and disease [33]. Despite the abundance of clinical evidence underpinning its use, there remains much debate regarding its fundamental physiological mechanisms, and the optimum irradiation parameters to best deliver its effects [23]. Hence, the aim of this study was to determine the effect of both polarized and non-polarized PBM on the viability, proliferation, ΔΨ and magnitude of apoptosis of fibroblasts undergoing oxidative stress. Overall, when compared to non-irradiated controls, P-PBM appeared to promote an increased proliferative, metabolic and protective effect when compared to NP-PBM and its matched controls.

Fibroblasts play a key role in mammalian wound healing, making them an attractive point of investigation for PBM research. Fibroblasts exist in a quiescent state throughout the body, until they are activated by chemoattractants and growth factors resulting from tissue damage

[44]. At this point they strongly interplay with the ECM through increased tensional forces contributing to the remodelling of the extracellular matrix, allowing increased cell replication, migration and differentiation [45]. Oxidative stress can be a driver of chronic dermal wounds, of which above optimal H_2O_2 levels can contribute to [46, 47]. Namely H_2O_2, can have a negative effect on fibroblast proliferation and migration, therefore negatively affecting wound healing [46, 48]. Interestingly, this work demonstrated that PBM, particularly when polarized, can negate some of the proliferative functional impairments that H_2O_2 can have on these cells, in the process highlighting potential advancements for this therapy. Furthermore, our results show an S-Phase block with the addition H_2O_2, with PBM appearing to attenuate this effect, particularly when polarized. This suggests that P-PBM may better help preserve cell metabolism and DNA structure so that more cells can bypass the G1 cell cycle checkpoint. Additionally, the NC group demonstrated a significant decrease in the percentage of cells in the G2 phase, which at first seems counterintuitive, but is likely due to the samples in this group undergoing further replication given they were uninhibited by the addition of H_2O_2.

Polarization of light is one of many PBM variables which remains under investigated [38]. Currently, there is evidence that suggests that compared to otherwise matched non-polarized light, polarized light may exert additional positive biological effects [38]. The results from this study appear to support this, demonstrating enhanced metabolic, proliferative and cytoprotective effects compared to both non-polarized and non-irradiated controls. When light interacts with biological tissue, it can be absorbed, reflected or transmitted, with absorption most prevalent in biological tissues [9]. Light absorption is also influenced by the total amount of light scattering, which is high in most biological tissues, particularly the dermis, due to the density and specific three-dimensional structure of collagen [49]. Previous research noted that in the superficial layers of the skin, polarized light can penetrate these tissues with minimal depolarization [50], but in denser biological tissues linear polarized light is maintained better than circularly polarized light [51]. Hence, it is thought that at the light-tissue interface, polarization may penetrate biological tissues more effectively, hence the enhanced biological effects. It has also been shown that when polarized light is aligned parallel to the orientation of biological tissue, it penetrates with more energy, than when perpendicular [42]. Despite this, and given the two-dimensional nature of this project, in addition to decreased light attenuation observed with polarization, there may be other biophotonic interactions which require further investigation responsible for the observed effects in this study.

Not only can PBM increase proliferation, it can also have a cytoprotective effect on cells under oxidative stress [33]. The present results demonstrate a higher proportion of healthy, non-apoptotic cells in the P-PBM group compared to NP-PBM, and a significant decrease in

late-stage apoptotic cells when comparing the P-PBM to the PC group, highlighting the potential benefits of polarization. There is limited research discerning the effects of PBM on cellular apoptosis, but these findings align with other studies that demonstrate changes to CASP enzyme signalling pathways when cells are exposed to H_2O_2 [29, 35]. ΔΨ on the other hand, is more extensively investigated, with multiple studies demonstrating PBMs ability to enhance cellular ΔΨ [11]. Following a similar pattern across all analyses in this paper, P-PBM again demonstrated an increased ΔΨ compared to the NP-PBM and PC groups. Given the relationship between cellular apoptosis, ΔΨ and cytochrome c, future research should explore these pathways in greater detail.

Cellular viability assays in PBM *in vitro* research are controversial due to them often being quoted as proliferation measures when they do not directly measure proliferation and due to the large variability in reported results [11]. The results of this study appear to exemplify this, although capable capturing large differences, such as that between the NC and PC groups, at this level, the effects of PBM may be more subtle, and the viability assay as appeared to miss important changes in cellular metabolism, which the EdU assay later detected. Although cellular viability assays can be used as a cheap and easy 'screening' assay at the outset of *in vitro* PBM research, they may have limited utility in the present setting.

Despite this study being designed to model a chronic wound environment using fibroblasts exposed to oxidative stress, *in vivo* wounds are much more intricate and dynamic than the *in vitro* setting, and hence the difference between settings should be taken into account when interpreting our findings. Additionally, when wounds are treated in the clinical setting, they are often treated multiple times weekly, over a number of weeks [52]. In the present study the cells were irradiated only once, due to the rapid pace at which dermal fibroblasts can reach confluency in culture. We suspect that the single dose was responsible for the small, but significant effects seen, which when extrapolated into the clinical setting, demonstrate promise. Furthermore, there was only one set of irradiation parameters used in this work, additional fluences may be beneficial in determining the optimum dose-response parameters of both polarized and non-polarized PBM. Several exciting further avenues could be explored from this research. Although we have demonstrated several mechanisms by which polarization may exert its effects, more specific genetic and metabolic pathways should be explored to further elucidate these. Ongoing translation into 3D *in vitro*, animal, and clinical studies are required to understand the full spectrum of the effects of polarization on PBM therapy. Although previous research has indicated that PBM only undergoes a small amount of depolarization in the early layers of skin [38], the amount of depolarization occurring and the cellular monolayer in this study is unknown, and could be a topic of future investigations.

Finally, further photonic investigations need to be performed to better understand the fundamental light-tissue interactions of polarized and non-polarized PBM alike.

5. Conclusion

PBM is a therapy that has a wide range of clinical applications, however research outlining its fundamental biological effects is lacking. Additionally, there remain a host of possible application variables that remain under investigated. This study demonstrated that despite having no effect of cellular viability, polarized PBM demonstrated increases in cellular proliferation and $\Delta\Psi$ compared to non-polarized and non-irradiated otherwise matched controls. Additionally, polarized PBM decreased the magnitude of cellular apoptosis brought about by oxidative stress. Taken together, these findings indicate that polarization may be a way to further augment the biological effects of PBM. Further research is needed to understand the full spectrum of effects brought on by PBM and polarization at both a biological and photonic level.

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Conflict statement

The authors have no conflicts of interest to declare.

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Chapter 8: Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts

Chapter context and preface

Few studies have investigated the changes in gene expression in human dermal fibroblasts brought about by PBM, with none investigating this in the context of polarization. This chapter, which is currently under review, details how polarized PBM can influence the transcriptome of fibroblasts, in the process uncovering novel mechanisms of action, which have broad implications for the fundamental mechanisms of PBM more broadly.

Post review notes: A typographical error was pick up by one of the examiners. The 0.5uM H_2O_2 concentration stated in the methods section was in fact 0.5mM. The publishing journal has been made aware of this error.

Additionally, the paper was reviewed and published post-initial thesis submission. The minor revisions from the peer reviewers have been actioned in this chapter, with a full pdf version of the paper located in Appendix 1.

Chapter 8: Declaration of Co-Authorship and Co-Contribution

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2. CANDIDATE DECLARATION

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Vasso Apostolopoulos	5	Writing - Review and Editing, Supervision	[redacted]	24/1/22
Fotios Sidiroglou	5	Writing - Review and Editing, Supervision		24/1/22
Jack Feehan	10	Analysis, Writing - Original Draft, Review and Editing, Supervision		24/1/22

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Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts

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Abstract

Background: Photobiomodulation (PBM), the therapeutic use of light, is used to treat a myriad of conditions, including the management of acute and chronic wounds. Despite the presence of clinical evidence surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear. There are several properties of light that can be altered in the application of PBM, of these, polarization—the filtering of light into specified plane(s)—is an attractive variable to investigate.

Aims: To evaluate transcriptomic changes in human dermal fibroblasts in response to polarized PBM.

Results: A total of 71 Differentially Expressed Genes (DEGs) are described. All DEGs were found in the polarized PBM group (P-PBM), relative to the control group (PC). Of the 71 DEGs, 10 genes were upregulated and 61 were downregulated. Most DEGs were either mitochondrial or extracellular matrix (ECM)-related. Gene Ontology (GO) analysis was then performed using the DEGs from the P-PBM vs. PC group. Within biological processes there were 95 terms found ($p < 0.05$); in the molecular function there were 18 terms found ($p<0.05$); while in the cellular component there were 32 terms enriched ($p<0.05$). A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p<0.05$). Finally, there were 24 significantly enriched reactome pathways when comparing the DEGs of the P-PBM vs. PC groups ($p<0.05$).

Discussion and Conclusions: The P-PBM DEGs were almost always down regulated compared to the comparator groups. This may be explained by the P-PBM treatment conditions decreasing the amount of cellular stress, hence causing a decreased mitochondria and ECM protective response. Alternatively, it could point to an alternate mechanism, outside the mitochondria, by which PBM exerts its effects. Additionally, PBM appears to have a more widespread effect on the mitochondria than previously thought, opening up many new avenues of investigation in the process.

Keywords: Photobiomodulation; low level light therapy; polarized light; fibroblasts; wound healing; RNA-seq; transcriptome

1. Introduction

Chronic wounds are a major burden on the health systems globally, with an estimated prevalence of 2.21 persons in every 1000 [1]. There are a number of treatments for chronic wounds including, debridement, standard and bioengineered dressings, and anti-microbial agents [2, 3]. Given the high prevalence, there have been several investigations into new, low-cost and minimally invasive therapies to aid in the management of these conditions, one of these being phototherapies [4]. There are numerous clinical applications of light therapy in use today, none more so than that of photobiomodulation (PBM). PBM is used to treat numerous of conditions in clinical practice—from wound healing to sports injuries [5]. Despite a significant body of clinical knowledge surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear [5, 6]. Currently the leading mechanistic model centres on mitochondrial Cytochrome c Oxidase (CcO), oxygen and nitric oxide (NO) [7]. In this model the red and infrared photons emitted during PBM interact with the chromophore CcO, in the process dislodging NO molecules, leaving oxygen to bind with CcO in their absence. This is thought to lead to an increase in overall ATP production by the mitochondria, subsequently responsible for the clinical effects seen with PBM [7, 8]. However, there has been no direct photonic interactions observed between CcO and PBM, and recent evidence suggests that PBM can exert its effect in the absence of CcO [9, 10], raising questions surrounding the fundamental mechanisms of PBM.

Regardless of the fundamental mechanisms underpinning PBM, there have been cellular effects resulting from its use in *in vitro* studies across a number of settings [4], however one area of significant study is the treatment of wounds. When examining the specific effects of PBM on wound healing, many PBM studies have investigated the effect of PBM on fibroblasts, due to their critical role in the process. Fibroblast survival and proliferation are crucial in the process of wound closure [11], and so have been widely investigated in the PBM field. While a range of fluences (Joules/cm^2) have been shown to increase these metabolic parameters, there are many conflicting findings, particularly when it comes to viability, highlighting the need for more stringent experimental parameters [4]. For example, PBM can influence multiple genes related to cell proliferation and wound healing such as vascular endothelial growth factor (VEGF) and genes related to collagen production (COL1AI, COL4A1, COL5A1) [4]. However, similar studies have also shown PBM to have no effect on these genes [12, 13], while some even decrease their expression [14]. Additionally, *in vitro* proliferation assays have been further established via work showing increased cellular migration brought on by PBM [4]. Given ATP production in the mitochondria is at the heart of the proposed mechanisms of PBM [6, 15], how it affects functional measures of mitochondrial substrate and energy production have been reported. Again, these studies

demonstrate that a range of fluence are able to increase both ATP production and mitochondrial membrane potential [4]. In addition, genes related to mitochondrial energy metabolism, have shown that PBM contribute to genes influencing the function of complexes I, IV and V, and hence energy production [16]. However, there remains debate around the illumination dose needed to illicit the maximum amount of mitochondrial function [17].

Beyond the fundamental mechanisms of PBM, there is conjecture surrounding the optimal method of delivering PBM both *in vitro* and clinically [10]. There are many variables that can be altered during the application of PBM, and include: beam area, irradiation time, fluence, power, polarization, wavelength, pulse parameters and treatment number, all which may modulate treatment outcomes [10]. Of these, polarization—the filtering of light waves whose electric field vectors move in a specific plane or planes—presents as an interesting variable to investigate [5, 18]. There is a small but growing body of research demonstrating polarization of light may provide additional biological efficacy in PBM [5, 19, 20]. This is thought to occur due to the polarized light having a greater level of tissue penetration, compared to equivalent non-polarized PBM [18]; however, further research is required to determine the therapeutic mechanisms of polarized light. Hence, the aim of this work was to profile the transcriptome of human dermal fibroblasts using RNA-seq to provide novel insights into how polarization of PBM affects gene expression.

2. Methods

2.1. Setting

All experiments were undertaken in standard laboratory conditions, in a PC2 facility at a public university in Victoria, Australia.

2.2. HFF2 fibroblast cell culture and experimental treatments

The human caucasian foetal foreskin fibroblast (HFFF2) (Cell Bank Australia NSW, Australia) cell line was used for all experiments. The cells were cultured according to the manufacturers recommended protocol, documented in previous works [21]. Due to the scattering of light that occurs in standard, clear-walled plates, cells were plated at $4x10^4$ cells per well in 500 µL of growth media in black-walled, 24-well plates (Eppendorf, Germany) [22]. To induce oxidative stress, the cells were treated with 0.5 µM of hydrogen peroxide, twenty-four hours after seeding [23]. Immediately after peroxide treatment, the cells were exposed to PBM at a fluence of 1 J/cm^2 (A full description of the light parameters used is presented in Table 1). Three treatments were used to compare effects, with four technical replicates used in each. The treatments were polarized light (P-PBM); non-polarized light (NP-PBM); and a no-light

control (positive control - PC), with all exposed to the hydrogen peroxide stressor. 24 hours post irradiation, the RNA extraction was performed as described below.

2.3. Light Source

The light source used for experimental treatment was a fiber coupled 670 ± 5 nm BWF laser diode (B&W Tek, Delaware, USA) (Table 1). The fluence dose used in the treatments was calculated as described previously [21]. A linear, 25 mm glass filter (Edmund Optics, New Jersey, USA) was used to polarize the laser diode. The laser output power was appropriately adjusted in both polarized and non-polarized treatment setting to ensure consistent light treatment parameters across all experimental wells. [18, 21].

Manufacturer	B&W Tek
Model	BWF1
Emitter	Laser Diode
Class	III B
Pulse Mode	Continuous wave
Wavelength	670 nm
Distance from target	80 mm
Target spot size	1.9 cm^2
Power at target site (mW)	11.2
Exposure Duration (sec)	169
Total Fluence per site (J/cm^2)	1

Table 1: Laser system and fluence parameters

2.4 RNA Sequencing

RNA was extracted with an RNeasy mini-kit according to the manufacturer's instructions (Qiagen, USA), and immediately stored at -80°C until sequencing. RNA sequencing was performed by the Micromon genetics facility (Monash University, Melbourne, Australia). RNA quality was assessed via Agilent Bioanalyzer electrophoresis and Qubit fluorometer (Invitrogen, USA). A minimum of 2 μg of total RNA underwent library preparation and sequencing. Secondary quality control pf the RNA was performed using the AATI fragment analyzer prior to sequencing to asses for possible degradation of the samples during transport and/or preparation (Invitrogen, USA).

2.5 Statistical Analysis

Raw files were analysed using the RNAsik pipeline [24] utilising STAR aligner [25] with the Genome Reference Consortium Human Build 38 (GRCh38; Homo sapiens) genome reference. Feature Counts was employed to quantify the reads [26] producing the raw genes count matrix and various other quality control metrics. Raw counts were then analysed with Degust [27], which performed the normalisation using trimmed mean of M values [28], and differential expression analysis using limma/voom [29]. Differentially expressed genes (DEGs) were obtained using a False Discovery Rate (FDR) ≤ 0.05. Functional enrichment analysis (GO, KEGG and reactome pathways) was performed using STRING-db [30], where the data were exported and plotted using either SR plot and ggplot packages. Enrichment groups were considered significant at $p<0.05$.

3. Results

3.1 RNA Quality Control

The RNA integrity number of all samples was ≥9.9, representing high sample quality (Supplementary Figure 1). The mean Phred score was 36 across the samples, indicating >99.9% accuracy across sequencing reads (Supplementary Figure 1). Additionally, the size of each RNA library, distribution of p-values and normalized expression were all within acceptable limits across all samples (Supplementary Figure 1). The fourth NP-PBM was excluded as it was an outlier in the MDS analysis.

3.2 Screening Analysis of DEGs

There were a total of 71 (from 16280) DEGs when each experimental group was compared only to the control group (FDR <0.05). All these DEGs were found in the PPBM group, relative to the PC group (Figure 1). Of the 71 DEGs, 10 were upregulated and 61 were downregulated (Table 2).

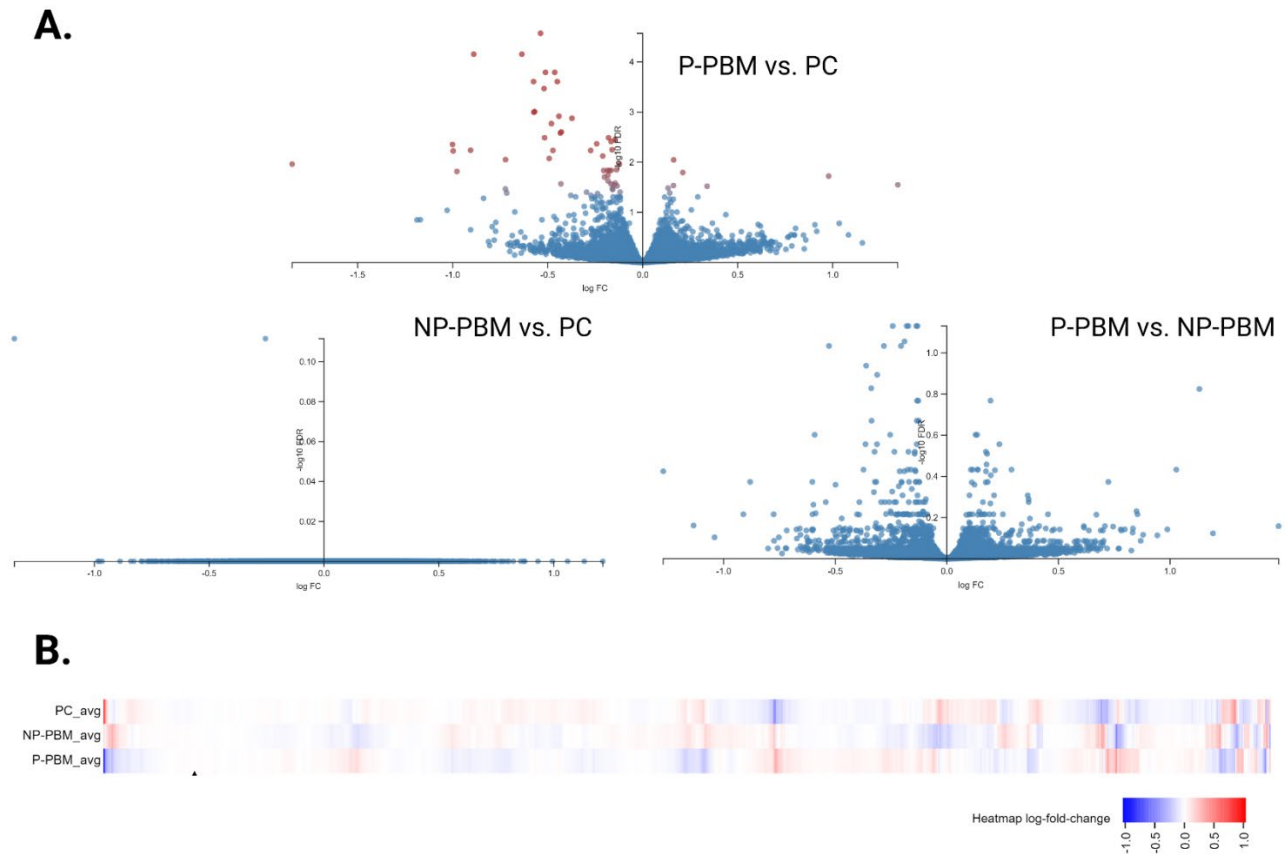

Figure 1: A. Volcano plot analysis of all genes analyzed across all groups. **B.** Heat map analysis of all genes analyzed across all groups. Figure sourced from the Degust bioinformatics platform.

Group Comparison	Upregulated DEGs	Downregulated DEGs	
P-PBM vs. PC	AC048341.2	ACTC1	MT-CYB
	AKR1B1	AMOTL2	MT-ND1
	AKR1C1	C1orf198	MT-ND2
	CLU	CLDN1	MTND2P28
	LAMB3	COL1A1	MT-ND3
	MIR199A1	COL4A1	MT-ND4
	PCNA	COL4A2	MT-ND4L
	PHLDA3	COL5A1	MT-ND5
	S100A4	CTGF	MT-ND6
	Z74021.1	CYR61	MT-RNR1
		DCLK2	MT-RNR2
		DDAH1	MT-TC
		DIO2	MT-TE
		FZD7	MT-TH
		GOPC	MT-TI
		IGFBP3	MT-TS2
		LAMA4	MT-TV
		LDLR	MT-TW
		LMO7	MT-TY
		LMOD1	NRBP2
		MARCKS	P3H2
		MIR100HG	PCNA
		MRVI1	SMAD3
		MSRB3	SSBP4
		MT-ATP6	SULF1
		MTATP6P1	TAF10
		MT-ATP8	THBS1
		MT-CO1	THBS2
		MTCO1P12	TPM1
		MT-CO2	TXNDC5
		MT-CO3	

Table 2: Full list of both upregulated and downregulated DEGs.

3.3 Network Pathway Analysis

There were two main gene association clusters found on network pathway analysis using the DEGs from above. The first involved mitochondrial genes associated with energy production, whilst the second involved genes associated with the ECM and collagen production (Figure 2).

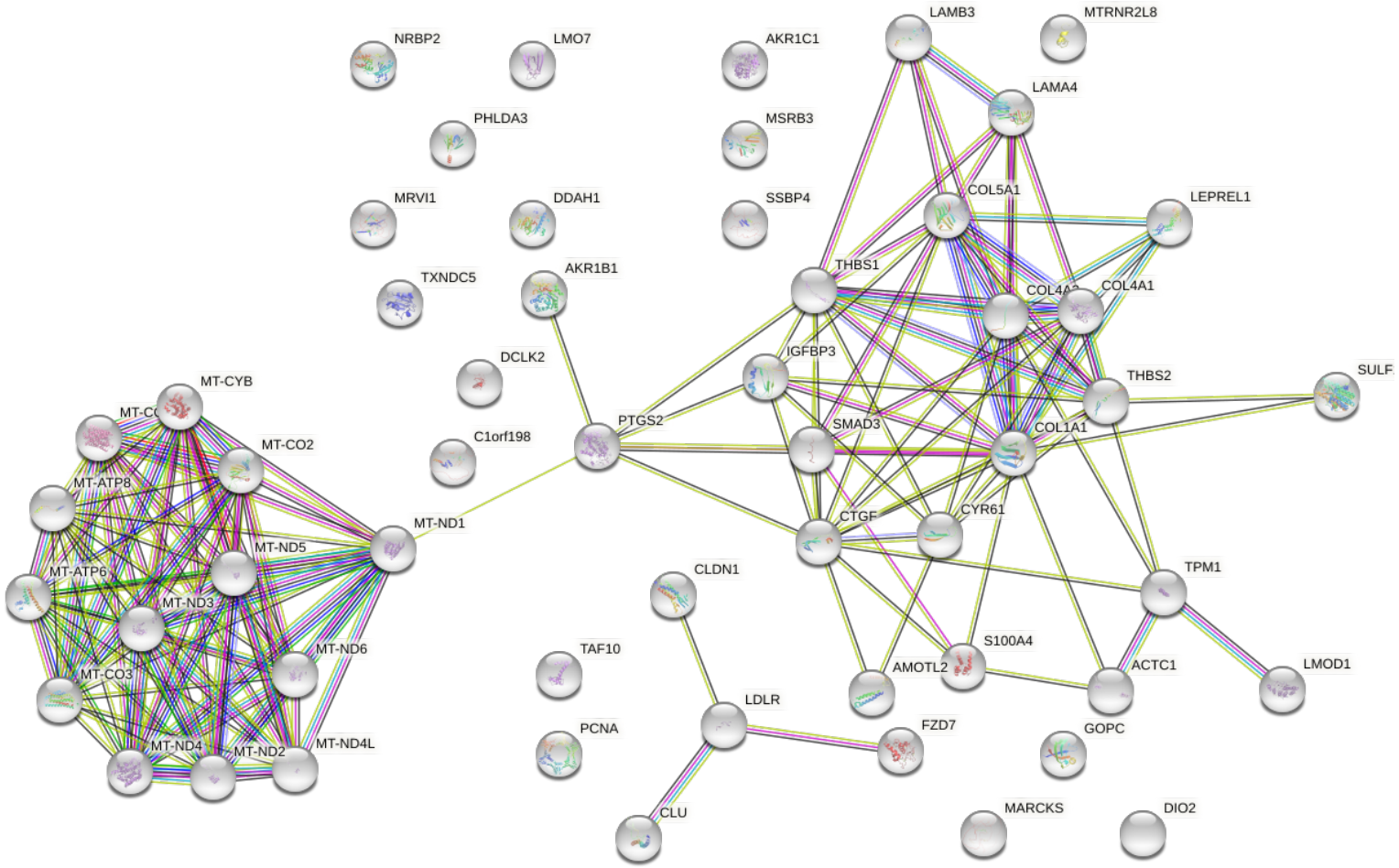

Figure 2: StringDB Network Analysis using DEGs. Light blue lines indicate known interactions from curated databases; Pink lines indicate experimentally determined known interactions; Dark green lines indicate gene neighbourhood predicted interactions; Red lines indicate predicted interaction from gene fusions; Dark blue lines indicate gene co-occurrence predicted interactions; Light green lines indicate text mining interactions; Black lines indicate co-expression interactions

3.4 Functional Enrichment Analysis

Gene Ontology (GO) analysis was performed using the DEGs from the P-PBM vs. PC group. In the biological process ontology there were 95 significant terms found ($p < 0.05$); in molecular function there were 18 terms ($p<0.05$); and in the cellular component ontology there were 32 terms found ($p<0.05$) (Figure 3).

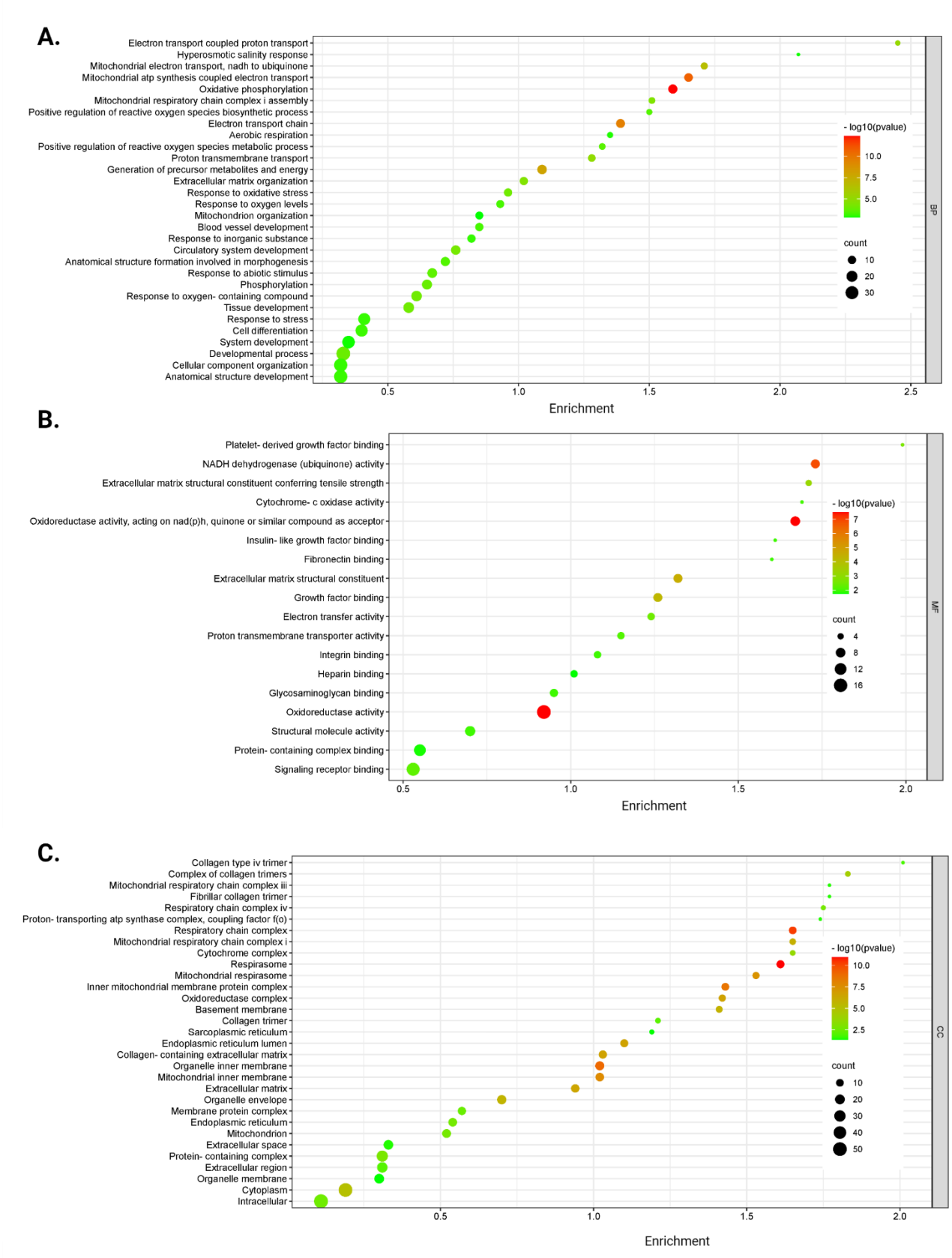

Figure 3: A. Top 30 Biological Process (BP) GO terms. **B.** Significantly enriched Molecular Function (MF) GO terms. **C.** Top 30 Cellular Component (CC) GO terms. Figure created with https://www.bioinformatics.com.cn/en

A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p<0.05$) (Figure 4).

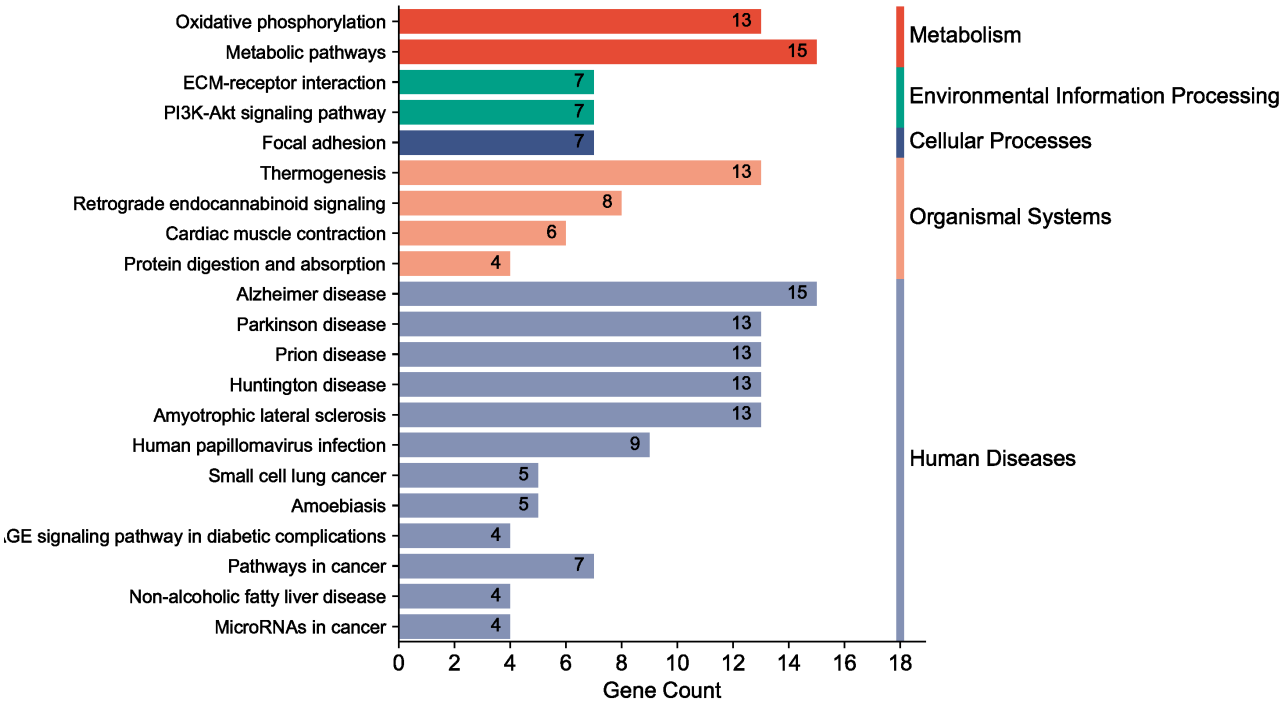

Figure 4: KEGG pathway analysis using DEG count. Figure created with https://www.bioinformatics.com.cn/en

Finally, there were 24 significantly enriched reactome pathways found when comparing the DEGs of the P-PBM vs. PC groups ($p<0.05$) (Figure 5).

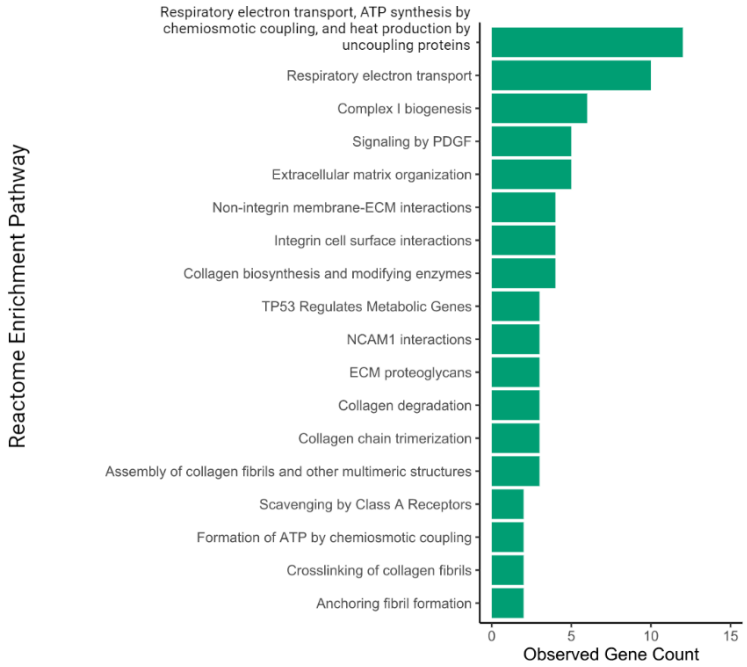

Figure 5: Reactome pathway analysis using DEG count. Figure created using ggPlot.

4. Discussion

PBM is a commonly employed intervention across multiple areas of clinical practice, often producing tangible clinical benefits. Despite this widespread use, there remains conjecture around the fundamental biological mechanisms responsible for the clinical effects observed [10]. As such, the transcriptome of human dermal fibroblasts were profiled following their exposure to oxidative stress, in response to both polarized and non-polarized PBM. The overarching results demonstrated that, P-PBM can influence the expression of multiple genes, mostly associated with the mitochondria and ECM, which relate to a number of important ontological and functional pathways.

The current leading mechanistic model of PBM centres on the mitochondria. To our knowledge, this is the first study which has investigated the mitochondrial transcriptome of human dermal fibroblasts in response to PBM. Interestingly, all the mitochondrial DEGs were downregulated when exposed to P-PBM. Previous research demonstrated that in healthy cells, and cells grown in ischaemic and diabetic models, PBM produces an upregulation in genes encoding for enzymes involved in ATP synthase and complexes I and IV [16]. That said, the previous works analyzed nuclear

mitochondrial-related genes, as opposed to the specific mitochondrial genes analyzed in this study. Given how susceptible the mitochondrial genome is to oxidative damage [31], in addition to the known cellular protective effects of PBM [21, 32], we propose that the downregulation of mitochondrial DEGs may have been caused by PBM ameliorating some of the effects brought on by the addition of an oxidative stress-inducing agent—H_2O_2. Recent findings have also cast doubt on the CcO-NO-ATP model of PBM as the sole mechanism underpinning its effect, demonstrating that PBM increased cellular proliferation and other metabolic parameters similarly in cells both with and without CcO [9]. Taken together with our findings, it appears that PBM fundamentally influences mitochondrial function, but it may be that it influences other areas of the mitochondria equally, or more so than CcO.

Currently, much research has focused on the efficacy of PBM in the treatment of dermal wounds [33-35]. Fibroblasts play a key role in this, by being stimulated from a mostly dormant state, in response to factors released in response to tissue damage [36]. They play an integral part in the integrity of the ECM in healing tissue by increased tensional forces brought about by their contractile capacity [37]. Importantly, one of fibroblasts chief functions is to produce the collagen matrix—the main structural component of connective tissue, which ultimately helps form focal adhesion complexes, which have important regulatory and structural functions [38]. The collagen-related and other ECM-related DEGs in this study, were universally downregulated, which conflicts with some, but not all of the findings relating to ECM-related gene expression in PBM exposed fibroblasts [4]. Several studies within the field, have demonstrated that collagen, and other ECM-related genes can either be upregulated, unchanged, or downregulated by PBM within acceptable fluence levels [4, 13, 22]. This is likely due to experimental inconsistencies, chiefly being, irradiation timings and cellular growth conditions [4, 10, 39]. It appears that the timing of PBM in response to cellular stress or damage is important, as the known protective effects of PBM, such as apoptosis inhibition [21, 40, 41], may more effectively inhibit cellular damage when applied closer to the initiating cellular stressor. This raises important clinical implications for the treatment of both acute and chronic dermal wounds, with timing of PBM application in relation to these conditions remaining underexplored.

This work has identified several functional ontological pathways which are influenced by PBM and relate to both cellular metabolism and wound healing. All the mitochondrial DEGs which were downregulated contribute to the ontological processes and pathways concerned with oxidative phosphorylation, ATP synthesis and the electron transport chain. More specifically, the Mitochondrial respiratory chains I, III, and IV cellular

component ontological pathways, as well as the Reactome pathway Complex I biogenesis were significantly enriched with the downregulated DEGs, further supporting the notion that PBM can influence multiple parts of the mitochondria, not only CcO [9]. Furthermore, there were multiple significantly enriched pathways associated with the ECM and wound healing processes including ECM organisation, structure, and interactions, collagen formation and biosynthesis, and integrin binding and interactions. Taken together these pathway analyses demonstrate that PBM has a strong influence on multiple areas of mitochondrial energy production, and pathways associated with wound healing, revealing many avenues for further research.

Interesting among the findings of this study, was the superiority of polarized light over equivalent non-polarized PBM. Work by ourselves and others has demonstrated that when compared to non-polarized, otherwise matched PBM, P-PBM can increase cellular viability and proliferation, decrease apoptosis, increase mitochondrial membrane potential and increase functional outcomes post-spinal injury in mice [5, 18, 21, 42]. The present results follow this trend, with P-PBM demonstrating the most profound influence on gene expression. The mechanisms underpinning these changes in PBM efficacy that polarization can affect are not fully understood, but currently it is thought that polarized light may present a way to better penetrate biological tissue through minimizing light attenuation, possibly through reduced light scattering, and therefore, be able to exert its effects more efficiently [5, 18]. This effect may be further enhanced when the plane of polarization is aligned to the tissue histological orientation [18]. Despite these findings, further research is required to determine the exact biophotonic interactions at play.

4.1 Limitations and future research

Although the methodological processes of this project being stringently controlled, there are some limitations we would like to acknowledge. One of the NP-PBM replicates was excluded from the analysis due to it being an outlier. This may have influenced the magnitude of gene expression in the NP-PBM group, however, the results in this study reflect our previous work demonstrating that P-PBM has a greater effect on cellular metabolic and regenerative function compared to NP-PBM and experimental controls [21]. Furthermore, the *in vitro* model of wound healing that was employed in this study, may not fully reflect the clinical treatment of wounds, with them often undergoing multiple exposures to PBM. These findings open many exciting avenues for future research. Firstly, the numerous significantly enriched ontological pathways found could be further explored to confirm if they translate to functional

cellular changes. Secondly, these experiments could be replicated with other wavelengths and intensities, as well as being translated to 3D *in vitro* cell cultures, animal studies and clinical translation studies to determine the full scale of effects that P-PBM and NP-PBM can have on wound healing. Finally, it is important to note that changes in gene expression doesn't necessarily reflect changes in downstream protein expression, hence, these could be further explored in future research.

5. Conclusion

PBM is a widely used therapy for a number of clinical conditions, including wounds, however, both the exact fundamental mechanisms underpinning its effects, as well as the optimum irradiation conditions remain unclear. The leading mechanistic theory of PBM is centred on increasing the efficiency of mitochondrial CcO. This study has shown that PBM, specifically when polarized, can have a more generalized effect on mitochondrial energy production, affecting multiple mitochondrial complexes, not only complex IV, which aligns with more contemporary PBM research. Additionally, this work supports other fundamental and clinical literature by identifying that PBM can strongly influence the pathways that influence the ECM and therefore wound healing. Further research should explore the cellular and molecular pathways identified herein, to continue to build a better understanding of the fundamental mechanisms of PBM.

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Conflict statement

The authors have no conflicts of interest to declare.

Author contributions

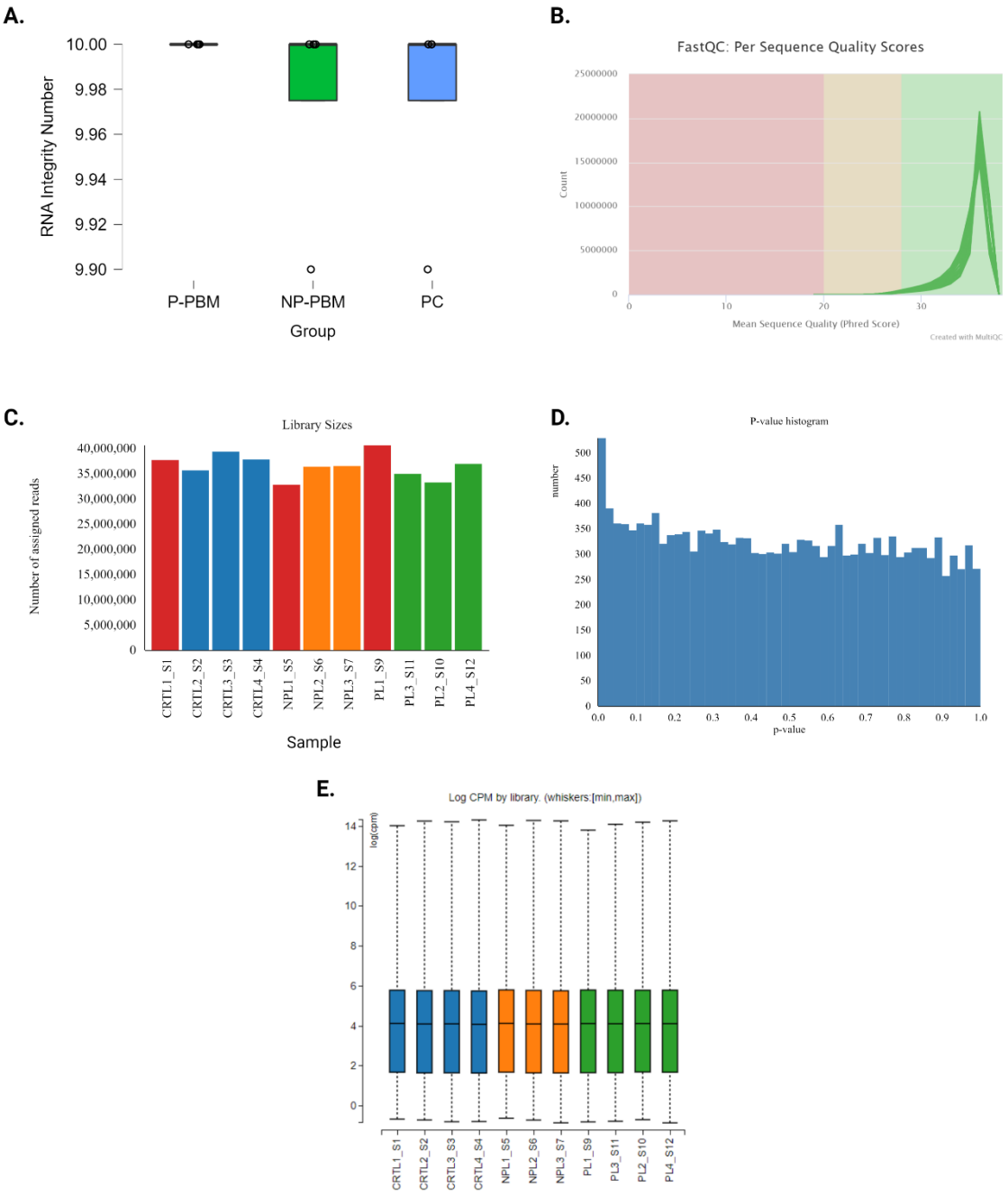
Nicholas Tripodi: Validation, methodology, Formal analysis, resources, data curation, writing – original draft, Writing- review and editing, project administration.

Fotios Sidiroglou: Formal analysis, Supervision, Writing – review and editing

Vasso Apostolopoulos: Formal analysis, Writing – review and editing, resources, supervision, and project administration

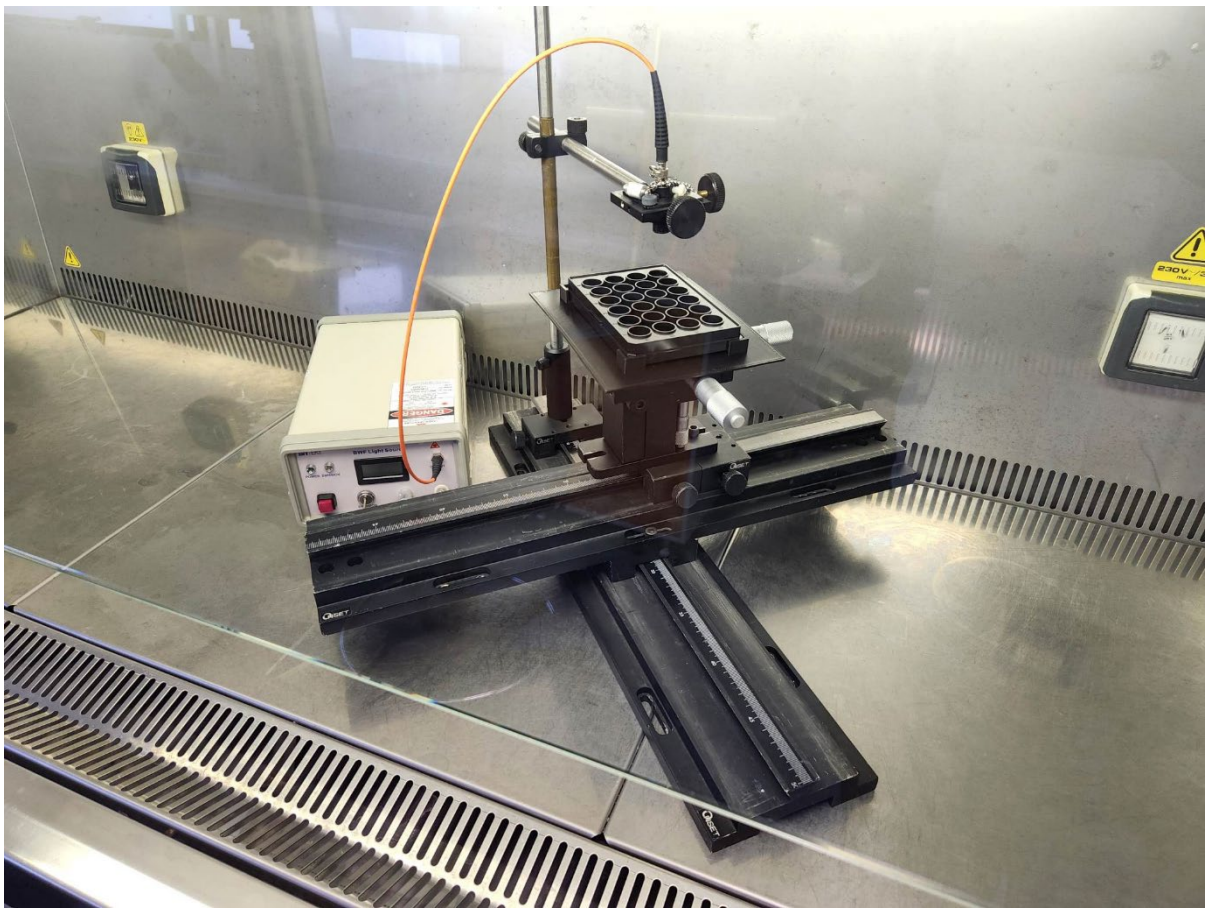
Jack Feehan: Validation, methodology, Formal analysis, resources, data curation, writing – original draft, Writing- review and editing, supervision, and project administration.

Appendix



Supplementary Figure 1: A. RNA Quality Assessment; **B.** Phred quality score of analysis of base calling; **C.** Sequencing library size distribution; **D.** Distribution of p-values throughout the sample; **E.** Normalized expression intensity. P-PBM: Polarized Photobiomodulation; NP-PBM: Non-Polarized Photobiomodulation; PC: Positive Control.

Supplementary Figure 2 – Image of PBM Irradiation Procedure



Supplementary Figure 2: Custom stage built for PBM irradiation

Supplementary Figure 3 – List of Abbreviations

ATP	Adenosine Triphosphate
BP	Biological Process
CC	Cellular Component
CcO	Cytochrome C Oxidase
DEGs	Differentially Expressed Genes
ECM	Extracellular Matrix
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide
HFFF2	Human Caucasian foetal foreskin fibroblast
J/cm^2	Joules per Centimeter Squared
KEGG	Kyoto Encyclopedia of Genes and Genomes
MF	Molecular Function
NO	Nitric Oxide
NP-PBM	Non-Polarized Photobiomodulation
PC	Positive Control
PBM	Photobiomodulation
P-PBM	Polarized Photobiomodulation
RNA	Ribonucleic acid
VEGF	Vascular Endothelial Growth Factor

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Chapter 9: General Discussion

Chapter context and preface

Given that each chapter has its own detailed discussion, to avoid repetition, the general discussion of this thesis will highlight the key findings and their implications by tying each chapter together in a cohesive narrative. It also discusses, in detail, the project limitations and importantly, what future research should be done to further elucidate the fundamental mechanisms of PBM.

General Thesis Discussion

This thesis details a project that has comprehensively explored the fundamental biological mechanisms of photobiomodulation (PBM), as well as the effect of altering its photonic properties, in this case, polarization. Beginning with a detailed review of the PBM field of literature, it then explored and documented a number of cellular and molecular pathways modulated by PBM, as well as establishing a reproducible and reliable set of irradiation and cell culture protocols and. Ultimately, it is hoped that this work will improve the understanding of PBM at both a fundamental and clinical level, and lead to improvements in the management of many diseases in which PBM might be used as both a primary and adjunctive therapy.

The rate of chronic wounds is on the rise [1]. This is due to a number of factors, with the ageing population and associated co-morbidities being two of the more important drivers [1]. With the economic burden of chronic wounds growing at an alarming rate, clinicians and policymakers alike have cast their eye to less invasive and cheaper therapies to deflate the ballooning costs associated with treatment. Phototherapy, and more specifically PBM is one of the non-invasive and cost-effective treatments which have the potential to contribute to the improved management of a wide-range on conditions, as well as saving tax payers millions of dollars globally. With phototherapy and PBM being used to treat a wide range of conditions, from chronic wounds and neonatal jaundice all the way to sports injuries [2], it is clear that they have a wide clinical utility. Despite this broad range of clinical applications, there remains a disconnect between the clinical evidence and the physiological underpinnings of PBM, with research yet to fully elucidate the full spectrum of PBMs effects at a mechanistic level [3, 4]). Furthermore, there remain many questions surrounding which light properties should be manipulated to best attenuate the biological effects.

Mitochondrial effects

The most widely reported mechanistic theory concerning the biological mechanisms of PBM is centred on the stimulation of the Cytochrome c Oxidase (CcO) molecule, also known as complex IV, contained within the mitochondria [5]. CcO is the terminal enzyme within the mitochondrial respiratory chain, with its main function being to facilitate the reduction of oxygen, and therefore ultimately contribute to energy production [6]. Nitric Oxide (NO), a known inhibitor of CcO [7], is thought to be displaced from CcO in the presence of red and near-infrared (600-1000nm) light [3, 5].

However, there is limited research to support this notion [5]. More recently, research has cast doubt on this model demonstrating that in both a mouse model of CcO knockout, and in human cells with a CcO mutation, the enzyme was not required to increase cellular proliferation and ATP production [3]. Adding to this, as described in chapter 8, we are the first to demonstrate, that in human dermal fibroblasts, PBM regulates expression in the mitochondrial genome, while also influencing the functional enrichment of pathways related to multiple parts of the mitochondria concerned with energy production. Specifically, we have shown PBM influences mitochondrial complexes I, III, and IV as well as ATP synthase, all of which play critical roles in metabolic energy production. These findings are further strengthened via the downstream effects of increased mitochondrial membrane potential ($\Delta\Psi$) demonstrated in chapter 7. Taken together, these results are important, as when contextualised within the current field of research, demonstrate that the effect of PBM on the mitochondria is more widespread than previously thought, in the process opening up a number of future avenues for further research.

Extracellular Matrix (ECM) effects

Throughout the PBM literature, there are a myriad of results describing the effects it can have on the ECM and its related processes. This has been shown across multiple *in vitro* measures, such as gene and protein expression and cellular proliferation and migration [8], as well as clinically, with PBM being used to improve tissue deposition in wounds [9]. This work, centred on fibroblasts, a cell that plays a key role in the ECM and production of collagen, and by extension wound healing, in a model designed to cause oxidative stress. This work has broadly reflected the positive biological effects that PBM can have on ECM healing and collagen remodelling seen in the literature. The transcriptome analysis done in this work has discovered a number of novel significantly enriched ontological pathways related to the ECM and its constituents. Specifically, pathways such as ECM organisation, structure, receptor organisation, and tensile strength, integrin cell surface interactions, collagen degradation, cross linking and trimer, amongst others were all significantly enriched. These novel findings were again further reinforced with the work done in chapter 7, which demonstrated that PBM, especially when polarized, brings about an increase in cellular proliferation, and also favourable cell cycle progression, namely at the G1 cell cycle checkpoint. Taken together, these results clearly demonstrate PBM's applicability to wound healing

interventions, and show that polarization maybe be able to further improve the clinical efficacy of PBM.

Cytoprotective Effects

PBM is also thought to have cytoprotective properties, chiefly through its effect on cellular apoptosis, and its associated pathways [10, 11]. Although there is limited research in this area, previous work has shown that when cells are exposed to H_2O_2 *in vitro*, PBM results in a decrease in apoptotic enzyme activity—namely CASP 3 and CASP 8 activity [12, 13]. These previous works, in part, informed the model used in the current project to simulate oxidative stress, similar to that seen in a chronic wound environment. Although specific apoptotic pathways were not investigated in this project, it did show that when polarized PBM was applied, it resulted in a significant decrease in the percentage of apoptotic cells, when compared to both the non-polarized and non-irradiated control groups by Annexin V/PI assay. Two further findings in this project also closely follow the findings of the Annexin V/PI assay. Firstly, it is known that decreases in ΔΨ can be a marker of increased cellular apoptosis [14]. Given the increases seen in ΔΨ within this work, it appears likely that the influence of PBM on the mitochondria, is at least in part responsible for the cytoprotective effects seen. Secondly, all of mitochondrial genes that were differentially expressed in chapter 8 were down regulated. This suggests that again, the cytoprotective effect brought on by PBM may alter the gene regulation via attenuation of oxidative stress, however, more research is need to elucidate the exact pathways and processes involved, specifically, how PBM may attenuate potential damage to the mitochondrial genome, given its high susceptibility to oxidative stress [15].

Polarization effects

Beyond the fundamental biological mechanisms of PBM, there remains much conjecture about the optimum set of light properties to apply both *in vitro* and *in vivo*. In this, it is important to consider a well-known and accepted concept within the field of PBM—the dose-response curve [16]. Fundamentally, it states that the biological efficacy of PBM will trend positively up until a given intensity or fluence, at which point it will plateau, and if the intensity continues to rise, will start to trend negatively [16]. In the cell line utilised, the optimum dose-response appears to be from 0.5-10 J/cm^2 [8]. This appears to be consistent across many cell lines and clinical settings, and barring

the extremes of light power intensities, fluence seems to be the main driver of the dose-response curve [17]. Hence, this project cast its eye towards other light properties of PBM to investigate. Properties such as wavelength, pulse characteristics, treatment cycles, beam area and polarization were all considered in the project design. Of these, the potential of polarization, given some of the preliminary evidence surrounding its possible efficacy was chosen to investigate.

Across virtually all biological measures performed in this project, the group that was subject to polarized PBM consistently, and significantly, outshone both the non-polarized, and non-irradiated groups, despite being exposed to otherwise identical fluence conditions. Researchers have proposed that the increase in biological efficacy seen from polarization, results from increased tissue penetration, particularly when applied parallel to the tissue orientation [2, 18, 19]. While this may be true for animal and clinical models, it does not fit within the two-dimensional cell culture setting of this thesis, and therefore raises the strong possibility that there are other biophotonic interactions that may be responsible for the effects seen in this work. Although generally not present in mammals, there are some species of both vertebrates and invertebrates that have developed linear-polarized light vision [20]. Based on a number of anatomical and neurological adaptations, researchers postulate that these creatures developed this ability to improve the contrast of their vision and hence provide added navigation clues [20]. Knowing this, in addition to the positive effects seen in polarized PBM, it has been postulated that there could be similar adaptation housed within PBM photoreceptors, namely dermal opsins, mitochondrial cytochromes and nanostructured water clusters [21]. Each of these photoreceptors in principle, could become ordered in structure to enhance their ability to absorb polarized light, given their geometric constraints [21]. Therefore, future research should investigate each of these receptors to better understand the results seen in this study.

Limitations

Throughout the PBM literature, there are a host of experimental inconsistencies and underreported application variables that have plagued the field for a number of years, making experimental replication challenging [4, 22]. Although, there is an accepted set of clinical treatment standards—the World Association for Photobiomodulation Therapy (WALT) guidelines [23], no such guidelines exist for *in vitro* PBM research [4, 8]. It is recommended that all *in vitro* PBM research report the variables of: wavelength, power,

irradiation time, beam area (at the culture surface), fluence, pulse parameters, number of and intervals between treatments, polarization and make and model of light device used [4]. It is also recommended that all PBM studies have input from an optical physicist, to ensure proper oversight and calculations of all light parameters [4]. This project accepted and implemented all these recommendations to improve experimental consistency and also to allow other research groups to replicate this work. Although there are some inevitable variabilities between experiments, this project implemented a number of protocols to limit possible sources of inconsistency. Firstly, the fully customised light stage was not only extensively piloted and calibrated (Chapter 5), the light stage measurements were all calculated under tolerances of less than a tenth of a millimetre. Furthermore, before each experiment the power of the light source was checked using an appropriate power meter, to ensure reliable and consistent fluence deliveries across all experiments.

Although there can be some well-to-well and plate-to-plate variability with all *in vitro* work, the experiments conducted in this project were all undertaken methodically and systematically, to make sure each time the cells where cultured and treated, they were exposed to identical conditions. Another source of potential result variability came from the addition of H_2O_2 to the cell treatment protocol. This was done to induce oxidative stress in the cell cultures, to model what can occur in chronic wounds clinically. This was introduced in Chapter 6, and refined in Chapter 7 as well as being informed by the literature [13]. Given the results described in Chapter 7 onwards, the addition of H_2O_2 does not appear to be a major limitation in this study, and in fact, seems to have amplified the efficacy of PBM further, when compared to a normal cell culture model. That said, the model used in this project, is exactly that—a model, and may not fully represent the multi-cellular and complex nature of chronic wounds *in vivo*. Fibroblasts are a heterogeneous cell population, and while many lineages have been identified, their functional nuances remain under investigated [24]. Therefore, it may be that the results in this project are only applicable to the specific fibroblast cell line used.

Finally, most PBM *in vivo* applications undergo multiple treatment cycles, while each experiment in this project was subject to only one treatment cycle. The single treatment cycle was used for two reasons: 1. The short amount of time it took the cell type used to reach confluency, and 2. To eliminate a possible source of well-to-well and plate-to-plate variability. This may account for the small to moderate, but significant effects of polarized PBM observed in this work, and likely, can be extrapolated when using

multiple treatment cycles. This may also be the reason behind many of the experiments in this thesis demonstrating a non-significant effect of non-polarized PBM compared to the positive controls.

Future Research

The novel discoveries in this project open up a host of possible and important future research directions. Firstly, although this project looked at some of the possible downstream functional cellular changes in response to the transcriptomic effects, there is significant scope to look further into these flow on effects. Specifically, detailed proteomic and metabolomic studies could provide a wealth of knowledge about how the observed transcriptome changes influence cell functionality. Further to this, given the multiple changes seen at the mitochondria, additional functional mitochondrial studies such as real-time metabolic analysis and specific protein expression assays would advance the understanding of these effects. The cytoprotective effects of PBM, in the form of decreased apoptosis were a key finding in this work. Although the same observation has been reported by a small number of other studies [12, 13], there remains a sparsity of understanding around the pathways which promote these cytoprotective effects, and hence should be investigated in further detail. While there is broad acceptance of the dose-response curve in PBM applications [16], and an acceptable amount of biological evidence demonstrating it both *in vitro* and clinically, the effects of both over and under-dosing PBM at a gene and protein level, and especially their underpinning pathways, remain under-investigated. This project not only sought to uncover new fundamental PBM mechanisms, but also test the efficacy of polarization as a light property. That said, there remain many other under-investigated light properties such as: wavelength, pulse structure, treatment cycles, and beam area that should be more deeply considered to contribute to the development of optimal *in vitro* and clinical irradiation protocols.

An interesting development in recent years is the advent of 3D *in vitro* cell culture, which is now more affordable, efficient and accurate than ever [25]. Given that 3D cell cultures are generally a better representation of biological processes *in vivo*, where possible, future research should adopt this methodology, which will facilitate better translation to clinical studies. Finally, there remains the conjecture around the specific biophotonic interactions between PBM and its target tissue. Although, as stated previously, this is said to occur predominately at CcO within the mitochondria, there is

limited evidence to support this notion, with more recent research casting doubt on this assertion [3]. The previous suggested future research suggested in this section will lead to the discovery of potential light-mediated pathways and photoreceptors that have either not being investigated, or that even remain currently undiscovered. Of these, opsins—a group of light sensitive G protein-coupled receptors appear to be [26] an intriguing future pathway for further investigation. Traditionally, thought to only exist in the eye, recent research has found they exist in multiple cell types (both human and non-human), with five types being found in fibroblasts (opsins 1-5) [27]. Opsin 3 in particular, has been found to play an important role in UV-induced skin ageing [27], and if conducted, future research probing the effects of PBM on opsin activity may yield important discoveries in the quest to fully uncover the full spectrum of PBMs fundamental effects.

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Chapter 10: General Conclusion

General Conclusion

Photobiomodulation (PBM), despite its widespread clinical use, lacks a set of fully described mechanisms by which it exerts its effects at a cellular and molecular level. Furthermore, there remain a number of underexplored properties of light that can be manipulated during the application of PBM to alter its effects. Hence, the aim of this thesis was to investigate the molecular and cellular effects of polarization on PBM *in vitro*, in a cell type known to be critical in the wound healing response, namely fibroblasts.

This thesis made a number of important discoveries. Firstly, it demonstrated that when compared to non-polarized PBM and non-irradiated controls, polarized PBM caused an increase in mitochondrial membrane potential, and altered the expression of many genes related to the mitochondrial genome. Importantly the effects on the mitochondrial genome were more widespread than previously thought. Secondly, P-PBM brought about a significant increase in cellular proliferation and modulated a number of genes related to the extracellular matrix and collagen production. Thirdly, PBM demonstrated a cytoprotective effect, in the form of reduced cellular apoptosis, when cells were exposed to oxidative stress. Finally, across virtually all biological measures performed in this project, polarized PBM consistently outperformed both the non-polarized PBM and non-irradiated control groups. Taken together, these results indicate a more widespread effect on the mitochondria than previously thought, and that polarization appears to improve *in vitro* measures related to wound healing more significantly than non-polarized PBM.

Chronic wounds and their associated quality of life impacts account for a significant economic and humanistic burden on the global health care system. The work performed in this thesis has shown that the polarization of PBM has the potential to improve an already efficacious therapy used to treat a myriad of costly and debilitating conditions, including chronic wounds. It is hoped that future research will build on the work done in this project by further investigating the fundamental mechanisms of both PBM and polarization and attempt to clinically translate this research to ultimately improve patient health outcomes, and contribute to easing the burden chronic wounds place on the health care system more broadly.

Chapter 11: Appendices

Appendix I: Published versions of manuscripts included in this thesis (in order presented)

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REVIEW ARTICLE

Good, better, best? The effects of polarization on photobiomodulation therapy

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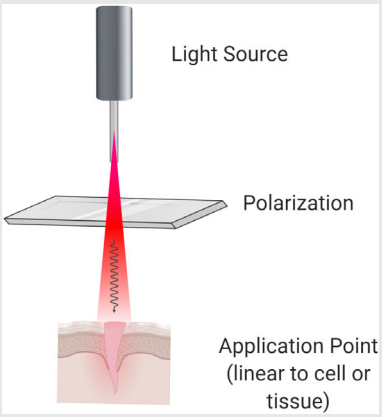
Abstract



Photobiomodulation therapy (PBMT) is a widely adopted form of phototherapy used to treat many chronic conditions that effect the population at large. The exact physiological mechanisms of PBMT remain unsolved; however, the prevailing theory centres on changes in mitochondrial function. There are many irradiation parameters to consider when investigating PBMT, one of which is the state of polarization. There is some evidence to show that polarization of red and near-infrared light may promote different and/or increased biological activity when compared to otherwise identical non-polarized light. These enhanced cellular effects may also be present when the polarized light is applied linear to the tissue direction. Herein, we synthesize the current experimental and clinical evidence pertaining to polarized photobiomodulation therapy; ultimately, to better inform future research into this area of phototherapy.

KEYWORDS

low-level light therapy, photobiomodulation, polarization, polarized light therapy

1 | INTRODUCTION

Phototherapy encompasses a broad spectrum of therapeutic modalities, all designed to bring about a positive biological effect. The earliest documented evidence of phototherapy dates back to the ancient Egyptians, who worshipped the sun god Ra. Through Ra's perceived power, the worshippers would expose themselves to direct sunlight to increase their energy levels and vitality [1]. In more recent times, a diverse group of phototherapeutic devices have been developed aimed at treating of a range of conditions, spanning from skin lesions to neurodegenerative diseases. These include: UV therapy, commonly used to treat dermatological conditions such as psoriasis, acne, vitiligo and lichen planus [2, 3]; polarized light therapy, which is used to treat musculoskeletal and dermatological conditions [4, 5]; and broad-spectrum fluorescent light-boxes, which are used to treat seasonal affective disorder [6–10]. Amongst all the phototherapies used clinically, Photobiomodulation

Therapy (PBMT), appears to be the most widely used and accepted. PBMT is a system of phototherapy that uses low-intensity, non-destructive laser and/or light emitting diode (LED) to create a therapeutic effect [11]. This type of phototherapy dates back to the 1960s, and like many scientific breakthroughs, was discovered by mistake. While working at Semmelwies University in Budapest, Hungary, Endre Mester assessed whether laser could cause cancer in mice. To his surprise, not only did the mice exposed to lasers not develop cancer, the experimental wound inflicted on them healed faster [11]. From this point onward, the medical application of lasers and LEDs has slowly grown, as has the evidence base [12]. PBMT has also been referred to as "cold-laser," "soft-laser," "low-level laser/laser therapy" or "biostimulation" [11, 13]. All of these use red and/or near-infrared (NIR) light commonly to create a biological effect. The known efficacious wavelengths that have been investigated range between 600 nm and 1000 nm [12], thus spanning both red and NIR. The full mechanistic effects of PBMT are currently not clear, but its effects are known to occur at both the cellular and molecular level [14].

PBMT has been shown to be clinically effective across a range of pathologies, many of which cause a significant burden to global health services and society more broadly. Given the theorized biological effects of PBMT on cellular factors related to tissue healing, research has been completed that shows PBMT can accelerate the healing of chronic diabetic ulcers [15]. PBMT has also been shown to assist in the treatment of various dermatological conditions such as psoriasis [16], hypertrophic scars and keloids [17] and may have the capacity to modulate various acne-inducing pathways [18]. PBMT has also been used in treating conditions associated with the nervous system. Another key focus of clinical research into PBMT is that of the treatment of pain. Multiple trials have shown PBMT to be effective in promoting analgesia in patients with diagnosed neuropathic pain [19] as well as both chronic and acute low back and neck pain [20] [21] [22]. Trials have also found PBMT to be of benefit in the treatment and management of various forms of osteoarthritis [23, 24] and tendinopathy [25, 26]. Finally, PBMT can also be applied to the sporting population. In fact, PBMT can provide immediate pain relief in sports injuries [27] and when used before exercise, can cause a significant performance improvement in both strength and endurance sports [28].

Despite plausible biological mechanisms and widespread use, there is still more research needed to better quantify the biological effects of PBMT and develop an accepted set of evidence-based guidelines for its use [14]. The application of PBMT is a delicate balance; too little energy will not create any detectible effect and too much can cause negative effects. This is known as the biphasic dose response or Arndt-Schulz effect [12, 13]. There can be a number of variables manipulated that can contribute to the summation of PBMT dosage, which include: wavelength, irradiance, pulse structure, coherence and polarization [12]. Light waves normally travel across all different planes. Light can be polarized by blocking or absorbing specific planes of light propagation, so the remaining photons travel in a specified plane or planes. There are three main types of polarization: linear polarization, where light travels in a single plane only; circular polarization, where light travels in two distinct linear planes that are perpendicular to one another; and elliptical polarization, where the light travels in an elliptical fashion, by combining two linear segments of light at different amplitudes [29]. Research suggests that linear or circular polarization may induce different or more pronounced cellular effects when compared to otherwise identical, non-polarized light, potentially being more pronounced, when polarized light aligns parallel to its target tissue [30, 31]. Currently, there is a small amount of evidence documenting the effects of polarized PBMT (PPBMT) and fewer still comparing non-polarized PBMT (NPPBMT) and PPBMT. Given that red and NIR light has the largest underpinning body of evidence, it makes sense to investigate the differences between polarized and non-polarized light within this spectrum, before expanding to polychromatic polarized light sources. Therefore, this review will synthesize the current experimental and clinical evidence surrounding narrow-band, monochromatic PPBMT (600-1000 nm), ultimately to better inform this potential area of advancement within the field of PBMT, and help to inform other, broader-spectrum phototherapy research.

2 | REVIEW METHODOLOGY

Searches were conducted using CINAHL (Cumulative Index to Nursing and Allied Health Literature), MEDLINE, PUBMED, The Cochrane Library and Google Scholar. The following search terms were used: low-level light therapy; photobiomodulation; photobiomodulation therapy; low-level laser therapy; polarization; polarized light; polarized PBMT; polarized low-level light therapy; polarized low-level laser therapy; polarized laser; polarized laser irradiation; polarized light therapy; polarized phototherapy; polarized photobiomodulation; polarized photobiomodulation (Figure 1). American and English spellings were used for all terms. Studies from all years were included. The inclusion criteria were peer reviewed original research, reviews and case studies related to the search topics. Studies that examined non-polarized light

FIGURE 1 Summary of search strategy and paper exclusion

only, polychromatic light, or light outside of the 600 to 1000 nm range were omitted. Non-English articles that were not able to be translated were excluded. Initial search identified 7590 entries. After exclusion of duplicates and conference abstract titles, an abstract analysis was used to identify potential items. Full-text analysis of all papers was performed to assess appropriateness for inclusion in this review. Reference lists of included articles were also used to locate additional relevant articles. In total 16 number of studies were found related to red and NIR PPBMT (Figure 1). No ethical approval was required for this review.

3 | A PRIMER ON LIGHT-TISSUE INTERACTIONS

Light is made up of packets of energy known as photons, which constantly travel at the speed of light throughout the known universe. The more photons in number, the brighter the light is. The perceived colour of light is determined by its wavelength on the electromagnetic spectrum. Visible light to humans, is generally defined as a wavelength between 400 and 700 nm. When light interacts with living tissue, it can be absorbed, reflected or transmitted [14]. Generally, only a small amount of light is reflected from biological tissue, this is said to follow Snell's law, which describes the change in direction of a light wave as it transitions between two media. Most light however, is absorbed. Light absorption by biological tissue is characterised by the absorption coefficient (μ_a). It is also important to consider the scattering of light within tissue, which is the precursor to light absorption. Scattering is described by the scattering coefficient (μ_s). To determine total light attenuation (μ_t)—the reduction in the intensity of light due to absorption and scattering—the scattering coefficient is added to the absorption coefficient. Hence, total light attenuation is expressed as:

$$\mu_t = \mu_s + \mu_a.$$

Focussing on the components of light attenuation, an "optical window" model has been develop to explain the relatively high levels of light penetration of red and NIR light [12]. As wavelengths get closer to the blue end of the spectrum, light is absorbed and scattered more readily in biological tissue. Additionally, at wavelengths greater than 1150 nm, water starts to absorb a significant amount of light energy. PBMT, demonstrated mainly for wavelengths from 600 to 1000 nm, exploits this optical range by generating maximum light penetration and minimum light attenuation [14]. It is important to note that this optical window refers to in vivo applications, and may explain why otherwise wavelengths of light show positive effects in vitro, yet do not translate to human and animal studies. Considering polarisation in this context, it may represent a method of achieving improved light penetration in biological tissues within the 600 to 1000 nm range.

4 | PBMT MECHANISMS OF ACTION

As there is scant mechanistic evidence pertaining to PPBMT we will prelude this review by describing the current theoretical mechanisms of NPPBMT (Figure 2). At a cellular level, PBMT appears to interact principally with the mitochondria [32]. The functions of the mitochondria are well known and are being increasingly investigated as a source of pathology [33]. Within mammalian mitochondria, cytochrome c oxidase (CCO)—an enzyme of the mitochondrial respiratory chain, which assists in the transfer of electrons from CCO to molecular oxygen [34]—has been shown to absorb red and NIR light, which then affects its structure and/or function [35]. This molecular photoacceptor is known as a chromophore [36]. When red and NIR light interacts with the CCO chromophore it increases its available energy and thus, increases the mitochondrial ability to generate adenosine triphosphate (ATP) [14]. The precise mechanism of how PBMT affects CCO remains unknown, but the current prevailing theory is based on the interplay between, nitric oxide (NO), oxygen and CCO [12]. It has been shown that NO competes with oxygen to interact with CCO,

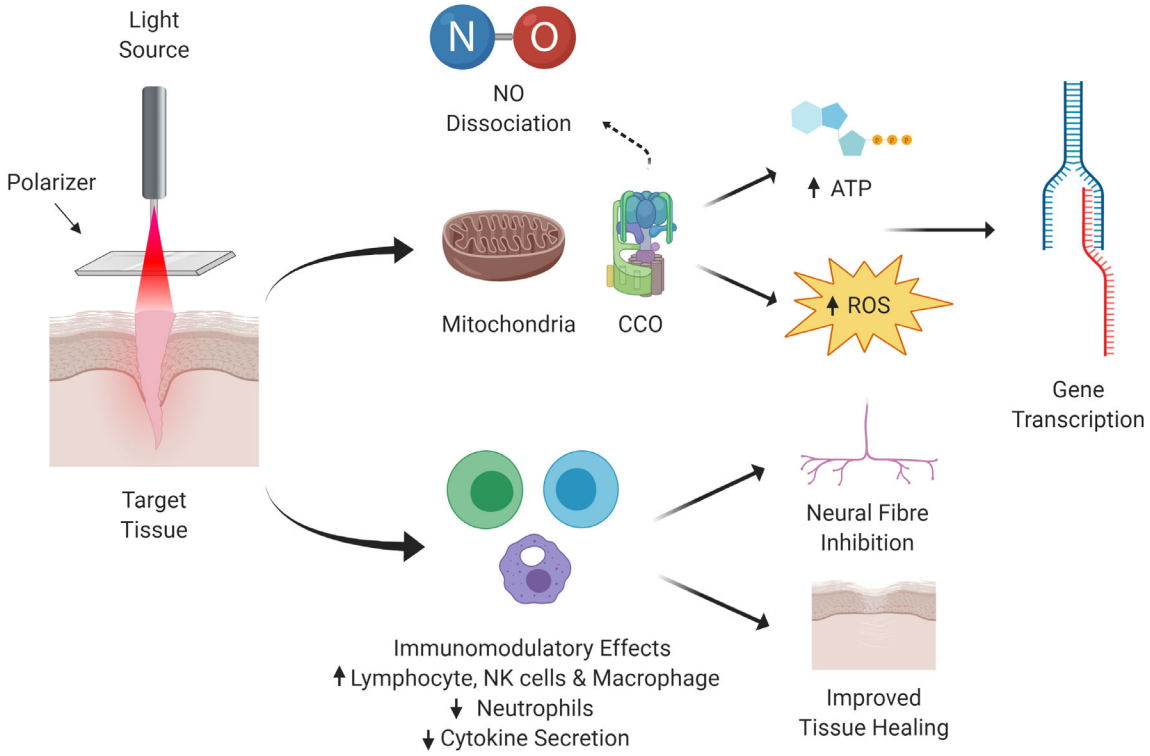


FIGURE 2 Proposed mechanisms of PBMT diagram. PBMT, photobiomodulation therapy

resulting in lowered cellular respiration and decreased ATP production [37]. Polychromatic light has been demonstrated to acutely reverse the inhibition of CCO by NO [38]. Moreover, exogenous NO has been shown to directly inhibit the functional cellular effects of PBMT in vitro [39]. These processes inform this mechanistic theory of PBMT whereby red and NIR light causes the dissociation of NO from CCO at a mitochondrial level, resulting in a higher rate of cellular respiration and increased ATP production [40].

PBMT appears not only to affect mitochondrial function, it has also been shown to have an effect on cellular reactive oxygen species (ROS) [14]. ROS are molecules that are important in redox signalling, oxidative stress, cell signalling, enzyme activation, regulation of cell cycles, and protein synthesis [14, 41, 42]. During many cellular processes, a portion of the oxygen metabolised is converted to ROS. PBMT promotes the metabolism of oxygen, presumably through its effects on the mitochondria, which can lead to an increase ROS production [14]. This has been demonstrated in vitro with PBMT changing the redox potential of a cell toward greater oxidation [43] and increasing ROS generation within the cell [44]. ROS can also activate nuclear factor kappa B (NF-κB) [45]. NF-κB is a transcription factor that can activate a number of genes, including those coded for cytokine and chemokine release, cell adhesion, cell surface receptors, anti-apoptosis and cellular proliferation [46, 47]. PBMT has been shown to increase NF-κB, presumably through the generation of ROS [45]. NF-κB is generally considered pro-inflammatory and PBMT anti-inflammatory. On face value this does not appear to compatible, however, it is proposed that both ROS and NF-κB may play a role in the dose-response relationship in PBMT. In the right amount NF-κB can cause reduced apoptosis, and increased cell proliferation and migration—responses thought to be beneficial in tissue healing [48]. Overexposure though, causes an undesired increase in ROS and NF-κB, which could potentially cause the downturn in cellular function when tissue is overexposed to PBMT [48]. More generally ROS can cause the modulation of DNA transcription and thus, may activate genes that play stimulatory or protective roles within the cell [14, 42, 47]. These changes in gene expression have been demonstrated across multiple cell lines. For example, in vitro experiments on fibroblasts have shown that PBMT promotes upregulation of multiple genes involved in DNA repair (MPG), inflammation (LENG5), growth and proliferation (CDK5R1) and metabolism (CANX) [49–51]. Similar changes to key genes involved in adaptation and healing have also been shown in muscle and tendon tissue in vitro and in vivo [52–57]. PBMT is also thought to play a major role in regulating the immune system by modulating many key cells affecting the immune system.

Specifically, PBMT has been shown to alter M1-related cytokine and chemokine expression via mitochondrial biogenesis and histone modification [58] and to enhance proliferation of peripheral blood mononuclear cells [59]. Additionally, PBMT can cause increased macrophage proliferation and altered differentiation [60], an increase in CD45 lymphocytes and natural killer cells [61] and interestingly, a decrease in the number of neutrophils in areas of inflammation [62]. These immune changes are key mechanisms across other forms of phototherapy [4] and further, are fundamental in producing the pain suppressing effects of PBMT. PBMT is known to modulate multiple substances related to the inflammatory drivers of nociception, which include: Prostanoids (prostaglandins, leukotrienes, eicosanoids); Kinins; Serotonin; Histamine; Cytokines; Neuropeptides; ROS; and ATP [63]. Additionally, PBMT can decrease nociceptive input by inhibiting A and C neural fibres by decreasing axonal flow, thought to work in conjunction with the aforementioned molecular changes [64–66]. It is currently thought that PPBMT works via the same pathways as NPPBMT, however, these effects may be enhanced through polarization (Figure 2).

5 | PPBMT *IN VITRO* EXPERIMENTS

The effect of PPBMT has been evaluated in both connective tissue and immune cell lines with the aim of quantifying PPBMT's effect on tissue healing and the immune response. Collagen is the most abundant protein in mammals and plays a critical role in the wound healing process [67]. One study measured the effect of the polarization angle on NIH/NT3 fibroblasts. It specifically measured vascular endothelial growth factor (VEGF) secretion, differentiation to myofibroblasts and collagen organization after irradiation with a 800 nm polarized light. Cells were irradiated at a 0°, 45°, 90° and 135° polarization angle for 6 minutes daily, for 6 days. This was compared against both a population that was exposed to light polarized in all orientations and a non-irradiated control. The results demonstrated increased cell viability, VEGF secretion and myofibroblast differentiation in all irradiated groups and compared to the non-irradiated control. In addition, the degree of polarization influenced collagen organization. The 0° to 135° samples showed increased collagen alignment at 30° and 130°. This contrasts the "all degree" and control sample that demonstrated peaks at 110° and 180°. However, as there was no NPPBMT sample, this study could not demonstrate a clear advantage of PPBMT [68].

Further, the effects of PPBMT and NPPBMT on Wharton's jelly derived mesenchymal stem cells was assessed. Following a 24-hour incubation period, the cells were irradiated once for 2, 4 or 6 minutes. There was a NPPBMT, PPBMT and control (non-irradiated) group. Cells that were irradiated for 6 minutes showed significantly increased levels of proliferation from the control group, however no significant difference was observed between the PPBMT and NPPBMT group. Furthermore, it was clear that cell counts and colony formation were both significantly higher after PPBMT when cells were plated at higher confluency (500 cells, per 35 mm well). However, scratch wound assays showed no significant improvement in wound closure rates in any group [69]. A limitation of this study includes that only one round of irradiation was performed; other analogous studies have shown that multiple doses of PLLLLT tend to show better outcomes compared to NPPBMT [31, 70]. Nevertheless, this study does provide evidence of some small advantage of PPBMT over NPPBMT.

In addition, the effects of PPBMT on the immune system have been studied. A study found that linearly PPBMT and NPPBMT caused an immunosuppressive effect, in terms of cellular proliferation, on human lymphocytes when compared to a halogen irradiated control sample. In addition, the immunosuppressive effect of the linear PPBMT was found to be 20% greater than the NPPBMT sample [71]. A major limitation of this study was a lack of exact protocol reporting, making replication impossible.

Despite the previous experiments showing possible advantages of PPBMT over NPPBMT there are studies casting doubt on the increased efficacy of PPBMT over NPPBMT. One study investigated the effects of irradiating HeLa cells with linearly polarized red laser light (637 nm). The experiment contained four trial groups; three groups were irradiated with a 99.4%, 60.9% and 34.2% polarization coefficient respectively, while a non-irritated group was used as a control. Despite the number of cells adhering to the glass surface (a measure of their biological activity) being significantly higher in the irradiated groups, there was no difference between the two experimental groups. This led to the conclusion that degree of polarization had no additional effects [72]. That said, the absence of comparison to a 0% polarization and the high exposure radiation intensity could have been confounding factors in the study.

6 | PPBMT ANIMAL MODELS

There have been a few studies showing positive effects of PPBMT on wound healing in animal models. One

experiment measured the effects of PPBMT on the healing of artificially induced wounds in mice. The mice were irradiated with either linear or perpendicular PPBMT (632.8 nm), with the angle of polarization being relative to their spinal cord. Each mouse had their own control wounds that were not irradiated. The results demonstrated that the irradiated wounds healed faster than the non-irradiated wounds and additionally, that parallel polarization caused faster and more complete healing compared to perpendicular [73]. The same research group used a similar methodology to assess collagen birefringence in skin repair in response to PPBMT (632.8 nm). The results demonstrated that the wounds irradiated with parallel PPBMT with respect to the rats spinal cord showed higher birefringence, indicative of a higher degree of collagen organisation and therefore wound healing, when compared to perpendicular polarization [74]. Researchers have also studied the differences in light-tissue interaction between healthy and healing rat skin. An experiment found that in the first 3 days of healing, the polarized laser lost significantly more intensity when passing through the healing tissue when compared to the non-irradiated, injured control as well as healthy tissue. The authors suggested that this effect was possibly due to the large number of inflammatory cells and debris in the healing tissue [75]. A similar methodology to assess collagen birefringence in healthy rat tendons. One Achilles tendon was irradiated with PPBMT and the other no exposed to light as a control. The PPBMT was orientated parallel relative to the tendon. It was found that the irradiated tendon exhibited enhanced collagen alignment relative to the control and the authors suggested that this effect may be applicable in the treatment of pathological tendons [76]. However, there was no comparison to non-parallel PPBMT or NPPBMT and therefore it is uncertain if the reported effects are due to the incident polarisation or PBMT more broadly.

The effects of PPBMT on healing of rabbit tissue was also noted. A comparison of parallel, perpendicular and 45-degree PPBMT relative to the wound against a non-irradiated controls was assessed. It was clear that, the fastest healing wounds were those irradiated with the parallel polarized light, followed by the perpendicular and 45 degree light respectively [70]. Despite positive results, as there were only four animals examined in this experiment, making the results less reliable - more wound models could have been used for a stronger result. PPBMT has also been shown to have an effect on the viscoelastic properties of soft tissues. A soft tissue sample was taken from the pleura of an animal and irradiated with PPBMT either perpendicular or parallel to the direction of tissue stretch. Tissue viscoelasticity was assessed via displacement sensor and stretch load cell before and after radiation. The results showed that the sample irradiated parallel to the stretch direction exhibited the greatest increase in viscoelastic capacity. The authors hypothesized that this effect could be due to changes in collagen organisation, however no direct mechanistic evidence of this was reported, nor was the type of animal sample [77].

There has also been a combined in vivo and in vitro study conducted on would healing in mice. Researchers took NIH3T3 fibroblast cells from wild mice and irradiated them with a 627 nm LED device at varied intensities. The experiment used five groups: an unlit control, a non-polarized light, and three types of polarized light: linearly polarized, right circularly polarized and left circularly polarized. In vitro, the linearly and right circularly polarized group demonstrated the greatest cellular proliferation. The authors suggested these changes were due to an increase in the irradiation absorbance value. The most efficacious intensity was reported to be between 2 and 8 J/cm^2. In vivo, a full thickness skin defect of 20 mm in diameter was created in mice. These wounds were irradiated using the same protocols as the in vitro study. It was found that the linearly and right circularly polarized light demonstrated the best healing effect at 7 days post-injury. Additionally, the right circularly polarized light promoted significantly increased expression of the type 1 procollagen mRNA compared to the control. However, there was no significant difference in type 3 procollagen mRNA expression between groups [30]. Interestingly, the authors did note a small temperature change 0.1°C per/min. The authors were confident that this small change did not influence their results, however analysis of heat-shock proteins would have been pertinent here to support this claim.

The effects of PPBMT on spinal cord injuries (SCI) have also been noted. One protocol induced an artificial spinal cord contusion using a with a weight-drop device. Before the injury site was surgically repaired the contusion was irradiated with either parallel or perpendicular PPBMT relative to the spinal cord. These rats were compared to a control group that was injured but did not receive any irradiation. The spinal cord was re-exposed and irradiated for five consecutive days. The results demonstrated that both irradiated groups recovered faster from the injury, with the parallel polarization group demonstrating a significantly better functional evaluation compared to the perpendicular group. Both irradiated groups also demonstrated a significantly smaller cavity formation induced by the contusion compared to control and that parallel polarization caused an approximate 40% greater light transmission through the spinal cord, compared to perpendicular irradiation. Interestingly, they also showed that there were no significant differences

between irradiated and control groups in spinal cord ATP content. This contradicts the key proposed mechanism of PBMT in which it acts on mitochondrial synthesis of ATP, implicating other biological mechanisms at play generating a therapeutic effect. The authors hypothesized that the improved functional recovery of the parallel irradiation was due to more efficient tissue light propagation [31]. However, the light penetration was measured on a healthy rat spinal cord, limiting its application to SCI. Given that other research has found that light penetration through injured tissue is less than in healthy tissue [75], the findings would be more applicable if demonstrated on injured spinal cord tissue. All these studies demonstrate the plausible effects of PPBMT in animal wound healing but raise further questions about the underpinning mechanisms of PPBMT and the optimum dosage at different stages in healing processes.

7 | LIMITATIONS

While the research above paints a thought-provoking picture of the efficacy and mechanisms of PPBMT, there remain many key limitations and questions. Firstly, there are conflicting findings pertaining to the light-tissue interactions of polarized light. Human and animal tissue exhibits anisotropic mechanical behaviour, meaning that their mechanical properties can vary in a three-dimensional space throughout the body. This is thought to be mainly due to the variation of collagen fibres in tissues [78, 79]. A key limiting factor in the transmission of light through tissues is scattering, particularly in the dermis due to collagen fibre density and its three-dimensional structure [80]. One study found the orientation of polarization that causes the least light scattering in human skin is correlated to the alignment of collagen tissue, and may have significant implications for phototherapy [80]. Another study found that in denser biological tissues, linearly polarized light is maintained better than circularly polarized light [81]. Furthermore, it has been shown that the more superficial layers of the skin (epidermis, papillary dermis) allow penetration of polarized light with only a small amount of depolarization [82].

There is also conflicting evidence regarding the effects of PPBMT in vitro. One study found no change in cell function with PPBMT and have suggested that polarization does not change the efficacy of PBMT [72]. However, as this study used HeLa cells, which are not linearly cylindrical structured like collagen fibres or axons, a hypothesis might be that the morphology of a specific cell renders them susceptible to PPBMT. Polarized light penetration can also be affected by the anisotropic nature of the skin and can be depolarized after about 1 mm [76]. However, evidence has shown that polarized light can penetrate healthy human skin to at least 1.2 mm with only marginal depolarization [83]. Furthermore, it has been demonstrated in animal nerve tissue that PPBMT applied perpendicular to the axis of the white matter tracts caused a significant increase in light penetration when opposed to perpendicular PPBMT [84]. In an attempt to model in vivo circulatory conditions one study looked at the amount of depolarization through animal tissue with and without fluid flow through the tissue. The results demonstrated that polarization was largely unaffected when passing through static tissue or, when the fluid flow was parallel to the polarization direction. Polarization was partially lost when flow was perpendicular to the polarization direction and when the rate of fluid movement was increased [85]. Considering all this, in conjunction with the known effects of PPBMT in animal models [31, 70] it seems plausible that polarized light aligned parallel to cylindrical, or linear biological microstructures such as myofibrils, axons or collagen fibres [79] may represent a more efficacious method to administer PBMT. With the advancement of 3D cell culture and 3D bioprinting, the potential advantages of PPBMT may be able to be quantified in vitro, representing a cost saving and ethical advantage over traditional animal research. However, more in vitro research is required to confirm this, and to reveal whether any advantages of PPBMT found in vitro, would persist in vivo.

Secondly, most of the experiments did not compare PPBMT to NPPBMT and further, did not use a light control outside the 600 to 1000 nm range, only a non-irradiation control. Therefore, it is impossible to confidently state whether the reported effects of PPBMT are significantly different from NPPBMT or even polychromatic, visible light sources. It is also unclear if the reported increases in efficacy are due to the increased penetration of PPBMT or if they are caused by the increase in relative irradiation intensity caused by the polarization effect. Thirdly, it remains unclear if the plane polarized light emitted by some helium-neon (he-ne) lasers is a factor to consider when interpreting the findings within this field [86]. Few, if any, PBMT research using he-ne lasers report their polarization state. Given that there is a potential biological difference caused by this effect, any future research using he-ne lasers, should report if they emit plane polarized light or not, and how that light is orientated to the target tissue. Finally, to our knowledge, there have been no human studies conducted that compare PPBMT and NPBMT, making clinical generalization of the relative efficacy impossible based on the current evidence.

8 | CONCLUSION

PBMT has been shown to be an efficacious system of phototherapy for treating varied common conditions that affect the population. Its proposed mechanisms are centred on increasing available ATP and changes in gene expression. The polarization of PBMT presents as an interesting variable to investigate further. Some evidence has shown when compared to NPPBMT, PPBMT can cause quicker and more organised wound healing and that it may be able to penetrate biological tissue more effectively when applied in a parallel orientation relative to the tissue being irradiated. However, more detailed mapping of cellular and molecular responses to the therapy is required to show a clear differentiation between PPBMT and NPPBMT, and other phototherapy modalities more broadly. Future research should be directed at ascertaining more detailed mechanistic evidence in vitro and in vivo, as well as comprehensively examining light-tissue interactions. Overall, PPBMT appears to be a promising advancement in phototherapy, though more research is needed to validate these claims to allow for its clinical utilization.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with this article.

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The effects of photobiomodulation on human dermal fibroblasts *in vitro*: A systematic review

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ABSTRACT

Photobiomodulation (PBM) is reported to impart a range of clinical benefits, from the healing of chronic wounds to athletic performance enhancement. The increasing prevalence of this therapy conflicts with the lack of understanding concerning specific cellular mechanisms induced by PBM. Herein, we systematically explore the literature base, specifically related to PBM (within the range 600-1070 nm) and its influence on dermal fibroblasts. The existing research in this field is appraised through five areas: cellular proliferation and viability; cellular migration; ATP production and mitochondrial membrane potential; cellular protein expression and synthesis; and gene expression. This review demonstrates that when fibroblasts are irradiated *in vitro* within a set range of intensities, they exhibit a multitude of positive effects related to the wound healing process. However, the development of an optimal *in vitro* framework is paramount to improve the reliability and validity of research in this field.

1. Introduction

Chronic wounds are a significant burden to the global healthcare system, costing upwards of 30 billion USD *per annum* [1,2]. In the United States alone, chronic wounds affect approximately 6.5 million people and account for up to 25 billion USD of healthcare expenditure annually [3]. With an aging population, the prevalence of chronic wounds is expected to increase [4], posing a significant challenge to healthcare systems globally. Historically, wound healing interventions have consisted of standard medical procedures such as: surgical debridement, topical antibiotics and skin substitutes (*e.g.* peptide coated mesh) [5,6]. As medical technology has advanced, lesser-known and lower-cost therapies, such as phototherapy have emerged, offering novel treatments for a variety of conditions. Phototherapy has long been proposed to aid in tissue healing since its inception in the mid-20th century [7]. Since this time, laser, and more recently LED, collectively known as photobiomodulation (PBM), has been applied to a growing variety of wound and skin conditions [8]. However, the lack of an accepted set of optimal parameters has led to inconsistency in reported PBM experimental outcomes, resulting in a disparate range of procedural standards and results [9].

Wound healing is a complex physiological phenomenon the body undergoes in response to tissue damage. The interrelated and dynamic nature of tissue healing can be broken down into three fundamental phases: inflammatory, proliferative and remodeling [10]. The inflammatory phase consists of vascular and cellular cascades in response to damage. Local vasodilation results in blood and extravasated fluid entering the extracellular space, inhibiting local lymphatic function. This influx causes the cardinal signs of inflammation: pain, redness, heat and edema. Simultaneous to this influx, hemostasis begins with platelet aggregation, growth factor released and chemotaxis of immune cells (primarily neutrophils and monocytes). Following the inflammatory phase, which may continue for up to two weeks, is the proliferative stage. This stage is characterised by three specific functions: re-

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epithelisation (barrier creation); angiogenesis (blood vessel regeneration); and fibroplasia (formation of granulation tissue). The proliferation phase can take weeks to months to complete, after which remodeling returns the tissue to its original histological state, or as close to it as the specific injury allows. Remodeling is typified by the replacement of type III collagen for type I. Type III collagen is predominantly secreted by fibroblasts during the proliferative phase of healing, whereas type I collagen is the typical pre-injury phenotype that lends itself to greater dermis strength [11,12]. It is important to note that while each of the tissue healing phases has a specific function, they occur in a contiguous and overlapping fashion [13].

Fibroblasts are the most abundant cell type found in connective tissue [14]. They exist in a quiescent state until stimulated by chemoattractants released by tissue damage, at which point they begin proliferating within the fibrin clot, which it degrades by secreting extracellular matrix (ECM) factors [15]. From here, when wounded, they undergo differentiation to myofibroblasts, which is initially triggered by transforming growth factor-β1-3. This differentiation is crucial as the increased actin content of the myofibroblast increases the migration and wound contracting ability of the cells [15]. Regardless of the tissue, myofibroblasts contribute significantly to the ECM *via* tensional forces that assist in remodeling the ECM [15].. The ECM is a key player in cell replication, influencing not only cell structure and shape, but proliferation, migration, survival and differentiation [16]. As the wound is closing and once the wound ECM has a similar tensile strength to the tissues surrounding it, they undergo apoptosis [15]. In sum, fibroblasts and the ECM directly influence each other in a symbiotic relationship, which occurs throughout all body systems [14].

PBM was initially developed by Hungarian physician Endre Mester in 1967 when he noticed an unexpected acceleration of hair regrowth whilst studying the effects that laser light exposure caused cancerous cell growth in rats [17]. Naturally, light therapy has evolved significantly since its origins and has expanded immensely in its application. PBM has been used successfully in the treatment of dermatological conditions, non-healing wounds, scarring, ulcers, musculoskeletal conditions, chronic pain, analgesia and immune modulation [18–22]. Despite its increasing use, many of the underlying physiological mechanisms of PBM remain unknown [8,23,24], prompting the necessity for further investigation.

The most common forms of PBM use wavelengths of 600 nm to 1070 nm to create a therapeutic effect [7]. At low intensities, changes observed in exposed tissues are believed to be attributable to photochemical, rather than thermal effects—hence the term 'Cold Laser' [19]. While the wavelength is the primary characteristic of PBM, other variables that can have an effect on its application include: fluence, polarization and pulse structure [8,23]. While the many of the mechanisms of PBM are still unclear, there are a number of documented cellular and molecular effects. Tissues exposed to PBM have been shown to have altered mitochondrial metabolism, specifically increasing the efficiency of cytochrome C oxidase (COX), and hence, stimulating adenosine triphosphate (ATP) production and generating reactive oxygen species (ROS) [18]. This increased efficiency is thought to occur by PBM promoting the disassociation of nitric oxide (NO) from COX, therefore allowing increased oxygen reduction [8]. These processes are thought to produce subsequent effects on gene expression, cell signaling, cell cycle regulation, enzyme activation and downstream protein synthesis [18]. Additional proposed effects include modulation of calcium, potassium and sodium ion transportation, which are vital for cellular physiology, analgesia and immunomodulation [25,26].

2. Objectives

This review systematically examines the current evidence describing the effects of PBM on dermal fibroblasts *in vitro,* with a focus on cellular viability and proliferation, cellular migration, ATP production and mitochondrial membrane potential, protein expression and synthesis, and gene expression. We aim to collate the demonstrated photobiological effects, as well as to summate the effects of differing fluence on these changes. We also analyze the strengths and weaknesses of the existing literature, suggesting ways in which novel research can be directed, and ultimately contribute to the development of a widely accepted experimental standard for future *in vitro* PBM research, to facilitate effective clinical translation.

3. Methods

This review was conducted according to the PRISMA statement guidelines [27]. The search strategy used for this review is located in Appendix 1. Databases that were searched for in this review were: PUBMED; EMBASE; CINAHL; SCOPUS; and web of science. The search was completed in January 2020, and updated in October 2020. Studies from all years were included. Inclusion criteria were: use of low-intensity (<1 W) red and near-infrared PBM/LLLT (600 nm-1070 nm), use of *in vitro* models with human dermal fibroblasts tissue (primary or cell lines), and investigation of at least one of: cellular viability; cellular proliferation; cellular migration; ATP production; mitochondrial membrane potential; protein expression and synthesis; and gene expression. Studies that examined polychromatic light, or light outside of the 600-1070 nm range were excluded. Non-English articles that were not able to be translated were also excluded. The initial search yielded 4929 results. Once duplicates were removed, title and abstract screening was performed to identify appropriate studies by two of the authors, with a third resolving any conflicts. From there, full texts of included studies were assessed for eligibility by two of the authors, with a third resolving any conflicts. There were 112 full-text articles were assessed for eligibility. Review of titles, abstracts and text led to the ultimate inclusion of 46 studies in the qualitative synthesis (Fig. 1). The data extracted from the papers were cell line, irradiation parameters (light source, fluence, power, total exposures) and experimental results. Studies that reported fluence in mW/cm^2 were converted to J/cm^2, so that consistent inter-study comparisons could be made. As no risk of bias assessment for this type of *in vitro* research has been developed, none was used.

4. Results

4.1. Cellular Viability

There have been several investigations into the cellular viability and proliferation effects of PBM, using a variety of assay methods, namely: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), WST-8 and Vision Blue assays. The literature regarding optimal fluence levels for dermal fibroblasts gives a mixed picture. Doses of 0.5, 1, 5 and 5.5 J/cm^2 demonstrate increased viability when compared to non-irradiated controls [28–33], while similar fluences of 1.5, 2.5, 3, 5, 6, 10, 12, 15, 16, 20, or 25 J/cm^2 showed no change [30,34–40], and doses of 0.5 and 10 J/cm^2 have even shown a decrease in viability [34]. Interestingly, a higher dose of 30 J/cm^2 have been shown to increase viability [41], seemingly contradicting dose-response principles in PBM. This inconsistency in experimental wavelength and fluence selection casts uncertainty on the optimal parameters for PBM, and emphasizes the demand for homogeneity in application and reporting. While the existing evidence suggests PBM exposure increases cellular viability, the outcomes appear dependent on multiple variables including cell condition, wavelength, fluence and duration (Fig. 2).

4.2. Cellular Proliferation

Common measures of PBM-induced cell proliferation include: Neutral Red, Trypan Blue, Bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU), ethynyl-2′-deoxyuridine (EdU), Propidium iodide (PI) and Methylene blue assays. In contrast to viability, the literature describes a clearer dose-response effect with doses of 0.45, 0.5, 0.75, 1, 2, 2.5, 3,

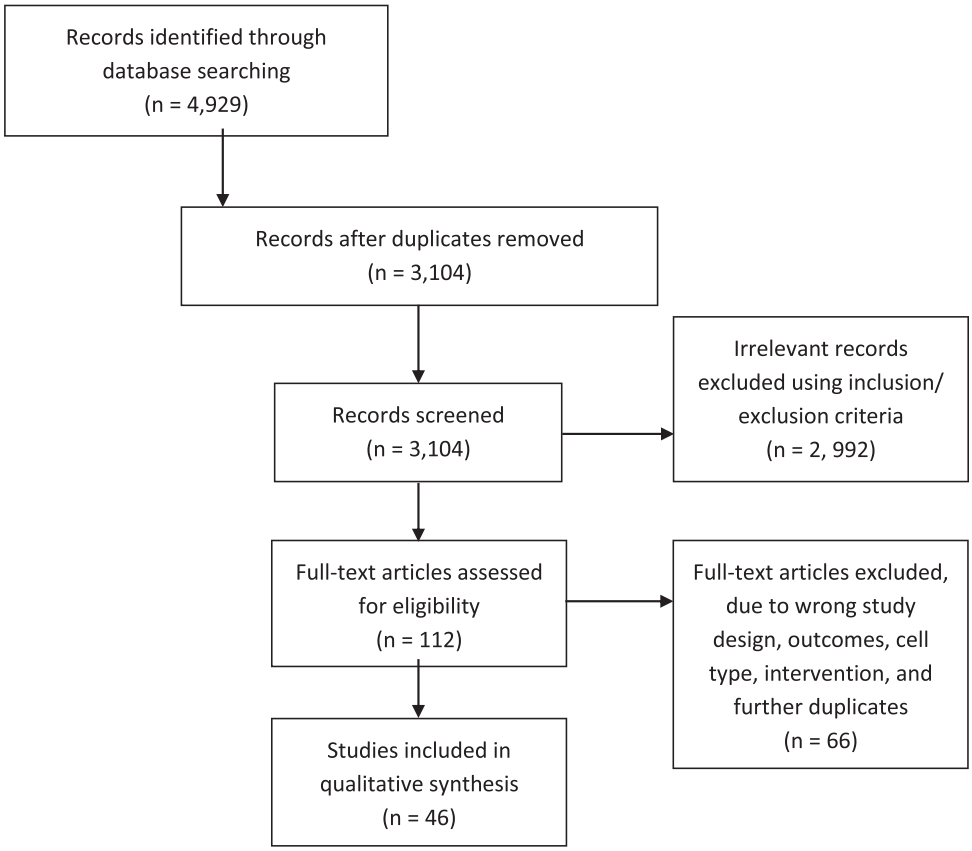

Fig. 1. Literature search process according to the PRISMA guidelines.

3.16, 3.61, 4, 5 and 10 J/cm^2 demonstrating increased proliferation [28,29,32,42–53]. Unlike cellular viability, evidence regarding the positive effects of PBM on proliferation are relatively consistent. There are only two studies showing unchanged proliferation at fluences of 2.4, 2.5, and 4 J/cm^2 [44,54], and higher doses of 10, 16 and 20 J/cm^2 show either unchanged, or decreased proliferation [38,44,45,48,49]. This suggests that proliferation may be a more sensitive and accurate measure than viability when measuring the cellular effects of PBM *in vitro* (Fig. 2).

4.3. Cellular Migration

The assessment of wound convergence *via* a scratch wound assay is an accurate and affordable measurement of cellular migration, as well as

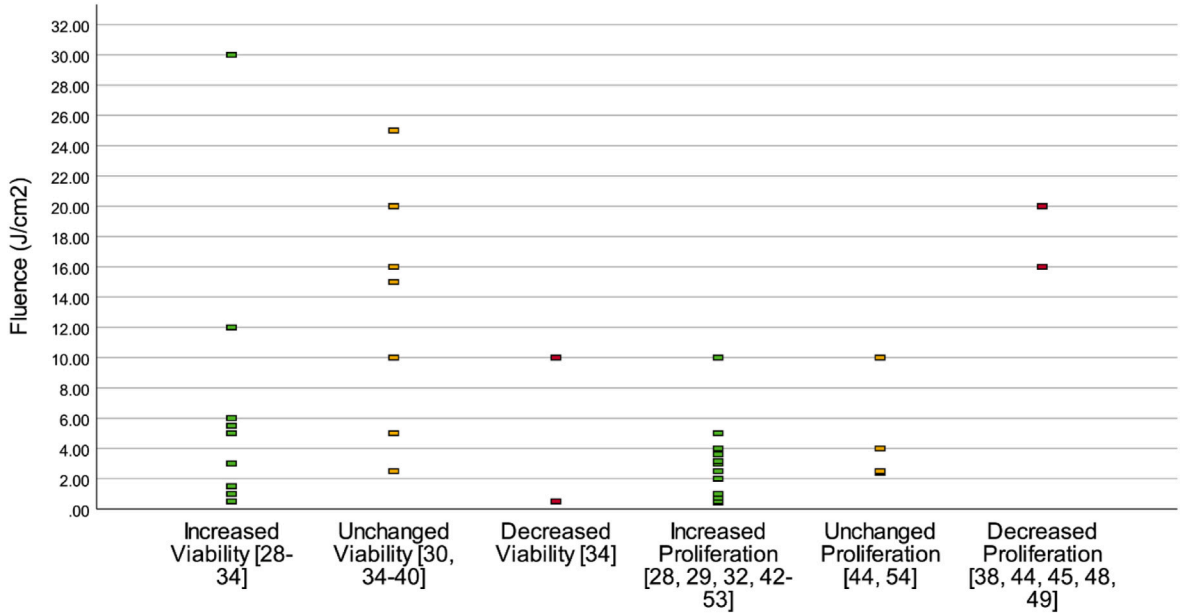

Fig. 2. Graphical summary of PBM's effect on dermal fibroblast cellular viability and proliferation.

proliferation and hypertrophy, and hence, is commonly employed to determine the efficacy of interventions *in vitro* [55]. Given the *in vitro* and *in vivo* focus on effects of PBM on wound healing, wound migration studies have proven popular within PBM basic science research [42]. The literature on this aspect of PBM research illustrates that fluences of 0.5, 3 and 5 J/cm^2 appear to promote faster and more complete cellular migration measured *via* scratch closure [28,30,37,40,43,45,53,56–58], while a higher fluence of 16 J/cm^2 caused a slowing of wound convergence compared to non-irradiated controls [30,45,58]. Despite existing investigations into PBM and wound migration appear positive, there is still a lack of clarity surrounding the precise parameters required to induce effective change to healing in human fibroblasts *in vitro*.

4.4. ATP Production and Mitochondrial Membrane Potential

The major proposed cellular and molecular mechanisms of PBM are centered on increases in bioavailable ATP and overall mitochondrial function [8]. Bioluminescence assays are the predominant method to assess changes in ATP level in fibroblasts and give a mixed picture of the optimum irradiation parameters for PBM in this setting. Fluences of 0.5 or 5 J/cm^2 have shown significant differences in ATP levels [44,59,60], while fluences of 2.5, 5, 15, or 16 J/cm^2 have shown no differences in ATP levels compared to controls [35,44,48,56,58,59]. Contrasting this, other analogous work demonstrated that a fluence of 5, 10 or 16 J/cm^2 resulted in a decrease in ATP production [44,48,58,60]. To further confuse the area, even higher doses of 45, 90 and 180 J/cm^2 have been demonstrated to cause no significant changes in ATP compared to non-irradiated controls [41], highlighting that ATP levels may not be well correlated to the PBM dose-response relationship.

Researchers have also investigated how PBM effects the mitochondrial membrane potential of dermal fibroblasts. One study described no changes to the mitochondrial membrane potential of fibroblasts under normal cell culture conditions after an irradiation of 5 J/cm^2, compared to a non-irradiated control, but showed a significant increase in mitochondrial membrane potential in wounded, hypoxic and acidotic cells exposed to the same treatment [56]. Another study found that both continuous wave (CW) and pulsed wave (PW) lasers created a dose-dependent decrease in mitochondrial membrane potential at a fluence of either 15 or 45 J/cm^2, while a fluence of 5 J/cm^2 created a slight increase in in PW mode, and slight decrease in CW mode, however, neither change reached statistical significance [61]. Interestingly, another study found PBM at 3 J/cm^2 caused a decrease in mitochondrial membrane potential compared to a non-irradiated control [39]. These results provide conflicting evidence as to an optimum dose response effect in this domain, again highlighting the need for greater consistency between PBM parameters in *in vitro* experiments.

4.5. Protein Expression and Synthesis

Increased cytokine expression is characteristic of accelerated wound closure and healing post-trauma [12]. While pro-inflammatory cytokines are beneficial in the short term, persistent production may delay wound healing overall [12]. Broadly speaking, the protein expressed in response to PBM in human dermal fibroblasts can be divided into inflammatory or matrix and cytoskeleton proteins. Of the matrix and cytoskeleton proteins, a fluence of 5 J/cm^2 has been demonstrated to upregulate CD90, extra domain A fibronectin (EDA-FN), α-smooth muscle actin (α-SMA), TGF-β1, p-Smad2/3, all crucial in fibroblast differentiation [62] and has demonstrated the inhibitory effect of TIMP1 on matrix metallopeptidases (MMPs), by simultaneously increasing TIMP1 and decreased MMP-3 and -9 [62]. Additionally 2, 2.5 or 5 J/cm^2 can increase the synthesis of epidermal growth factor (EGF) [35], basic fibroblast growth factor (bFGF) [30,48,58,60,63] and also collagen, type 1, alpha 1 [31,64].Conversely, a higher dose of 16 J/cm^2 has been shown to decrease the production of bFGF [30,48].

PBM has been demonstrated to influence the activity of macrophages and monocytes in their production of important cytokines such as tumor necrosis factor-α (TNFα), interleukin 6 (IL-6) and interleukin 8 (IL-8) amongst others [65]. A dose of 5 J/cm^2 has been shown to decrease the amount of TNFα, interleukin 1 beta (IL-1b), while having no effect on interleukin 6 (IL-6) [51], while conversely, another study found that 3 doses of PBM at a fluence of 3 J/cm^2 significantly increased TNFα, IL-1b, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [39]. Other studies however, found that doses of 0.5, 1, 2, and 5 J/cm^2 stimulated the release of IL-6 [29,45,63], with conflicting research also demonstrating that fluences of 3, 4, 5, and 16 J/cm^2 causing no change to its expression [45,52]. One of these studies also found that fluences of 3, 4, 5 J/cm^2 did not affect the levels of IL-1b, TNFα, and IL-6, while fluences of 6 and 8 J/cm^2 upregulated these pro-inflammatory cytokines [52]. Researchers have also found that fluences as little as 0.5 J/cm2 can affect cytokines and growth factors involved in cell communication and proliferation such as BDNF and FGF 6 and 7 [40]. Interestingly though, higher irradiation levels of 45, 90 and 180 J/cm^2 have been shown to increase the expression of heat shock proteins (HSP) 27, 60, 70 and 90 [41] which commonly suggests a stress response. These studies suggest that PBM appears to modulate cellular cytokine secretion, however, inconsistency between assessment methods—mainly from differences in cell culture conditions—presents a barrier to identification of the precise immunomodulatory effects that occur following irradiation [66] (Table 1).

Table 1
A summary of the known protein expression in human dermal fibroblasts by PBM.

Protein	Stimulatory Fluence - J/cm2	Neutral Fluence - J/cm2	Inhibitory Fluence - J/cm2
HSP 27, 60, 70, 90	45, 90, 180 [41]	–	–
TGF-β1	–	5 [63]	–
pTGF-β1R1	–	5 [63]	–
p-Smad2/3	–	5 [63]	–
Thy-1 (CD90)	–	–	5 [63]
EDA-FN	5 [63]	–	–
α-SMA	5 [63]	–	–
COL1A1	5 [31, 63]	–	–
TIMP1	5 [63]	–	–
MMP3	–	–	5 [63]
MMP 9	–	–	5 [63]
EGF	5 [36]	5 [36]	
bFGF	2 [64], 2.5 [48] 5 [30, 48, 58, 60], 16 [60]		16 [30, 44]
IL-6	0.5, 1, 2 [64], 5 [29, 45], 6, 8 [52]	3, 4 [52] 5 [51, 52], 16 [45]	–
IL-1b	3[39], 6, 8 [52]	3, 4, 5 [52]	5 [29, 51]
TNF-a	3 [39], 6, 8 [52]	3, 4, 5 [52]	5 [29, 51]
NF-κB	3 [39]		
BDNF, Eotaxin-3, FGF6, FGF7, Fractalkine, Fit3-ligand, and GCP2	0.5 [40]		

Table 2
A summary of the known gene expression in human dermal fibroblasts by PBM.* Indicates wounded cells in standard culture conditions, while ¤ indicates wounded cells in high glucose conditions. ◦ Indicates normal cells in high glucose cell culture conditions, while ˆ indicates ischemic cell culture conditions. ˜ Indicates hypoxic wounded cells and » indicates hypoxic wounded cells in high glucose conditions. ¨indicates that cells were taken from diabetic donors, and cultured under standard cell culture conditions. All genes demarcated # were found in standard cell culture conditions.

Fluence	Increased Gene Expression		Unchanged Gene Expression		Decreased Gene Expression		Ref
0.1 J/cm^2	*NCAM1*#		*COL11A*#*COL6A1*# *CD44*#	*MMP11*# *CTGF*#	–	–	[73]
0.67 J/cm^2	*RUNX1*˜ *PDGFRA*˜*EHBP1*˜*GPC3*˜ *AXIN2*˜*KDR*˜*GLMN*˜ *MSMB*˜ *EPHB2*˜*MSR1*˜ *KIT*˜		*ERCC5*#◦˜*PDE11A*#*CD96*#◦˜ *GPC3*#*MSG6*#*DKC1*#*TP5*# *HFE*# *NF1*#◦˜*EXT1*#*EPCAM*#◦˜ *FANCD2*#◦˜*KIT*#*BUB1B*#◦˜ *POLH*#*ESCO2*#◦˜*ANTXR2*# *FANCA*˜ *MET*˜ *BRCA2*˜ *BARD1*˜*RECQL4*˜*FANCI*˜*XRCC3*˜	*MSH6*˜ *PTCH2*˜ *GALNT12*˜ *ERCC6*˜*DIS3L2*˜*RAD51B*˜ *TMC6*˜ *MSR1*˜ *PDE 11A*˜*KDR*˜ *RET*˜*BMPR1A*˜*EPHB2*˜ *RUNX1*˜*PDGFRA*˜*EHBP1*˜ *EPHB2*˜*SDHC*˜ *TSC1*˜ *MSR1*˜ *ATM*˜ *BLM*˜ *BRIP1*˜ *BRCA1*˜	*NF1*#*NTRK1*#*MSR1*# *ANTXR1*#*ERCC5*#*FLCN*# *TP53*#◦˜	*GPC3*˜*TMC6*˜ *PTCH1*˜*DKC1*˜	[76]
0.88 J/cm^2	*CDK5R1*#*PDGFC*#*BCR*# *DAG1*#*P38Beta2*#*SRF*#*SEPW1*# *ATOX1*#*RIPK1*# *SSI-1*#*CANX*#*ZMPSTE24*#*BCAT2*# *AHCY*#*TOR1B*#*PSMB3*#*PPIH*# *APOC3*#*LYPLA2*#*NDUFB2*#*ETFB*# *ATP5H*#*ABC1*#*KCNG1*#*SCN4A*# *KCNJ13*#*DAG1*#*ARHD*#*MYH9*# *RANBP9*#*FMOD*#*TIP39*#	*CEACAM3*#*CDH12*# *OC81537*#*ADRM1*# *MPG*# *APRT*# *NUDT1*#*GCN5L1*#*GAS41*# *LOC51131*#*LENG5*# *AMSH*# *PENK*#, *GC20*# *PDE6D*# *AD-017*# *PELP1*#, *DSCR3*# *MPG*# *KERA*# *DUSP5*#*FLJ22625*#*KIAA0076*# *FKBP1A*#*MGC4251*#*YF13H12*# *FLJ20186*#*MCG13033*#*FLJ12886*# *KIAA0202*#*FUBP*#*KIAA0332*#	–	–	*CCNH*#*KNSL1*#*CUL1*# *HSPA1A*#*CASP6*#*STIP1*# *ELL2*#*CCT2*#*PAMCI*# *HDLBP*#*ENO3*#*ALDOA*# *NR2F2*#*CLIC4*#*ASNA1*# *ARPC2*#*LRRFIP1*#*TPM4*# *KRTHA1*#*FBN1*#	*MMP10*#, *CDH13*# *ZNF74*# *ZNF7*#*TSN*#*SEP2*# *ELF1*#*CSRP1*#*DDXL*# *PTTG1IP*#*LPP*#*YWHAB*# *RBMS2*#*PPP4R1*#*G3BP*# *PTMS*#*RES4-22*#*SERPINE1*# *TRIP10*#*SF3B2*#	[74]
1.5 J/cm^2	*COL 1*#*TGF-B*#*TIMP1*#	*TIMP2*# *IL-6*#	*COL3*#		*MMP1*# *MMP2*#*HSP70*#		[72]
4 J/cm^2	*FGF*# *VEGF*# *TGF-β1*# *TGFβR1*# *TGFβR2*#	*ACTA1*# *FN1*# *DCN*# *DDR2*# *MMP2*#	*TGFβR3*#		*CTGF*#	*ELN*#	[71]
5 J/cm^2	*ACTC1*# *ADAMTS1*#* *ADAMTS8*#*¤ *ADAMTS13** *ATP4B*ˆ *ATP5F1** *ATP5G2*ˆ *CD40LG*# *CD44*#* *CDH1*#*¤	*ITGA3*#* *ITGA4*# *ITGA5*#* *ITGA6*#* *ITGA7** *ITGA8*¤ *ITGAL*¤ *ITGAM** *ITGAV*#* *ITGB2**	*ADAMTS13*#¤ *CD44** *CDH1**¤ *CLEC3B*#*¤ *CNTN1*#*¤ *COL1A1*#¤ *COL4A2*¤ *COL6A1* *COL6A2* *COL7A1*	*ITGB5*#*¤ *ITGB5*# *LAMA1** *LAMA2**¤ *LAMA3** *LAMB1*#*¤ *LAMB3*#* *LAMC1*#* *MMP3*#*¤˜» *MMP7**	*ACTA2*# *ADAMTS1*¤ *ANGPT1*# *CD44*¤ *CDH1*# *COL1A1*# *COL1A2*# *COL3A1*# *COL5A1*#¤ *COL5A2*#	*KAL1*¤ *LAMA1*#¤ *LAMA2*# *LAMB3*¤ *LAMC1*¤ *MAPK1*# *MAPK3*# *MIF*# *MMP2*#¤ *MMP3*#*˜	[64,67–70,75]

(*continued on next page*)

Table 2 (*continued*)

Fluence	Increased Gene Expression		Unchanged Gene Expression		Decreased Gene Expression		Ref
	CFS2[#]	*ITGB3*[#*]	*COL8A1*[¤]	*MMP8*[#]	*COL6A1*[¤]	*MMP7*[#]	
	CNTN1[*¤]	*ITGB4*[*¤]	*COL11A1*[#*]	*MMP9*[*¤»]	*COL6A2*[¤]	*MMP8*[¤]	
	COL1A1[#*¤-»]	*KAL1*[#*]	*COL12A1*[¤]	*MMP10*[#*¤]	*COL7A1*[¤]	*MMP9*[#*¤-]	
	COL4A1[#]	*LAMA3*[#¤]	*COL14A1*[#*]	*MMP12**]	*COL12A1*[¤]	*MMP1*[#*¤]	
	COL4A2[#]	*MMP2*[#*]	*COL15A1*[#*¤]	*MMP13**]	*COL14A1*	*MMP12*[#¤]	
	COL4A3[#]	*MMP3*[¤]	*COL16A1*[#*]	*MMP14**]	*COL16A1*[¤]	*MMP13*[#]	
	COL5A1[#*]	*MMP7*[#¤]	*CTGF*[*¤]	*MMP15*[¤]	*CTGF*[#*]	*MMP14*[¤]	
	COL5A3[#]	*MMP8**	*CTNNA1*[*¤]	*MMP16*[#*]	*CTNNA1*[#]	*MMP16*[¤]	
	COL6A1[#]	*MMP9*[#¤]	*CTNNB1*[#*¤]	*NCAM1*[*¤]	*CTNNB1*[¤]	*PLAU*[#]	
	COL6A2[#]	*MMP9*	*CTNND1**	*PECAM1*[#¤]	*CTNND1*[¤]	*PLAUR*[#]	
	*COL7A1**	*MMP11*[#*¤]	*CTNND2*[¤]	*SELE*[#¤]	*CTNND2*[#*]	*PTEN*[#]	
	COL8A1[#*]	*MMP13*[¤]	*ECM1*[¤]	*SELL*[#*]	*CTSK*[#]	*PTGS2*[#]	
	COL11A1[¤]	*MMP14*[#]	*HAS1*[#¤]	*SELP*[#¤]	*CXCL1*[#]	*RHOA*[#]	
	COL12A1[#*]	*MMP15*[#*]	*ICAM1*[#*¤]	*SGCE*[*¤]	*CXCL5*[#]	*SERPINE1*[#]	
	COL14A1[#¤]	*NCAM1*[#*]	*ITGA1*[¤]	*SPP1**	*ECM1*[#*]	*SGCE*[#*]	
	COX6B2[¤]	*NDUFA11**	*ITGA2*[#*]	*TGFBI*[#*¤]	*FGF2*[#]	*SPARC*[¤]	
	COX6C[*¤]	*NDUFS7**	*ITGA3**	*THBS1**	*FN1*[¤]	*SPG7*[¤]	
	CSF3[#]	*PDGFA*[#]	*ITGA4*[#*¤]	*THBS2*[¤]	*HAS1*[¤]	*SPP1*[#¤]	
	*CTNNA1**	*PECAM1**	*ITGA6*[#¤]	*THBS3*[*¤]	*ICAM1*[¤]	*TAGLN*[#]	
	CTNNB1[#]	*PLAT*[#]	*ITGA7*[#*¤]	*TIMP1*[#*]	*IL1B*[#]	*TGFB1*[#]	
	CTNND1[#]	*PLG*[#]	*ITGA8*[#¤]	*TIMP2*[*¤]	*IL6ST*[#]	*THBS1*[¤]	
	CTNND2[¤]	*PPA1*[¤]	*ITGAL*[#¤]	*TIMP3*[*¤]	*ITGA1*[¤]	*THBS3*[#¤]	
	CTSG[#]	*RAC1*[#]	*ITGAM*[#¤]	*TNC*[¤]	*ITGA2*[#¤]	*TIMP1*[#]	
	CTSL2[#]	*SELE*[*¤]	*ITGAV*[#*]	*VCAM1**	*ITGA3*[¤]	*TIMP3*[#]	
	CXCL2[#]	*SELL*[*¤]	*ITGB1*[#*]	*VCAN*[*¤]	*ITGA5*[#¤]	*TNC*[#*]	
	CXCL11[#]	*SELP**	*ITGB2*[#*¤]	*VTN*[#¤]	*ITGA6*[¤]	*VCAM1*[#]	
	EGF[#]	*SGCE*[¤]	*ITGB3*[#]		*ITGAV*[¤]	*VCAN*[#*]	
	EGFR[#]	*SPARC*[#*]	*ITGB4*[#*¤]		*ITGB1*[#¤]	*VTN**	
	F3[#]	*SPG7*[#*]			*ITGB3*[¤]	*WNT5A*[#]	
	F13A1[#]	*STAT3*[#]			*ITGB5*[#*]		
	FGA[#]	*TGFA*[#]					
	FGF10[#]	*TGFBR3*[#]					
	FN1[#*]	*THBS1*[#*]					
	*HAS1**	*THBS2*[#*]					
	HGF[#]	*THBS3**					
	*ICAM1**	*TIMP1*[#*¤-»]					
	IFNG[#]	*TIMP2*[#]					
	IGF1[#]	*TNF*[#]					
	IL10[#]	*VCAM1*[¤]					
	IL2[#]	*VCAN*[¤]					
	IL4[#]	*VTN*[#*]					
	ITGA1[#*]	*WISP1*[#]					
	*ITGA2**						

4.6. Gene Expression

As the PBM research field grows, investigations into changes in gene expression and the transcription factors that govern this expression have been at the forefront of recent studies. Multiple *in-vitro* studies have been conducted to determine the regulation of genes involved in the wound healing process in response to PBM. While many genes likely undergo changes when irradiated, much of the current literature has focused on the modulation of genes related to cell proliferation and wound healing such as collagen type-I alpha (*COL1A1*), vascular epithelial growth factor (*VEGF*), and various MMPs encoding genes. Multiple studies have shown that a dose of 0.1, 0.88, 1.5, 3, 4 or 5 J/cm^2 can significantly modulate various genes related to wound healing and the ECM, in both normal, and healthy cell cultures [46,64,67–74], and furthermore a single study has shown modulation of similar genes at a fluence of 20 J/cm^2 [38]. Interestingly, research at a higher fluence of 45, 90 and 180 J/cm^2 has demonstrated the upregulation genes encoding for HSP 27 and 90 again suggestive of a stress response [41]. One study has also investigated the effects of PBM on mitochondrial energy metabolism genes, demonstrating that a fluence of 5 J/cm^2 can upregulate genes related to mitochondrial complexes I, IV and V such as *NDUFA11, COX6C, ATP5F1*, reinforcing the mechanistic mitochondrial hypothesis of PBM [75]. Researchers have also investigated the effect of PBM on fibroblast genes that predispose to cancer, demonstrating that a fluence as little as 0.67 J/cm^2 can affect a range of cancer genes such as *BRCA 1* and *2* [76]. While evidence appears to demonstrate an ability for PBM to alter the transcriptional profile of fibroblasts, translation of this into functional outcomes is problematic, with none of the studies investigating the downstream cellular effects of these changes (Table 2).

5. Discussion

While a growing body of research suggests that PBM appears to be an effective intervention for accelerating wound healing, many aspects of the exact molecular and cellular mechanisms underpinning these effects are still to be explored [8,18]. While plausible mechanisms of PBM have been proposed, there is no current evidence describing the complete molecular and cellular effects [7]. The fundamental aim of this review was to synthesize the current evidence describing the effects of PBM on human dermal fibroblasts, particularly within five specific domains: cellular viability and proliferation; cellular migration; ATP production and mitochondrial membrane potential; protein expression and synthesis; and gene expression. Overall, it was found that in addition to a lack of clarity surrounding established physiological mechanisms, there are no evidence-based guidelines or investigational consistency regarding the optimal light parameters for investigating the biological effects of PBM *in vitro*. As the body of evidence for PBM continues to expand and evolve, several obstacles will need to be overcome to improve the consistency of research within the field. As the cost and prevalence of chronic wounds likely to increase in the future [4], the shortage of translational research with consistent methodology presents an explicit requirement for new research, as currently, animal models are left to bridge the gap from research to clinical practice.

PBM appears to be able to have dose-dependent biological effects, with stimulatory changes with lower to moderate doses, and inhibitory effects at high dose [77,78]. It appears likely that total fluence and exposure time are associated with the efficacy of PBM [79]. On this basis, PBM appears to mostly accelerate physiological processes of fibroblasts *in vitro* that contribute to wound healing when applied at a fluence of 0.5–5 J/cm^2 [28,29,35,46,56]. Within these ranges, cellular proliferation, viability and migration appear to improve when compared to non-irradiated controls. Importantly, some of the research in this subsection fails to consider the distinction between migration, viability and proliferation, which must be explored further to determine the effectiveness of PBM interventions [80]. Having said this, these findings are generally in agreement with investigations on other cell types, suggesting a common underlying mechanism [81–87]. Furthermore, our review demonstrates that there appears to be a more defined dose-response relationship when testing PBM's effect on cellular proliferation, as opposed to viability *in vitro,* as illustrated by inconsistent cellular viability findings. Frequent intra- and inter-study inconsistencies regarding the optimum irradiation parameters required to produce a positive physiological effect may be in part responsible for these findings. Despite many experimental variables contributing to these inconsistencies in viability measurements, our findings do indicate that true proliferation assays may be better placed than viability assays to optimize *in vitro* PBM experiments before proceeding to more expansive experiments. Hence, the use of modern, sensitive and accurate proliferation assays, such as the EdU assay, should be utilized more frequently at this phase of PBM research.

While the majority of investigations found benefit within these lower fluence ranges, there is some inconsistency in results. Some studies report a positive physiological change *in vitro* when exposed to doses as high as 180 J/cm^2 [41] casting uncertainty on the suggested inhibitory threshold of 9–10 J/cm^2 [77]. This is possibly due to the many differences in cell culture conditions, methods of assessment and light parameters, leaving the optimal fluency for positive effects unclear, and necessitates further focused research on dose-responses. In the field more broadly, detailed parameters are sometimes misreported or completely lacking. Variables including radiation spot size, target well configuration, distance from target sight, continual measurement of power output, and controlling for light scattering, amongst others, are often lacking [9]. Until all parameters are consistently reported, discrepancies in the literature both *in vitro* and *in vivo*, are likely to hamper further progress.

Changes to mitochondrial function are central to the current mechanistic theory of PBM [25]. It mostly appears irradiation up to 5 J/cm^2 can have short-term positive effects on ATP production and mitochondrial function, in the form of complex enzyme regulation and mitochondrial membrane potential [44,59–61]. In cellular models of disease (physical trauma, hyperglycemia, acidosis and hypoxia), the optimal level of irradiation required to impact the mitochondria can change significantly or disappear entirely [59]. This finding is important, as it infers that different disease states and applications of PBM may warrant tailored application. In a clinical context, this is critical knowledge to allow the full therapeutic exploitation of PBM. Furthermore, despite indirect evidence of interactions between PBM and COX in the form of enzymatic reactions [59,88], no research has demonstrated direct, preferential photonic interaction with COX, or any other parts of the mitochondria. Understanding the minutia of light-biomolecule interactions will open a gateway to deep mechanistic understanding of the therapeutic use of light.

Multiple investigations have shown changes to both gene and subsequent protein expression downstream as indicators of a physiological effect induced by PBM. Modulation of protein and gene expression through PBM represents an important mechanism by which the treatment may influence inflammatory cytokine production, wound healing rates, and thereby promote cellular viability, proliferation and migration [35,46,62,63,68]. Several studies have demonstrated mixed results in modulating ECM, collagen and cytokine expression. However, the literature investigating these processes at large, exhibits vast heterogeneity of light application, leading to conflicting results amongst researchers. A fluence of up to 5 J/cm^2 appears to cause upregulation in the genes associated with healing processes [46,62,64,68]. However, other studies have reported similar results at fluences as high as 180 J/cm^2 [41]. Again, this contradictory evidence casts confusion on the optimal parameters required for influencing gene and protein expression. While PBM seems able to have an impact on transcription, the findings appear to lack consistency in terms of expression of specific groups of genes, or transcription factors. Furthermore, the pathways that underpin these transcriptional effects remain under investigated. Future reviews should investigate gene regulation more rigorously to provide

insight into the clinical effects of the treatment. Investigating the transcription factors and cellular signaling related to gene expression may also provide a better mechanistic understanding of how the genes responsive to PBM contribute to wound healing.

Our review focused on fluence as the main variable governing the physiological effects of PBM. As such, we did not explore the effect of wavelength on fibroblast physiology: namely those between red and infrared. There is much debate within the PBM field as to whether wavelength, specifically those between 600 and 1070 nm exert different physiological effects [8,88]. Using the *optical window* model, near-infrared light is said to penetrate deeper than red due to decreased absorption by melanin and hemoglobin [7]. Looking at this through the lens of *in vitro* research, this seems less important given the amount of light penetration needed for this application. Having said this, some of the *in vitro* work we reviewed has demonstrated different physiological effects when comparing red light to NIR wavelengths [30,38,58], with other research showing a synergistic effect using multiple red and NIR wavelengths [89]. However, there has been no clear evidence to show an optimal wavelength for *in vitro* research [90]. Given that current evidence points to importance of correct fluence, as oppose to wavelength, as a determinant of successful *in vitro* work [90], a universal consensus to a set of *in vitro* experimental standards tightly controlling fluence reaching the tissue, should be developed first. Although other light variables such as wavelength, pulse structure and polarization orientation are important factors in *in vivo* research [22], improved *in vitro* experimental standards and guidelines will contribute to improved translation research, leading to advanced patient outcomes, and the wider uptake of PBM by the medical field more broadly.

6. Limitations

In an attempt to limit the impact of divergent technical variables, search criteria excluded wavelengths existing outside of the defined PBM parameters of 600 nm-1070 nm, possibly neglecting potentially relevant literature. The exclusion animal and non-dermal cell lines, may also have overlooked key literature, however, we felt that this would have made the review too broad.

7. Future Directions

Once a more consistent set of *in vitro* parameters has been established for PBM research more broadly, there are many innovative methods that could be used to develop more valid scientific data. Three-dimensional (3D) cell culture is an emerging method used in biomedical research that better resembles the *in vivo* environment and represents a more efficacious way of translating *in vitro* research to animal and human studies [91]. Initial studies investigating the effects of PBM on fibroblasts using a 3D collagen matrix model have demonstrated promising results. This study found increased cellular viability and upregulation in gene expression responses when exposed to a 780 nm laser [92]. Furthermore, the advent of 3D bioprinting is an exciting technology that the field of phototherapy could take advantage of. This technology can allow research to produce detailed, 3D, multicellular models that may eventually supersede animal models [93]. Researchers should also consider next-generation sequencing (NGS) technology to create a more rapid and accurate representation of the genome, revealing potential modulatory effects of PBM. A significant shortcoming of NGS and its' widespread application is the significant cost associated with the technology. However, continued technological innovation is likely to make it more affordable and competitive [94]. Future research on PBM and wound healing should not only try to establish more concrete biological mechanisms behind the therapy, but also aim at developing more detailed models of photonic interactions with target cellular components.

8. Conclusion

This review found that PBM has the capacity for therapeutically significant effects on human dermal fibroblasts *in vitro*, particularly in the domains of cellular viability, proliferation and migration, ATP and mitochondrial function, as well as changes in protein and gene expression. With the research and application of PBM growing at an unprecedented rate, the development of an optimal, widely-accepted *in vitro* framework is vital to improve the validity and consistency of the research in this field. While existing research demonstrates varied benefits, the variance in light parameters, methodological assessment methods leads to challenges in interpretation of results. The experimental standards will help to improve understanding of the precise cellular and molecular mechanisms of PBM. This therapy may offer a safe, non-invasive intervention for a variety of chronic and debilitating conditions; making it an exciting area for future research. Its clinical exploitation has the potential to relieve healthcare systems globally of huge costs relating to slow, or non-healing chronic wounds and their complications.

Declaration of Competing Interest

The Authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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RESEARCH Open Access

The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: a systematic review and meta-analysis of randomized control trials

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Abstract

Background: Tendinopathy is a common clinical condition that can significantly affect a person's physical function and quality of life. Despite exercise therapy being the mainstay of tendinopathy management, there are many potential adjunct therapies that remain under investigated, one of which is photobiomodulation (PBM). PBM uses varied wavelengths of light to create a biological effect. While PBM is used frequently in the management of tendinopathy, high quality evidence supporting its utility is lacking.

Methods: A systematic search of the Pubmed, CINAHL, SCOPUS, Cochrane Database, Web of Science and SPORTSDICUS databases was performed for eligible articles in August 2020. Randomized Control Trials that used red or near-infrared PBM to treat tendinopathy disorders that made comparisons with a sham or 'other' intervention were included. Pain and function data were extracted from the included studies. The data were synthesized using a random effects model. The meta-analysis was performed using the mean difference (MD) and standardized mean difference (SMD) statistics.

Results: A total of 17 trials were included ($n = 835$). When compared solely to other interventions PBM resulted in similar decreases in pain (MD -0.09; 95% CI − 0.79 to 0.61) and a smaller improvement in function (SMD -0.52; 95% CI − 0.81 to − 0.23). When PBM plus exercise was compared to sham treatment plus exercise, PBM demonstrated greater decreases in pain (MD 1.06; 95% CI 0.57 to 1.55) and improved function (MD 5.65; 95% CI 0.25 to 11.04). When PBM plus exercise was compared to other interventions plus exercise, no differences were noted in pain levels (MD 0.31; 95% CI − 0.07 to 0.70). Most studies were judged as low-risk of bias. The outcome measures were classified as very low to moderate evidence quality according to the Grading of Recommendation, Development and Evaluation tool.

Conclusion: There is very-low-to-moderate quality evidence demonstrating that PBM has utility as a standalone and/or adjunctive therapy for tendinopathy disorders.

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Keywords: Tendinopathy, Photobiomodulation, Pain, Low-level laser therapy, Meta-analysis, Systematic review

Background

Tendinopathies represent a common presentation to clinical practice, particularly in active persons [1]. For instance, Achilles tendinopathy has been reported to occur at a rate of 2.35 per 1000 patients [2], whilst occurring between 6.2–9.5% in athletic populations [3]. Regardless of cohort, tendinopathy can profoundly affect a person's quality of life and ability to perform activities of daily living, and cause considerable economic impact [4]. Traditionally, tendon pain was known as tendinitis, referring to the pain and inflammation thought to be associated with this condition [4]. However, as research in this area advanced, it was noted that most painful tendon disorders are chronic disorders, lacking a primary inflammatory driver [5–7]. Hence, the next term that evolved to describe this disorder was tendinosis, referring to the deleterious histopathological changes that can occur within a painful tendon [5]. More contemporary research now advocates for the term tendinopathy when describing any painful tendon disorder [7, 8]. Despite the original definition being grounded in the histopathological and clinical findings [7], tendinopathy is now defined as persistent tendon pain and loss of function related to mechanical loading [8], which may be associated with radiological changes [9].

Despite extensive research efforts in recent years, the complete pathophysiological picture of tendinopathy remains poorly understood [1]. However, it is known that four key cellular changes typify tendon pathology: 1. Increased number and metabolism of tenocytes; 2. Large proteoglycan presence, causing increased water content; 3. Abnormal collagen alignment and 4. New blood vessel and nerve growth within the tendon [10]. Regardless of the exact pathophysiological mechanisms, diagnosis of tendinopathy is primarily clinical, rather than radiological [1]. Tendinopathy presents as localized tendon pain that is correlated to mechanical load, that is beyond the tendon's current capacity [8]. A clinician must pay close attention to changes in activity load and other rheumatological, metabolic and endocrine risk factors, with pain being produced during specific provocative movements, or by activities of daily living [1]. Furthermore, given the poor correlation between pain, function and histopathological radiological findings [10], and the absence of a defined nociceptive tendinopathic pathway [1], it is also important to consider the psychosocial influences of tendinopathy [1, 4, 11].

Due to the common prevalence of tendinopathy there is a large variety of treatment methodologies that have been employed, of which, exercise rehabilitation is the most well supported [1, 12, 13]. There are also a number of adjunct therapies used in the management of tendinopathy, including: Extracorporeal shock wave therapy (ESWT), Non-steroidal anti-inflammatory drugs (NSAIDs), injection therapies such as platelet rich plasma (PRP), corticosteroids (CS), and prolotherapy, transdermal application of CS through the method of Iontophoresis, and also passive interventions such as stretching and deep friction massage [1, 13]. While some of these treatments show promise, most have been shown to be no better, or worse that exercise rehabilitation [1].

An emerging and underexplored treatment in the management of tendinopathy is photobiomodulation (PBM) [14]. While the exact physiological mechanisms underpinning PBM are yet to be fully described, the prevailing theory is based on the interplay between adenosine triphosphate (ATP), nitric oxide (NO) and cytochrome c oxidase (complex IV of the mitochondria) [15]. It is thought that both red and near-infrared (NIR) light have a high affinity for CCO [15]. During routine metabolism, or in instances of cellular stress, NO may competitively bind to CCO, displacing oxygen, slowing or limiting ATP production. PBM has been suggested to displace the NO from CCO, allowing oxygen to more freely interact with CCO, thus enhancing ATP production [15]. Despite this mechanism being widely accepted, there is no evidence to date that shows a direct photobiological interaction with CCO [14, 16]. Additionally, there are many other secondary mechanisms by which PBM may exert its effects. These include an increased production of reactive oxygen species (ROS), which can lead to upregulations in gene transcription and downstream protein expression [14, 17], and additionally may modulate key immune cells leading to improved tissue healing and neural fibre inhibition [14, 18, 19].

At a more fundamental level, how PBM affects tendon tissue in vitro, and in animal models has been investigated. In vitro PBM appears to influence multiple mechanisms related to growth and proliferation. Specifically, PBM can increase the expression of genes related to proliferating cell nuclear antigen (PCNA) and transforming growth factor-β1 (TGF-β1) [20, 21]; Cyclins E, A, and B1 [21]; expression of genes related to type I collagen, decorin [22] and dynamin II [23], all of which are key regulators of the healing response. Interestingly, PBM has also been shown to decrease the expression of genes related to inflammation such as TNF-α [24] and IL-6 in

tenocytes [25]. The positive effects of PBM have also been observed in animal models of tendinopathy, showing mild improvements in functional healing compared to non-irradiated controls [26]. However, as with many areas of study within the field of PBM, a recent review article reported that the lack of a standardized process for treating animal tendons with PBM makes comparison difficult, and its further development and standardization should be given priority [27].

The impact of PBM on tendinopathy has been appraised with reviews on specific tendinopathies such as: lateral elbow tendinopathy [28]; Achilles tendinopathy [29]; and shoulder tendinopathy [30]; all of which demonstrated mixed effects, possibly due to a lack of consistent PBM application variables between studies. There has also been a systematic review and meta-analysis of the effects of PBM on all human tendinopathies, however it was reported in 2010, and included both randomized controlled trials (RCTs) and controlled clinical trials (CCTs) [31], and again mixed results were reported. Building on these previous works, and given the proposed universal effects of PBM, the aim of this work was to synthesize the current evidence describing the impact of low-intensity red and NIR PBM on pain and function in all tendinopathy disorders in human patients. Specifically, appraising only RCTs, we analyzed the effects of PBM on tendinopathy in three domains: Pain, PROMS and Strength.

Methods

Protocol and registration

This review was prospectively registered in the PROSPERO database (registration number: CRD42020202508). It was also completed and structured according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [32].

Eligibility criteria

Studies included in this review were any randomized controlled trials that used up to a class 3B power laser, or equivalent light sources within the 600 nm – 1100 nm spectrum, to treat any diagnosed tendinopathy or tendinopathy-related disorders. Given the proposed universal effects of PBM, and the wide-ranging appraisal aim of this review, all tendinopathy and tendinopathy-related disorders were pooled. Comparisons had to be made to placebo or other clinical interventions in human adults. Further, the trials needed to report Visual Analogue Scale (VAS), validated Patient Reported Outcome Measure (PROM) data and/or changes in muscle strength. Studies were excluded if they were produced before the year 2000 given the change in both the diagnosis and understanding of tendinopathy [7] and the changes in PBM application [33] in that time. Articles unavailable in English were excluded.

Information sources and search strategy

The search terms used in this review were: (Photobiomodulation OR Low-level laser OR LLLT) AND (tendon* OR tendin* OR epicond* OR teno* OR elbow OR bursitis OR subacromial). The databases that were searched were: Pubmed, CINAHL, SCOPUS, Cochrane Database, Web of Science, SPORTSDiscus. This search was completed by 1st August, 2020. An updated search was performed in April 2021 and yielded no additional results. Reference lists of relevant PBM reviews were also searched. A detailed description of the search can be found in Table 1 of Additional file 1.

Study selection

The titles and abstract of all the studies yielded in the initial search were screened by two of the authors (NT and JF) for eligibility using the Covidence (Melbourne, Australia) platform. Any disagreements were resolved by a third author (MH). From here, full-text analysis was completed by the two of the authors (NT and JF) and again resolved by a third (MH). The authors of studies which reported insufficient data for the meta-analysis were contacted by email, however, were excluded if no response was given.

Risk of Bias

Two of the authors (NT and JF) assessed the included studies for bias using the Cochrane Collaboration's risk-of-bias tool [34]. Publication bias was assessed by funnel plot analysis generated by Review Manager Version 4.5 (The Cochrane Collaboration, Denmark), where there were more than 10 studies to analyze.

Data collection process

Data of interest was extracted individually by two of the authors (NT and JF), with any disputes or inconsistencies resolved by the addition of a third author (MH), and then reaching a consensus decision.

Data items

The primary outcomes taken for this study were pain intensity, in the form of the VAS, validated PROMS and changes in muscle strength. Range of motion measurements were excluded as they are not considered to be a core domain of tendinopathy [35]. The secondary outcome taken was reporting of adverse effects.

Summary measures

As the primary measurements were all reported as continuous data, VAS and PROM data were combined using the mean difference (MD) statistic, while change in

muscle strength data was analyzed using the standardized mean difference (SMD) statistic (given the heterogeneity in measuring muscle strength), using the change scores between time points. As only three of the included studies reported the SD change score [36–38], the correlation coefficient was calculated to be 0.8 based on these studies [39]. The data then underwent a sensitivity analysis comparing the meta-analysis results using a correlation coefficient of 0.2 and 0.8. As no change in the results were detected with either coefficient, the correlation coefficient of 0.8 was used for the final analysis VAS data was reported on a scale of 0–10, with data reported on a scale of 0–100 transformed to the 0–10 scale. PROM data was reported on a scale of 0–100. Studies that reported multiple VAS sub-scales (i.e. VAS rest, VAS night, etc.) and strength testing measurements means were averaged, and their standard deviation pooled according to previously described measures [39]. Studies that reported a 95% confidence interval (CI), and not the SD, were converted to SD [39].

Synthesis of results

Two authors (NT and JF) completed the analysis using both Microsoft Excel (Microsoft, USA) and Review Manager Version 4.5 (The Cochrane Collaboration, Denmark). A random effects meta-analysis was used to analyze the results, with the I^2 statistic being used to assess study heterogeneity. The trials were grouped according to VAS, specific PROM and strength measurements. Given the variability in design amongst the included studies, multiple subgroupings were made according to time points analyzed and comparison treatments and controls. 'End of treatment' was defined as end of a 2–4 week course of the treatment intervention, while 'Follow Up' was defined as 3 months post-treatment.

The evidence quality of each outcome was subjectively assessed using the Grading of Recommendation, Development and Evaluation (GRADE) tool [40]. Using the criteria from Tomazoni, Almeida [41], five factors and threshold criteria were used to assess the evidence quality: Risk of Bias: > 25% of trials classified at high risk of bias; Inconsistency: $I^2 > 50\%$; Indirectness: > 50% of participants not related to trial's target audience; Imprecision: < 400 participants in the comparison for continuous outcomes; and Publication Bias: funnel plot if > 10 trials in same comparison [41]. The evidence quality could be categorized according to four ratings: High; Moderate; Low; and Very Low. Each time an outcome did not meet each of the threshold criteria it was downgraded one level per criteria. For example, if one measure did not meet the thresholds for risk of bias and Inconsistency it was classified as low-quality evidence, downgraded from high-quality evidence.

Results

Search summary

The detailed search strategy is shown in Table 1 of Additional file 1. The initial search strategy yielded 1230 results, after title and abstract screening of these results, 104 studies remained. When these were subjected to full-text screening 22 studies were eligible, of which 17 were included in the meta-analysis [36–38, 42–55] (Fig. 1). The five eligible, but excluded studies, were omitted due to insufficient data, which could not be obtained by contacting the authors [56–60]/ The pooled studies equated to a total of (n = 835) participants.

Included study characteristics

Participant diagnosis

Of the included studies, one investigated (n = 1) Achilles Tendinopathy (AT) [53]; one investigated De Quervain's Tenosynovitis (DQT) (n = 1) [51]; seven (n = 7) investigated Lateral Elbow Tendinopathy (LET) [36, 43, 45, 46, 48, 50, 52]; one (n = 1) investigated Patella Tendinopathy (PT) [38]; and seven (n = 7) investigated Sub-acromial Syndrome/Rotator Cuff Tendinopathy (SAS/RT) [37, 42, 44, 47, 49, 54, 55] (Table 1).

Interventions

There were a wide array of PBM application variables used within the included studies. All the studies used NIR light, ranging from 0.5-5 J/cm^2, and all studies irradiated multiple sites. Additionally, there were a number of studies that did not report all necessary light application variables [36, 42, 46, 47, 49, 51, 54, 55] (Tables 1 and 2). Other comparative interventions ("other interventions") included: Phonophoresis and Iontophoresis [43]; ESWT [46]; High-Intensity Laser Therapy (HILT) [48]; Passive Physiotherapy [37]; and US [51]; with the remaining studies using exercise alone [36, 42, 50, 52, 53, 55], or exercise plus another intervention [45, 54]. Only four studies used the WALT guidelines [33] to inform their treatment protocols [36, 51, 53, 54] (Tables 1 and 2).

Outcome measures

All the included studies used VAS as an outcome measure. Of the studies that used PROMS in their measures, four (k = 4) studies used the Disabilities of the Arm, Shoulder and Hand (DASH) measure [36, 45, 50, 55]; with one (k = 1) using the Quick DASH (Q-DASH) [48]; two (k = 2) used the Patient Reported Tennis Elbow Evaluation (PRTEE) [36, 43]; two (n = 2) used the Shoulder Disability Questionnaire (SDQ) [37, 55]; three (k = 3) used the Shoulder Pain and Disability Index (SPADI) [44, 47, 49]; and one (k = 1) study used the Victoria Institute of Sport Assessment-Patella Tendon (VISA-P) [38]. Due to the heterogeneous nature, and limited

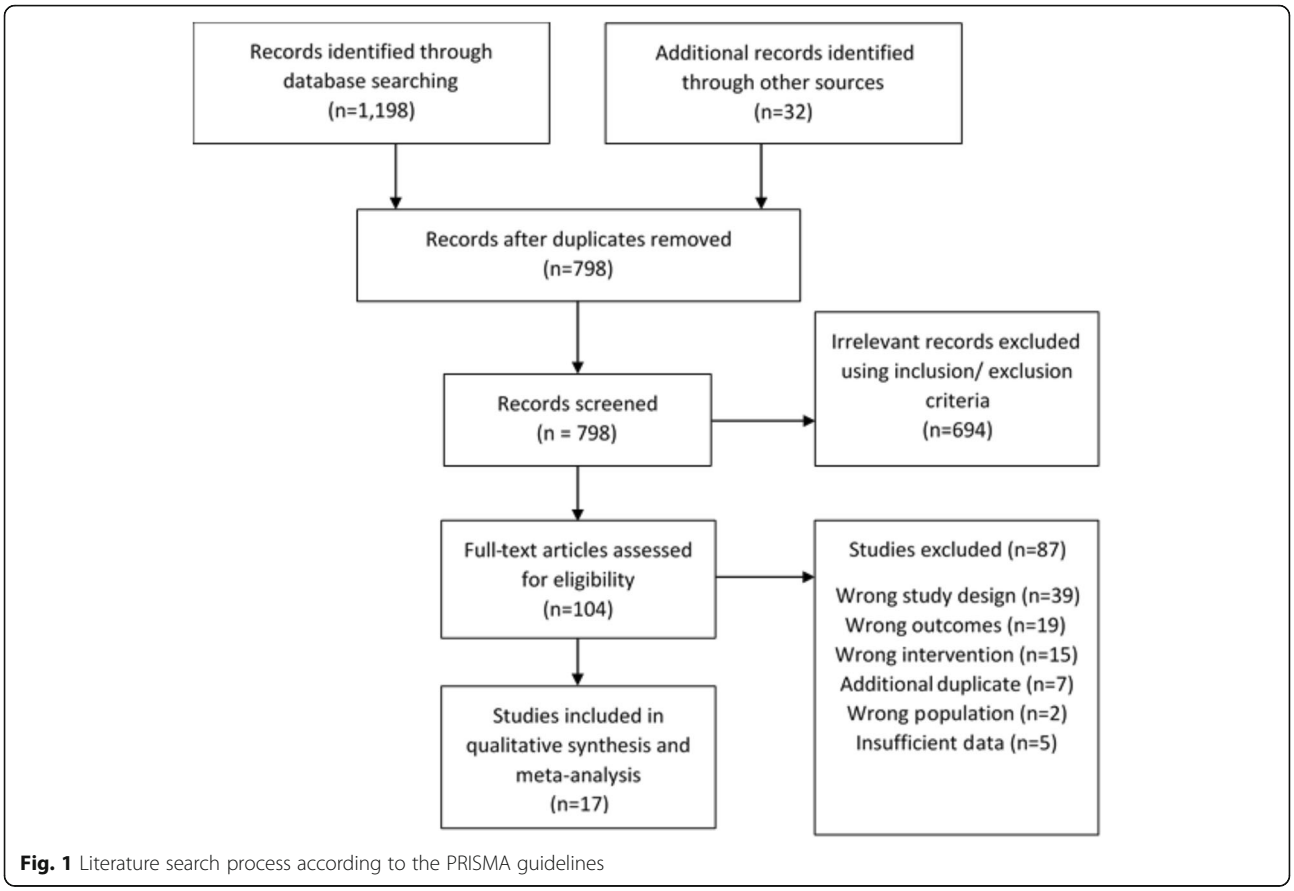

Fig. 1 Literature search process according to the PRISMA guidelines

numbers of study interventions, only the DASH scores could be subject to meta-analysis. Additionally, there were 10 ($k = 10$) studies that used muscle strength scores and an outcome measure [36, 38, 43, 45, 46, 48, 50–52, 55] (Table 1). Only five studies reported if any adverse effects occurred in the trial, of which there were none [42, 44, 47, 48, 55].

Risk of Bias

When pooled together the included studies were judged to a low risk of bias 68.1% of the time, an unclear risk of bias 23.5% of the time, and high risk of bias 8.4% of the time. Largely, the included studies tended to under report the randomization and blinding protocols, with some studies also failing to report all the required light parameters, hence being judged as being subject to 'other bias' (Fig. 2). Publication bias via funnel plot analysis was not completed as none of the individual forest plots contained > 10 studies [34].

VAS measures

PBM only versus other interventions only

When compared to other interventions only (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), PBM only, demonstrated similar effects from baseline-end of treatment (MD -0.09; 95% CI --0.79 to 0.61; $I^2 = 78\%$; $n = 105$). The studies in this outcome were downgraded to very low-quality evidence due to risk of bias, inconsistency, and imprecision (Fig. 3a).

PBM plus exercise versus sham plus exercise

Overall, PBM plus exercise demonstrated significant reductions in pain levels compared to sham plus exercise (MD 1.06; 95% CI 0.57 to 1.55; $I^2 = 82\%$; $n = 224$). The time period subgroup analysis showed similar results with, PBM plus exercise creating a more substantial decrease in pain at baseline-end of treatment (MD 0.96; 95% CI 0.27 to 1.64; $I^2 = 89\%$; $n = 154$), and baseline-follow up (MD 1.22; 95% CI 0.68 to 1.76; $I^2 = 35\%$; $n = 70$). There were no significant between-subgroup differences found ($p = 0.55$). The studies in this outcome were downgraded to low-quality evidence due to inconsistency and Imprecision (Fig. 3b).

PBM plus exercise versus other intervention plus exercise

No significant difference was found between PBM plus exercise and other interventions (ESWT and US) plus exercise (MD 0.31; 95% CI – 0.07 to 0.70; $I^2 = 0\%$; $n = 70$). The time period subgroup analysis demonstrated similar effects on pain within the baseline-end of

Table 1 Characteristics of included studies

Study First Author, Year	Diagnosis	Total Participants; Participants per group	Intervention Groups	Outcomes Extracted	Treatment Time	Measurement Time Points
Abrisham 2011 [42]	SAS	80; 40/40	PBM + Exercise, Sham; Laser + Exercise	VAS	Two weeks	1. Baseline; 2. Two weeks
Baktir 2018 [43]	LET	37; 12/13/13	PBM; Phonophoresis; Iontophoresis	VAS; PRTEE-t	Three weeks	1. Baseline; 2. Two Weeks
Bal 2009 [44]	SAS	44; 22/22	PBM + Exercise; Exercise Only	VAS; SPADI-t	Two weeks	1. Baseline; 2. Two weeks; 3. Three month follow up
Celik 2019 [45]	LET	43; 23/22	PBM + Exercise; ESWT + Exercise	VAS; DASH	Four weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Devrimsel 2014 [46]	LET	60; 30/30	PBM; ESWT	VAS	Four weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Dogan 2010 [47]	SAS	52; 30/22	PBM + Exercise; Sham PBM + Exercise	VAS; SAPDI-t	Three weeks	1. Baseline; 2. Three weeks
Emanet 2010 [36]	LET	50; 25/25	PBM + Exercise; Sham PBM + Exercise	VAS; DASH; PRETEE-t	Three weeks	1. Baseline; 2. Three weeks; 3. Three month follow up
Eslamian 2012 [37]	RT	50; 25/25	PBM + Passive Physiotherapy; Sham PBM + Passive Physiotherapy	VAS; SDQ	Three weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Kaydok 2020 [48]	LET	59; 30/29	PBM + HILT	VAS; QDASH	Three weeks	1. Baseline; 2. Three weeks
Kibar 2017 [49]	SAS	62; 30/32	PBM; Sham PBM	VAS; SAPDI-t	Three weeks	1. Baseline; 2. Three weeks
Lam 2007 [50]	LET	39; 21/18	PBM + Exercise; Sham + Exercise Only	VAS; DASH	Three weeks	1. Baseline; 2. Three weeks
Liu 2014 [38]	PT	21; 7/7/7	PBM; Exercise Only; PBM + Exercise	VAS; VISA-P	Four Weeks	1. Baseline; 2. Four weeks
Sharma 2015 [51]	DQT	30; 15/15	PBM; US	VAS	Two Weeks	1. Baseline; 2. Two weeks
Stergioulas 2007 [52]	LET	50; 20/20	PBM + Exercise; Sham + Exercise	VAS	Four and Eight Weeks	1. Baseline; 2. Eight weeks; 3. Two month follow up
Stergioulas 2008 [53]	AT	40; 20/20	PBM + Exercise; Sham + Exercise	VAS	Four and Eight Weeks	1. Baseline; 2. Four weeks; 3. Eight Weeks; 4. Three month follow up
Yavuz 2014 [54]	SAS	31; 16/15	PBM + Exercise; US + Exercise	VAS; SPADI-D	Four Weeks	1. Baseline; 2. Four weeks; 3. Three month follow up
Yeldan, 2009 [55]	SAS	60; 34/26	PBM + Exercise; Sham PBM + Exercise	VAS; DASH; SDQ	Three Weeks	1. Baseline; 2. Three weeks

treatment (MD 0.20; 95% CI − 0.34 to 0.74; $I^2 = 0\%$; $n = 35$), and baseline-follow up (MD 0.43; 95% CI − 0.12 to 0.97; $I^2 = 0\%$; $n = 35$) periods. There were no significant between-subgroup differences found ($p = 0.57$). The studies in this outcome were downgraded to moderate-quality evidence due to imprecision (Fig. 3c).

Proms

DASH: PBM plus exercise versus sham plus exercise

PBM plus exercise demonstrated a significant improvement in the DASH PROM score compared to sham plus exercise (MD 5.65; 95% CI 0.25 to 11.04; $I^2 = 78\%$ $n = 112$). The time period subgroup analysis showed no significant effect of PBM at baseline-end of treatment (MD 2.83; 95% CI − 4.56 to 0.70; $I^2 = 80\%$; $n = 69$), while PBM plus exercise demonstrated a significant positive effect at the baseline-follow up period (MD 9.47; 95% CI 5.63 to 13.31; $I^2 = 0\%$; $n = 43$). There were no significant between-subgroup differences found ($p = 0.12$). The studies in this outcome were downgraded to very low-quality evidence due to risk of bias, inconsistency and imprecision (Fig. 4).

Strength measures

PBM only versus other interventions only

When compared to other interventions only (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), PBM only, demonstrated a significantly decreased

Table 2 PBM variables of included studies

Study First Author, Year	PBM light source; Wavelength	Light source power output during treatment (mW)	Fluence per spot (J/cm^2)	Treatment spots	PBM sessions per week; Total PBM sessions	WALT recommendations informed trial?
Abrisham 2011 [42]	'Laser Device;' 890 nm	Not Reported	2–4	3	5; 10	No
Baktir 2018 [43]	GaAs Laser Diode; 904 nm	0.12	Not Reported	5	5; 15	No
Bal 2009 [44]	GaAs Laser Diode; 904 nm	13.2	2	4	5;10	No
Celik 2019 [45]	GaAs Laser Diode; 904 nm	40	2.4	6	3;12	No
Devrimsel 2014 [46]	'Laser;' 850 nm	Not Reported	Not Reported	Not Reported	2; 10	No
Dogan 2010 [47]	GaAlAs; 850 nm	Not Reported	5	5–6	4–5; 14	No
Emanet 2010 [36]	GaAs Laser; 905 nm	Not Reported	1	2	5; 15	Yes
Eslamian 2012 [37]	Ga-Al-As Laser Diode; 850 nm	100	4	Up to 10	3; 9	No
Kaydok 2020 [48]	Ga-Al-As Laser Diode; 904 nm	240	2–4	6	3; 9	No
Kibar 2017 [49]	Ga-Al-As Laser Diode; 850 nm	Not Reported	4	11	3; 9	No
Lam 2007 [50]	Ga-Al-As Laser Diode; 904 nm	25	2.4	Average 2.4	3; 9	No
Liu 2014 [38]	Ga-Al-As Laser Diode; 810 nm	200	Not Reported	3	6; 24	No
Sharma 2015 [51]	Ga-Al-As Laser Diode; 830 nm	30–40	3	Not Reported	3–4; 7	Yes
Stergioulas 2007 [52]	Ga-As; 904 nm	40	2.4	6	1–2; 12	No
Stergioulas 2008 [53]	Ga-Al-As Laser Diode; 820 nm	30	0.5	6	1–2; 12	Yes
Yavuz 2014 [54]	Ga-Al-As Laser Diode; 850 nm	Not Reported	3	5 maximum	2–3; 10	Yes
Yeldan, 2009 [55]	GaAs; 904 nm	Not Reported	Not Reported	5 Maximum	Not Reported	No

effect from baseline-end of treatment (SMD -0.52; 95% CI − 0.81 to − 0.23; $I^2 = 0\%$; $n = 105$) (Fig. 5a). The studies in this outcome were downgraded to low-quality evidence due to risk of bias and imprecision.

PBM plus Exercise versus Sham plus Exercise.

Overall, the results demonstrated that PBM plus exercise caused significant increase in strength compared to sham plus exercise (SMD 0.66; 95% CI 0.11 to 1.21; $I^2 = 81\%$; $n = 144$). The time period subgroup analysis however, demonstrated no significant effect for PBM plus exercise on functional strength measures within both the baseline-end of treatment (SMD 0.59; 95% CI − 0.13 to − 1.31; $I^2 = 83\%$; $n = 94$) and baseline-follow up period (SMD 0.82; 95% CI − 0.33 to 1.96; $I^2 = 87\%$; $n = 50$). There were no significant between-subgroup differences found ($p = 0.74$). The studies in this outcome were downgraded to low-quality evidence due to Inconsistency and Imprecision (Fig. 5b).

GRADE classifications

The quality of evidence classification for each outcome is located in Table 2 in Additional file 1.

Discussion

The overarching aim of this review was to investigate the effect of low-intensity red and NIR PBM on pain and function in patients with tendinopathy and tendinopathy-related disorders. It was found that when compared to other interventions, with or without exercise added (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US), that there is very low-moderate quality evidence to show that PBM with or without

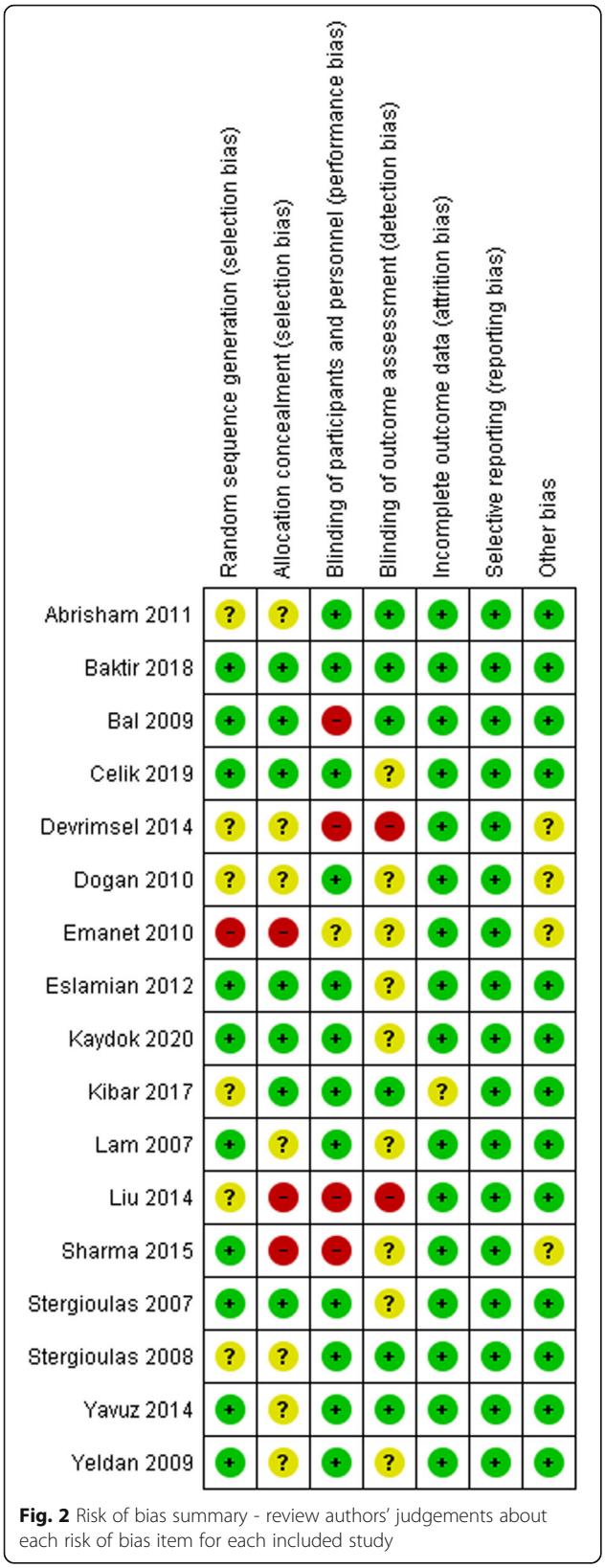

Fig. 2 Risk of bias summary - review authors' judgements about each risk of bias item for each included study

exercise were equally effective at reducing pain. This review also found very low-quality evidence demonstrating that when PBM is combined with exercise, it results in a significant improvement in PROMS compared to sham treatment plus exercise. There was also low-quality evidence demonstrating that other interventions (Phonophoresis, Iontophoresis, ESWT, HILT, CS Injection and US) were significantly better at improving functional strength measures compared to PBM, while when exercise was added to PBM therapy, it was significantly better at restoring functional muscle strength compared to sham treatment plus exercise.

Despite the small body of somewhat favorable evidence for PBM, as a whole, there were multiple limitations with the studies included in this review. Firstly, according to the GRADE classification system, all outcome measure assessed were classified as very low, low, or moderate quality of evidence. This was largely due to many of studies been classified as inconsistent ($I^2 > 50\%$) and imprecise (< 400 participants per outcome measure) and judged to be at high risk of bias (> 25% trials are classified as high risk). Although the imprecision could be addressed with the inclusion of more studies, the fact that we were not able to assess for publication bias, as no outcomes had more the 10 included trials, is something that will have to be addressed in future trials and reviews. Furthermore, 31.9% of the risk of bias variables assessed were judged to be of unknown or high-risk of bias, which should be taken into account when interpreting the results of this review.

It is well documented throughout the literature that the inconsistent nature of PBM experiments, both clinical [41, 61] and in vitro [14], are a significant hurdle in establishing both a concrete physiological mechanism, and a widely used and accepted set of clinical implementation guidelines. Appraising the studies included in this review, we see many differing forms of PBM application, including total number of treatments, treatment sites, and irradiation per site. This is understandable given they are treating different areas of tendon pathology, however, there were some studies that did not report all the required treatment variables [36, 42, 46, 47, 49, 51, 54, 55], making exact replication challenging, in the process affecting the quality of evidence. The WALT (World Association for Laser Therapy) recommendations are a set of therapeutic recommendations for clinical and scientific application of red and NIR spectrum PBM [33]. Only four of the trials in this review referenced the WALT recommendations in their study design [36, 51, 53, 54], further underlining the need for higher levels of inter-study consistency.

Heavy strength and plyometric training, in addition to training load management, appear to be the most efficacious exercise modalities to employ during tendinopathy

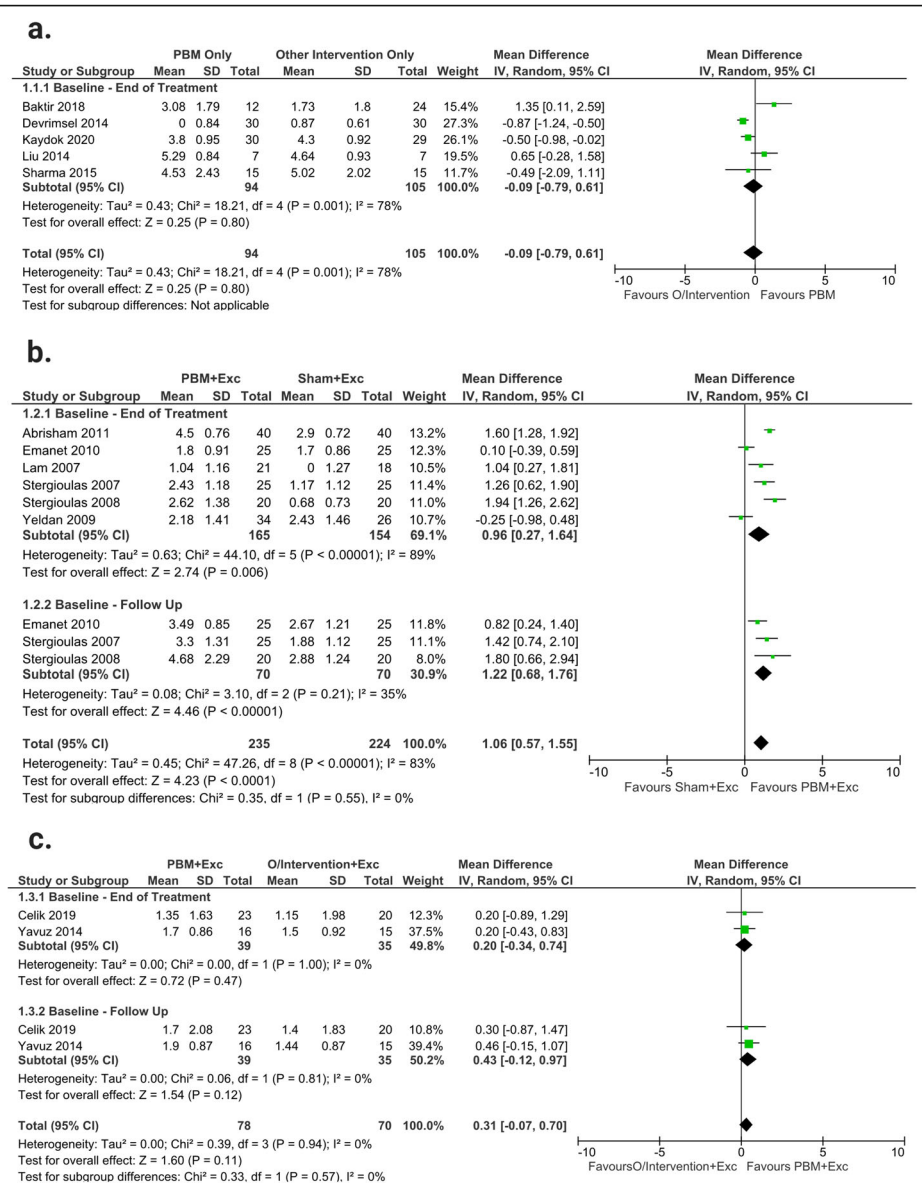

Fig. 3 VAS: **a**: Forest plot of comparing PBM only and other interventions (O/Intervention) only; **b**: Forest plot of the effects of PBM plus exercise (Exc) versus sham treatment plus exercise; **c**: Forest plot of the effects of PBM plus exercise versus other interventions plus exercise

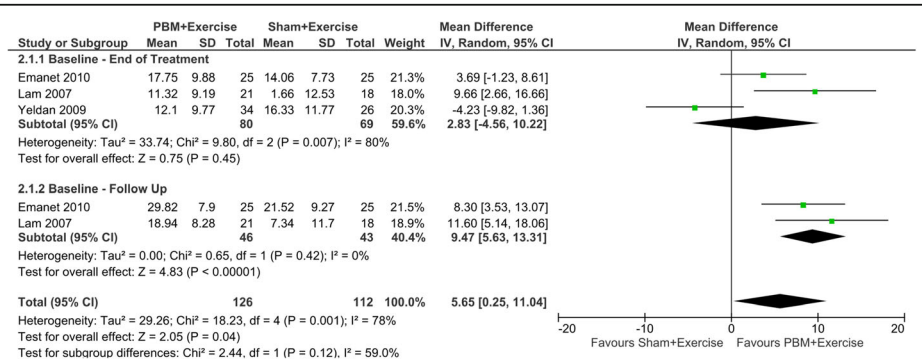

Fig. 4 PROMS: Forest plot of comparing PBM plus exercise versus sham + exercise

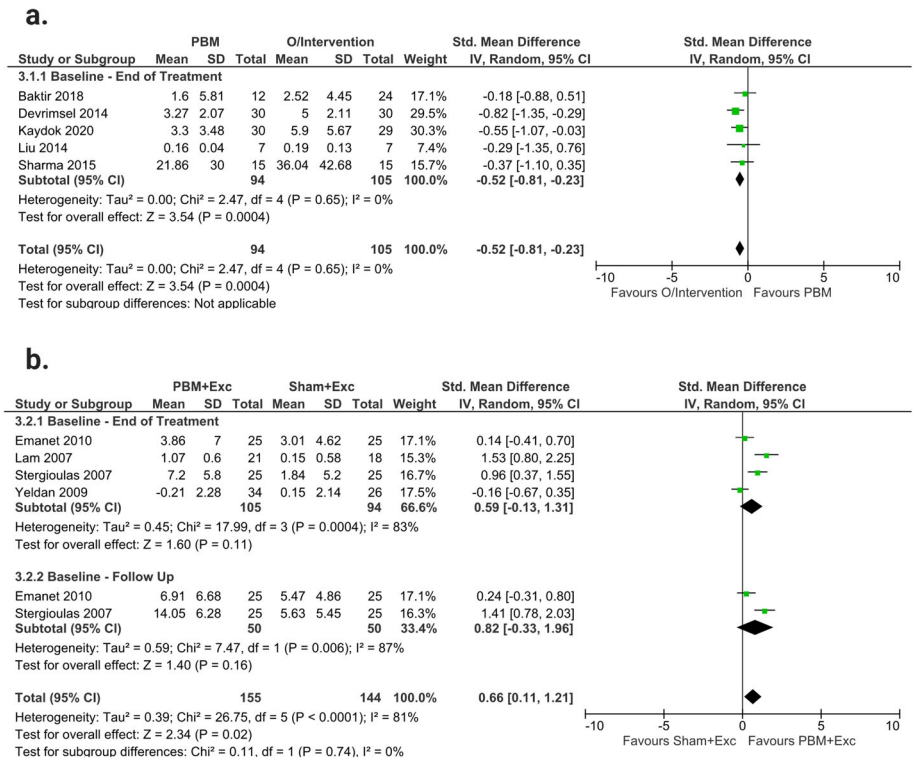

Fig. 5 Strength Measures: **a**: Forest plot of comparing PBM only and other interventions (O/Intervention) only; **b**: Forest plot of the effects of PBM plus exercise (Exc) versus sham treatment plus exercise

management [1]. This review demonstrated very low-quality evidence that PBM could be used as an adjunct therapy to enhance the effects of exercise rehabilitation. That said, a limitation of this analysis was that all the exercise modalities from each study were pooled in each outcome measure, hence different exercise prescriptions may have affected the results. Future research in this area should more stringently control the exercise prescription groups in line with tendinopathy best practice. Interestingly, this review also found that when compared to other interventions, PBM was equally as effective at decreasing pain, however, this was again limited by the pooling of all other interventions. Many of the other interventions that used a pharmacological anti-inflammatory agent, such as Phonophoresis, Iontophoresis and CS Injection, can cause unwanted patient side effects [62]. In fact, it is now recommended that practitioners move away from these methods, CS injections in particular, due to the long-term deleterious tissue effects they can have [62]. In light of this, PBM may represent a non-invasive, cost effective and safe alternative to the more traditional injection and anti-inflammatory based therapies used in tendinopathy management. However, more robust trials are needed to elucidate this effect.

To our knowledge only one other systematic review and meta-analysis has been performed on the effect of PBM on all tendinopathies previously [31]. This review demonstrated similar mixed results concerning the effects of PBM on pain and function in tendinopathy and similar issues with evidence quality to the present review, despite having fewer studies available for analysis. Tendinopathy specific systematic review and meta-analyses have been conducted for shoulder [30] and Achilles tendinopathy [29] and similarly to this review, found a mixed efficacy of PBM underpinned by trials of moderate-very low evidence. Taking these findings together, it is clear that more widespread and robust RCTs are needed to better inform the use of PBM in tendinopathy management.

The strengths of this review include a detailed search of multiple databases, as well as additional searches of paper reference lists. Further, two of the authors performed the entire search process and the risk of bias and GRADE categorization, with a third author resolving any disputes. Another limitation of this study was the fact that all tendinopathies were pooled together as a single diagnostic entity. Hence, the analysis may not have accounted for the heterogeneity of tendinopathy disorders. However, the analysis appeared to indicate similar effects of PBM, regardless of specific diagnosis. More specific-tendinopathy RCTs are needed to underpin more robust single-tendinopathy systematic reviews and

meta-analyses. Additionally, the exclusion of multiple studies whose required statistics were unobtainable from either the paper, or the contact authors may have changed the study results. As previously stated, the future research focus of PBM for the management of tendinopathy should be set on performing repeated robust RCTs that adequately report and justify all treatment parameters and follow the Consolidated Standard of Reporting Trials (CONSORT) guidelines. This will firstly better elucidate if PBM is an effective standalone and/or adjunct therapy for PBM, and secondly if high-quality evidence is found for this effect, it will underpin improved treatment guidelines, potentially translating to improved patient health outcomes.

Conclusion

PBM is an increasingly used treatment modality for a range of musculoskeletal disorders, however, there are many questions regarding its mechanisms and true effectiveness that remain under-investigated and unanswered. Currently, there is very-low-to-moderate quality evidence that low-intensity red and NIR PBM is an effective standalone and exercise-adjunctive treatment for tendinopathy disorders in humans. Further, a similar quality of evidence demonstrates that it may have utility as a less-invasive and more risk-averse adjunctive treatment to more traditional passive interventions. More robust RCTs that adhere to the CONSORT guidelines need to be performed to further elucidate its effectiveness.

Abbreviations

SAS: Subacromial syndrome; LET: Lateral elbow tendinopathy; RT: Rotator cuff tendinopathy; PT: Patella tendinopathy; AT: Achilles tendinopathy; PBM: Photobiomodulation; ESWT: Extracorporeal shock wave therapy; HILT: High-intensity laser therapy; US: Ultrasound; VAS: Visual analogue scale; DASH: Disabilities of the arm, shoulder and hand measure; QDASH: Quick DASH; PRTEE: Patient reported tennis elbow evaluation; SDQ: Shoulder disability questionnaire; SPADI: Shoulder pain and disability index; VISA-P: Victoria institute of sport assessment-patella tendon; WALT: World association for laser therapy; Exc: Exercise; O/Intervention: Other Intervention; MD: Mean Difference; SMD: Standardized mean difference; CI: Confidence Interval; mW: Milliwatt; J: Joules; NIR: Near-infrared light; RCTs: Randomized controlled trials (RCTs); CCTs: Controlled clinical trials; ATP: Adenosine Triphosphate; NO: Nitric Oxide; CCO: Cytochrome C Oxidase; PROMS: Patient reported outcome measures; PCNA: Proliferating cell nuclear antigen; ROS: Reactive oxygen species

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13102-021-00306-z.

Additional file 1: Table 1. Review Search Strategy and Results. **Table 2.** GRADE Classifications.

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Authors' contributions

NT was involved in conceptualization, methodology, analysis, writing and project administration. JF was involved in conceptualization, methodology, analysis, and writing. MH was involved in conceptualization, methodology, analysis, and writing. FS was involved in conceptualization, methodology, and writing. VA was involved in conceptualization, methodology, analysis, and writing. All authors read and approved the final manuscript.

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Availability of data and materials

The Pubmed, CINAHL, SCOPUS, Cochrane Database, Web of Science and SPORTSDICUS databases were searched for eligible articles in August 2020′. Additionally, this study was registered with the PROSPERO database (registration number: CRD42020202508). All data and analysis can be made available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The Authors have no competing interests to declare.

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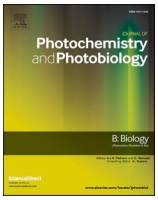

The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: Potential applications to wound healing

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ABSTRACT

Photobiomodulation (PBM) is a widely used therapeutic intervention used to treat several chronic conditions. Despite this, fundamental research underpinning its effectiveness is lacking, highlighted by the lack of a definitive mechanism of action. Additionally, there are many treatment variables which remain underexplored, one of those being the effect of polarization the property of light that specifies the direction of the oscillating electric field. When applied to PBM, using linearly polarized light, when compared to otherwise identical non-polarized light, may enhance its biological efficacy. As such, we investigated the potential biological effects of polarized PBM when compared to non-polarized and non-irradiated controls in the domains of cellular viability, proliferation, apoptosis and mitochondrial membrane potential ($\Delta\Psi$) within cells exposed to oxidative stress. It was noted that polarized PBM, when compared to non-polarized PBM and non-irradiated controls, demonstrated mostly increased levels of cellular proliferation and $\Delta\Psi$, whilst decreasing the amount of cellular apoptosis. These results indicate that polarization may have utility in the clinical application of PBM. Future research is needed to further elucidate the underpinning mechanisms of PBM and polarization.

1. Introduction

Photobiomodulation (PBM) is an umbrella term given to any light source used to treat clinical conditions [1]. Although there is some debate about which wavelengths and intensities constitute PBM, the most common form used is red and/or infrared light with <1 W (W) in power. PBM has demonstrated clinical benefits across a wide spectrum of conditions affecting the population, including: musculoskeletal and neuropathic pain [2], dermatological conditions [3,4], would healing [5,6] and is currently being evaluated as a treatment in neurodegenerative conditions and traumatic brain injury [7]. Despite a large, and growing body of evidence demonstrating the positive effects of PBM, a full understanding of its molecular and cellular effects is lacking [8].

The leading proposed mechanism underpinning PBM, is that red and near-infrared light specifically interacts with the cytochrome *c* oxidase (CcO) enzyme in the mitochondria. It is thought that light displaces nitric oxide (NO), which competes with oxygen at the CcO substrate binding site, and ultimately increases ATP production [9] (Fig. 1). While indirect evidence supports this, there is not yet any confirmatory evidence of a direct interaction between light and the aforementioned mitochondrial machinery [8]. Additionally, PBM is thought to promote the production of reactive oxygen species (ROS), presumably through the increase in oxygen metabolism [10]. This increase in ROS is thought to be a key driver in many of the observed changes in gene regulation and transcription factors [10]. Recently however, it has been shown that PBM can exert a proliferative effect on cells despite the absence of CcO, casting some doubt on the CcO/NO/ATP hypothesis of PBM [8]. This strongly underlines the need for more basic science research to gain

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further insights into the fundamental mechanism(s) of PBM.

Regardless of the precise photonic and molecular mechanisms of PBM, it has been shown to improve both cellular viability and proliferation across a wide range of *in vitro* settings [11]. However, there are mixed results on the effect of varying fluences (Joules (J)/cm^2) on cellular viability, with data showing increases, decreases and no effect across a range of light doses. Specifically, fluence between 0.5 and 5.5 J/cm^2 have shown to increase viability [12–15], whilst fluence of 1.5–25 J/cm^2 demonstrated no change in viability [16–21], and doses of both 0.5 and 10 J/cm^2 resulted in a decrease in cellular viability [22]. These inconsistencies are likely due to varying irradiation and treatment conditions, highlighting the need for a consistent set of experimental standards when it comes to *in vitro* PBM research [11,23]. Direct measures of cellular proliferation have also been conducted widely in this field with more consistent results. Measures of cellular proliferation appear to show a consistent dose-response relationship. PBM induces an increase in proliferation proportional to the light intensity, until a point, at which it plateaus and decreases the biological effect as power keeps increasing [24]. Multiple *in vitro* studies describe that a fluence from 0.45 to 10 J/cm^2 increases cellular proliferation [22,25–30], with fluences of 10–20 J/cm^2 shown to decrease proliferation [19,28,31]. These effects on cellular behaviour are thought to underpin the positive clinical effects shown by PBM, particularly in the context of wound healing [9,32].

Another mode by which PBM is thought to exert its biological effect is through cell protection, specifically in decreasing cellular apoptosis in response to cellular stress [33]. The caspase enzymes are known to be a key player in cellular apoptosis [34], and are of particular interest in PBM studies given their relationship to the mitochondria, where free cytochrome *c* within the cytosol helps generate caspase cellular machinery [34]. Indeed, it has been shown that when cells are exposed to cellular stress in the form of H_2O_2 *in vitro*, PBM caused a decrease in CASP 3 and CASP 8 activity when compared to non-irradiated controls [29,35]. There are also additional pathways by which PBM may induce an anti-apoptotic effect such as the Akt/GSK3β/β-catenin and Akt/YAPp73 signalling pathways and hepatocyte growth factor [32]. However, more research is required to elucidate the optimum dose and interaction between PBM and the apoptotic pathways. Given the known link between mitochondrial membrane potential (ΔΨ) and apoptosis [36], this has also been evaluated in the context of PBM [11]. In line with the effects of PBM on apoptosis, a mostly consistent dose-response curve, with a fluence of 5 J/cm^2 increasing ΔΨ has been noted, while a fluence of both 15 J/cm^2 and 45 J/cm^2 caused a decrease in ΔΨ [37]. However, there are some conflicting results, where a fluence of 3 J/cm^2 can cause a decrease in ΔΨ [20], further highlighting the need for more research.

In addition to the incomplete understanding of the fundamental photonic and physiological mechanisms underpinning its effects, and an absence of an accepted set of *in vitro* experimental standards, there are a number of technical properties of light that remain under investigated in PBM [23]. Variables such as wavelength, power, irradiation time, beam area, radiant energy, fluence, polarization, pulse parameters and treatment cycles, are all factors which can influence the outcome of PBM application [23]. Of these, polarization— the property of light that specifies the direction of the oscillating electric field —is an intriguing variable to investigate. Using linearly polarized light, when compared to otherwise identical non-polarized light, may increase its biological efficacy [38,39]. Specifically, polarized PBM has been shown to increase fibroblast proliferation and procollagen mRNA expression [40], alter immune cell function [41], and in animal models, improve the recovery time of rats exposed to spinal cord injury [42]. Despite, the promising biological effects of polarized PBM when compared to non-polarized PBM, more research is needed to fully uncover any potential benefits of polarized light in the field of phototherapy. Herein, we determined the biological effects of polarized PBM when compared to non-polarized and non-irradiated controls in the domains of cellular viability, proliferation, apoptosis and ΔΨ.

2. Methods

2.1. Setting

All procedures were performed in the PC2 laboratory facilities at Victoria University, Werribee campus, Australia, under standard

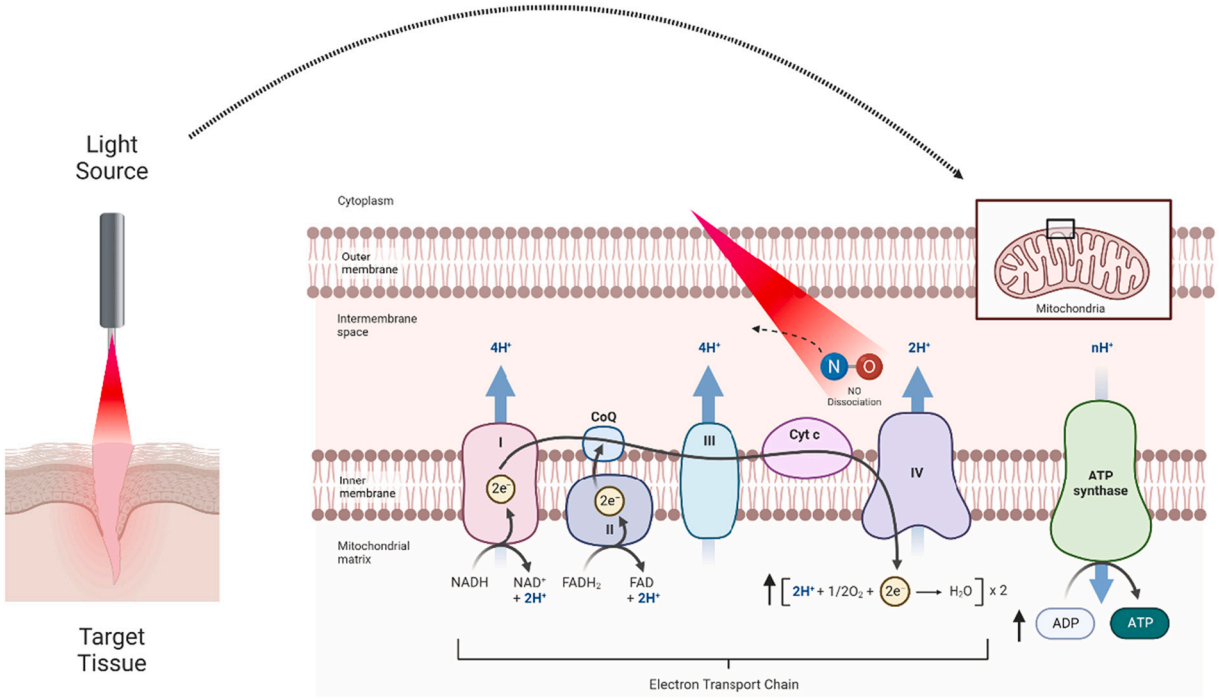

Fig. 1. Proposed PBM biological mechanisms. ATP: adenosine triphosphate; ADP: adenosine diphosphate; NO: nitric oxide; NAD: Nicotinamide adenine dinucleotide; FAD: Flavin adenine dinucleotide; H: Hydrogen; e, electron; O_2: Oxygen; H_2O: Water; Cyt c: Cytochrome c; I-IV: Cytochrome I-IV. Created with biorender.com

laboratory conditions, with aseptic technique.

2.2. Cell Culture and Treatments

All experiments were completed using the human caucasian foetal foreskin fibroblast (HFFF2) cell line. These cells were sourced commercially from Cell Bank Australia (NSW, Australia). Cells were cultured in low glucose (1000 mg/L) dulbecco's modified eagle medium (DMEM) with sodium pyruvate, without phenol red pH indicator dye (Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS) (Cell Sera, Rutherford, NSW, Australia), 1% 5000 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 1% 200 mM glutamax (Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown in a humidified incubator at 5% CO_2 and 37 °C. Cells were sub-cultured at 80% confluency until sub-culture 4, at which point they were transferred to 2 mL cryovials with 90% Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA, USA) and 10% FBS. Cells were frozen at 1×10^6 per aliquot in a Mr. Frosty Freezing Container (MilliporeSigma, Burlington, MA, USA) at −80 °C for 1 week before being transferred to liquid nitrogen storage. When the cells were required for experiments, cells were thawed into the same growth media used for cell culture. Each individual assay was exposed to identical treatment conditions. Firstly, the cells were plated at 4×10^4 cells per well in a black-walled, clear bottom 24-well plates (Eppendorf, Hamburg, Germany) in 500 μL of growth media to reduce possible light scatter and interference between adjacent wells [25]. After 24 h the cells were exposed to 0.5 μM of H_2O_2 to induce oxidative stress [43]. Immediately after H_2O_2 exposure, each well irradiated at a fluence of 1 J/cm^2. The full irradiation parameters are shown in Table 1. There were four experimental groups, all conducted in quadruplicates, unless otherwise indicated: 1: linearly polarized light + H_2O_2 (P-PBM); 2: non-polarized light + H_2O_2 (NP-PBM); 3. no-light + H_2O_2 (positive control - PC); and 4. no-light and no H_2O_2 (negative control - NC). 24 h post treatment the experimental assays were commenced. All experimental groups were otherwise exposed to the same conditions.

2.3. Light Source

For each experiment a 670 ± 5 nm BWF laser diode fiber coupled laser system (B&W Tek, Newark, Delaware, USA) was used. The details of this laser system are in Table 1. The experimental fluence was calculated by monitoring the optical power output using a Spiricon MPE-2500 power meter (Ophir-Spiricon, Utah, USA), at the same distance from the laser tip, as the cell monolayer. A 25 mm linear glass polarizing filter (Edmund Optics, New Jersey, USA) was used to produce linearly polarized light. The laser output power was recalibrated with the polarizer in place, to ensure matched fluence for both polarized and non-polarized treatments. The polarizer was oriented in the same position as calibrated for all treatments to ensure consistent light parameters [42].

Table 1
Laser system and fluence parameters.

Manufacturer and Model	B&W Tek, BWF1
Emitter Type	Laser Diode
Wavelength	670 nm
Class	III B
Pulse Mode	Continuous wave
Distance from target	80 mm
Target spot size	1.9 cm^2 (area of a 24 well)
Power at target site (mW)	11.2
Exposure Duration (sec)	169
Total Fluence per site (J/cm^2)	1

2.4. H_2O_2 Dilution

The Annexin V/propidium iodide (PI) assay was used to determine the optimum concentration of H_2O_2 to stimulate cellular stress and apoptosis. Cells were exposed to the following concentrations of H_2O_2: 16 mM, 8 mM, 4 mM, 2 mM, 1 mM, 500 μM, 250 μM, 125 μM, 62.5 μM, 31.25 μM, 15.63 μM and 0 μM for 24 h, before being stained with Annexin V-Alex Fluor 488 and PI according to manufacturer instructions to identify optimal conditions of cellular stress.

2.5. WST-8 Assay

To measure cellular viability, a WST-8 assay was performed. Briefly, 50 μL of WST-8 solution was added to each well immediately after irradiation and placed back in a humidified incubator set to 5% CO_2 and 37 °C for a period of 24 h. Any bubbles that formed during the addition of WST-8 were removed by centrifugation at 200 ×*g* for 2 min. Optical Density (OD) at 450 nm was measured at 24 h using a Bio-Red xMark microplate reader (Bio-Rad. Hercules, CA, USA).

2.6. EdU Assay

To measure cellular proliferation, the EdU assay was performed. Briefly, the cells were fixed and permeabilized, then 10 μL of EdU solution was added to each sample according to the manufacturer's instructions (BD Biosciences, San Jose, California) 24 h post treatment. 15 min before flow cytometry analysis, each sample was incubated for 15 min in 1 μL of PI to determine total DNA content. 24 h post-treatment the samples were prepared for flow cytometry analysis again using the manufacturer's instructions.

2.7. Annexin V/PI Assay

To measure healthy (non-apoptotic), dead and apoptotic cells, an Annexin V/PI assay was performed (BD Biosciences, USA). Briefly, 24 h post treatment each sample was prepared for flow cytometry analysis according to the manufacturer's instructions. 15 min before analysis 5 μL of Annexin V and 1 μL of PI were added into each sample and incubated at room temperature before being analyzed by flow cytometry.

2.8. MitoProbe JC-1 Assay

To measure the ΔΨ of the samples, a MitoProbe JC-1 assay was performed (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 24 h post treatment 0.5 μM of JC-1 dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample. A CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) control (50 μM) was also used to confirm that the JC-1 response is sensitive to changes in membrane potential. After 24 h, samples were analyzed by flow cytometry as described below.

2.9. Flow Cytometry

All flow cytometry was performed on an Accuri C6 flow cytometer (BD Biosciences, NJ, USA), with a 488 nm laser, and 530/30, 585/40 and 670LP filters. Automated sampling was used with regular sample agitation. Acquisition was performed with the BD Accuri C6 Plus Software (v1.0.23.1, BD Biosciences, NJ, USA), with post processing analysis performed on the software FlowJo (v.10.8.1). A sequential gating process was used to identify events of interest, using unstained, negative, and positive fluorescent controls. Firstly, size and density parameters were used to eliminate cellular debris, and forward and side scatter pulse processing used for doublet discrimination, before finally fluorescence of interest was analyzed.

2.10. Statistical Analysis

All raw data were exported into JASP (JASP, Amsterdam, The Netherlands) for statistical analysis. All data is expressed as mean and standard deviation. A one-way ANOVA followed with Tukey's post-hoc testing was used to analyse the differences between group means. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Determination of the Optimal H_2O_2 Dilution

An almost linear decline in cell apoptosis was observed as the concentration of H_2O_2 decreased. The effect of H_2O_2 appeared to diminish at a concentration of 0.25 mM (Fig. 2). Based on this an experimental concentration of 0.5 mM was selected for ongoing experiments, as previous work had used the lowest apoptosis-inducing concentration of H_2O_2 possible [43].

3.2. WST-8 Assay

There were no significant differences in cellular viability between any of the groups that were exposed to cellular stress ($p > 0.05$) using the WST-8 assay. As expected, the negative control group demonstrated a significantly higher cellular viability when compared to the P-PBM ($p = 0.001$), the NP-PBM ($p = 0.007$), and the PC ($p = 0.006$) groups (Fig. 3).

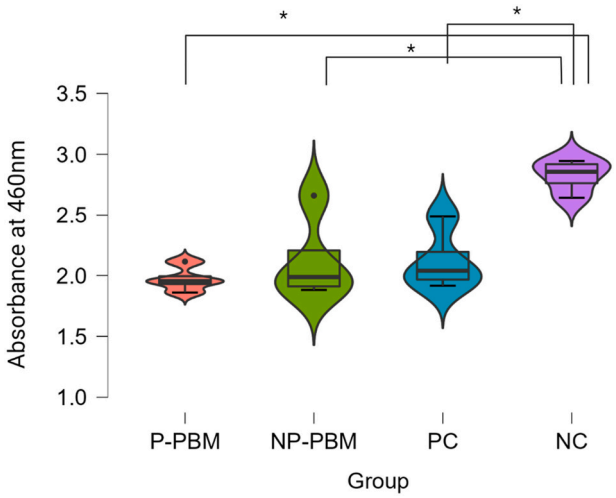

Fig. 3. HFFF2 cell viability using the WST-8 assay. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5 mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

3.3. EdU Assay

The P-PBM group demonstrated a significant increase in total proliferating cells compared to the NP-PBM ($p = 0.029$) and the PC ($p = 0.006$) groups (Fig. 4). The NC group demonstrated significantly increased proliferation compared to the P-PBM ($p < 0.001$), NP-PBM ($p < 0.001$), and PC ($p < 0.001$) groups.

Upon assessing the different stages of the cell cycle (Fig. 5) there were no significant differences between the groups with regards to percentage of cells in the G1 and Sub G1 phases. In the P-PBM group, there was a significant increase in the percentage of cells in the S-Phase compared to the NP-PBM ($p = 0.034$) and the PC ($p = 0.014$) groups (Fig. 5a), while the NC group demonstrated a significant increase in the percentage of cells in the S-Phase cycle compared to all other groups ($p < 0.001$). Additionally, the percentage of cells in the G2-Phase was significantly decreased in the NC group, compared to all other groups ($P < 0.001$) (Fig. 5b).

3.4. Annexin V/PI Assay

Annexin V/PI assay was used to determine apoptosis of cells following PBM with or without polarization. There was a significantly higher proportion of healthy (non-apoptotic) cells in the NC group compared to the PC ($p = 0.003$) and NP-PBM groups ($p < 0.001$). There was also a significantly higher proportion of healthy cells in the P-PBM group compared to the NP-PBM ($p = 0.005$) group (Fig. 6a). There were a significantly less proportion of early apoptotic cells in the NC group

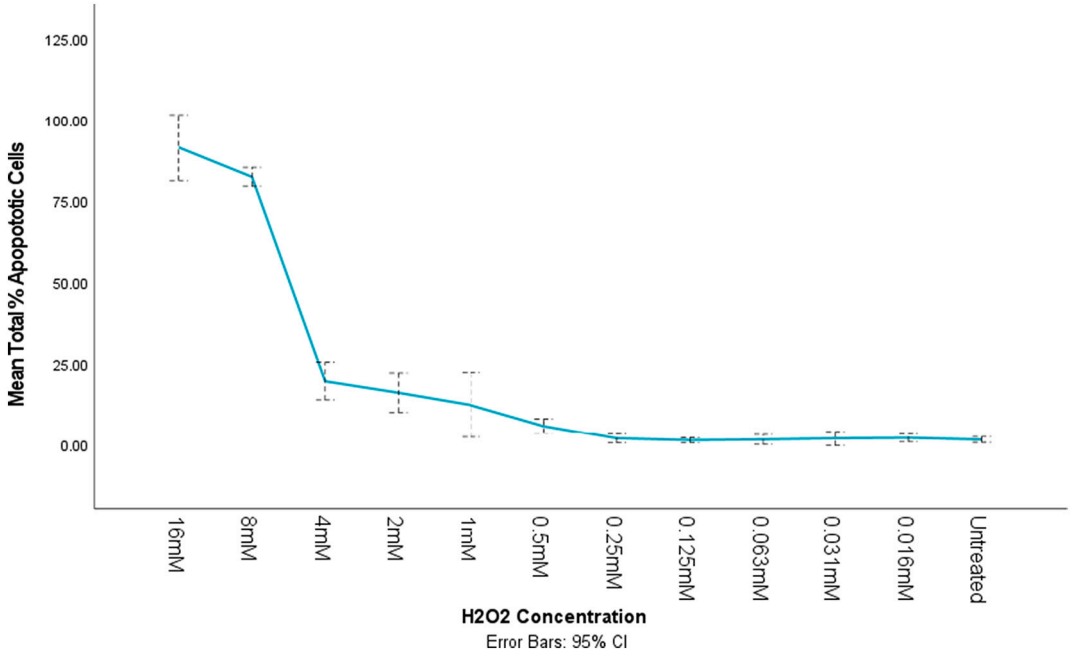

Fig. 2. Mean apoptotic human caucasian foetal foreskin fibroblast (HFFF2) cells as a percentage of total cells plotted with H_2O_2 concentration.

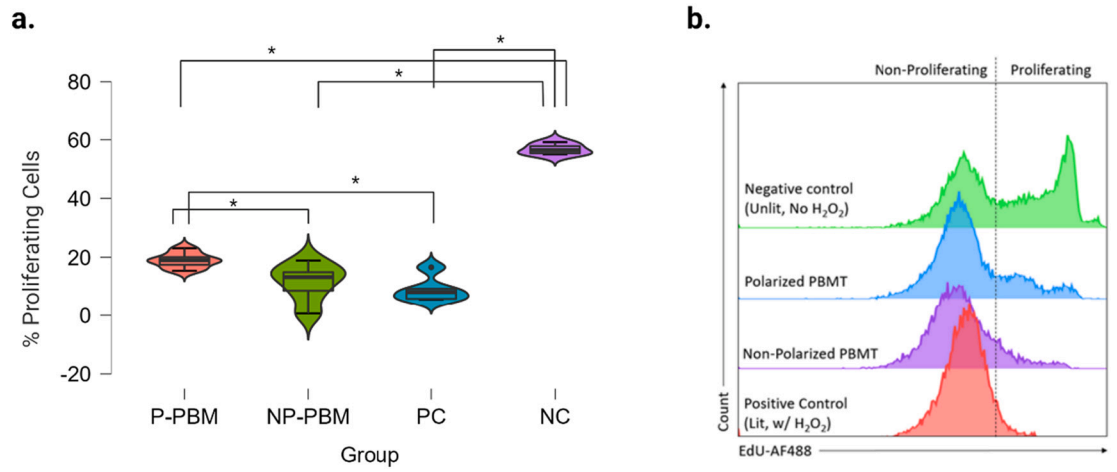

Fig. 4. HFFF2 cell proliferation using EdU assays. (a) Percentage of proliferating cells, and (b) histogram of proliferating and non-proliferating cells. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5 mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

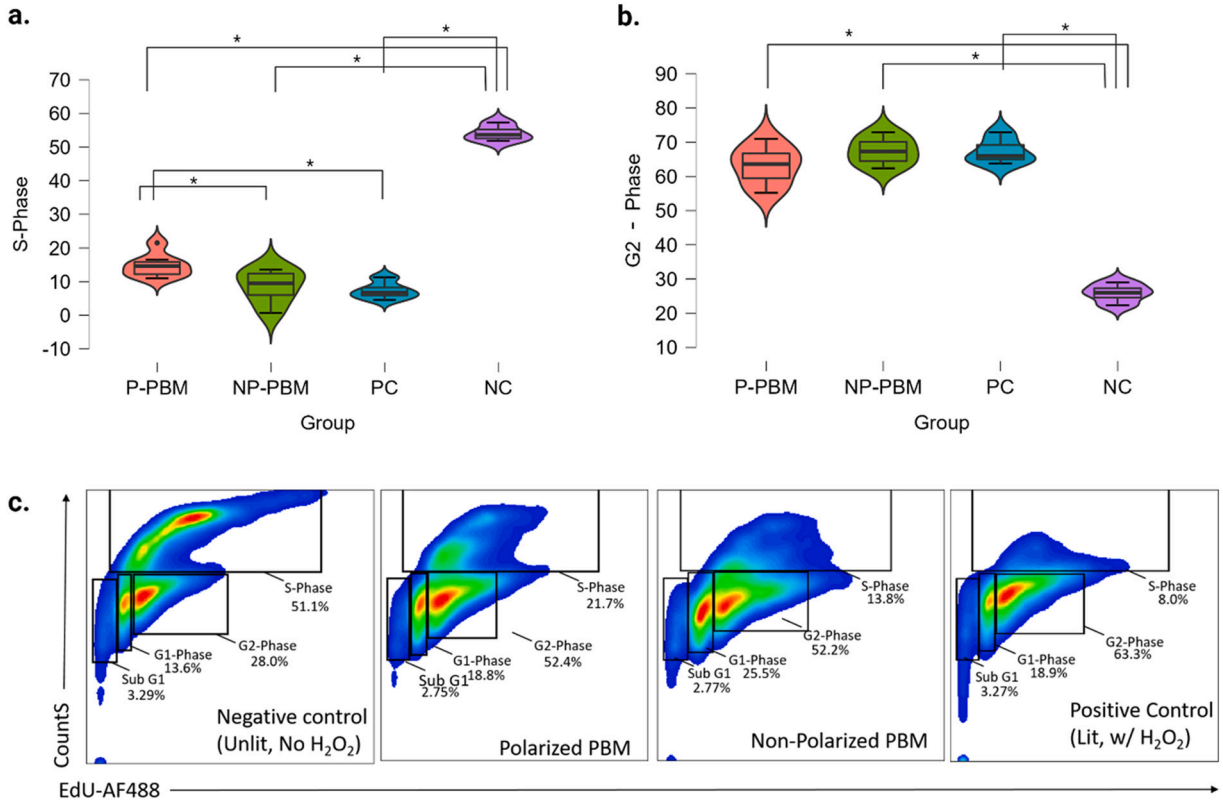

Fig. 5. (a) Percentage of HFFF2 cells in the S cell cycle phase; (b) Percentage of HFFF2 cells in the G2 HFFF2 cell cycle phase, and (c) fluorescent dot plots of all HFFF2 cell cycles by percentage. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5 mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

compared to the NP-PBM ($p < 0.001$) and PC ($p = 0.006$) groups (Fig. 6b). Additionally, there was a significant decrease in late apoptotic cells in the NC group compared to the PC group ($p < 0.001$), and a significant decrease in late apoptotic cells in P-PBM group compared to the NP-PBM ($p = 0.019$) and PC ($p < 0.001$) groups (Fig. 6c).

3.5. Mitoprobe Assay

Using the mitoprobe assay it was shown that P-PBM significantly increased ΔΨ when compared to the NP-PBM ($p = 0.003$) and the PC ($p < 0.001$) groups when analyzed as red/green fluorescence intensity ratio. The NC group was shown to have a significantly higher ΔΨ when compared to all other groups ($p < 0.001$) (Fig. 7).

4. Discussion

PBM is a widely-used clinical therapy, and in particular, has been used extensively in the treatment of wounds and musculoskeletal injury and disease [33]. Despite the abundance of clinical evidence underpinning its use, there remains much debate regarding its fundamental

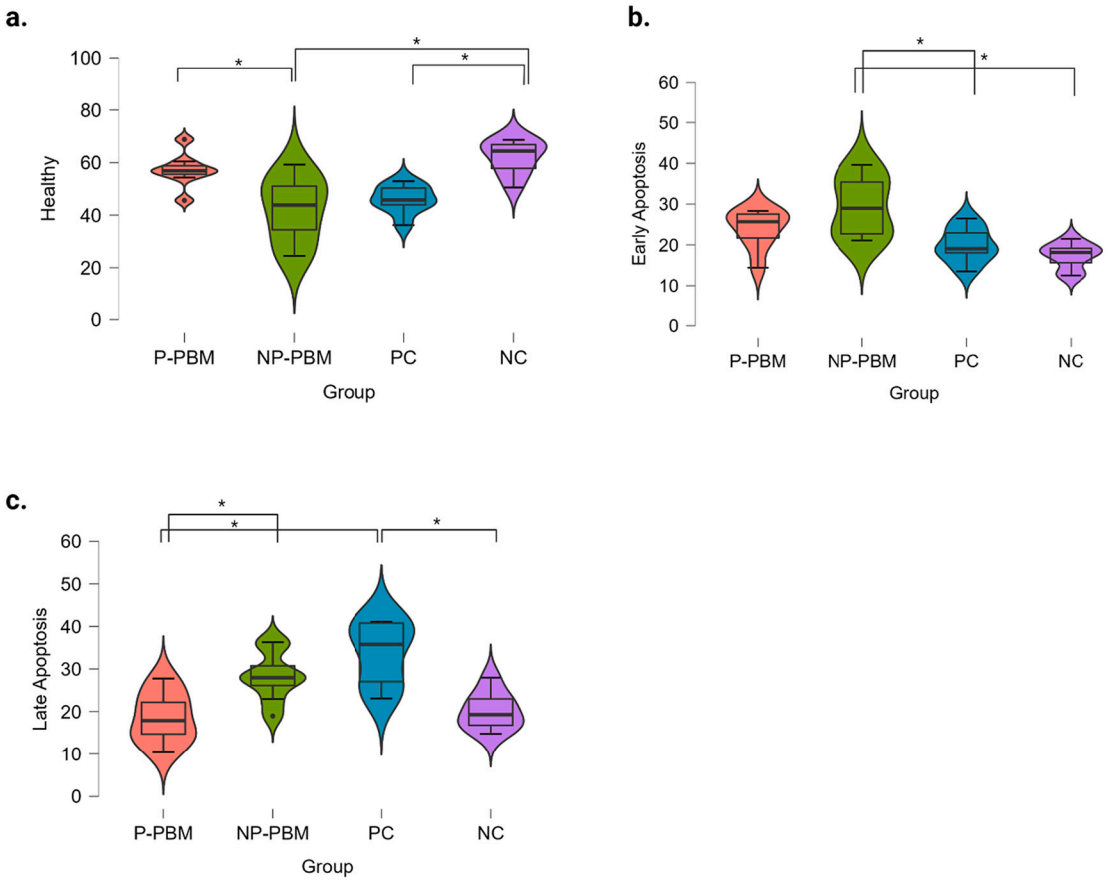

Fig. 6. (a) Percentage of healthy (non-apoptotic) HFFF2 cells, (b) percentage of early apoptotic HFFF2 cells and (c) percentage of late apoptotic HFFF2 cells across all the groups. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5 mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$.

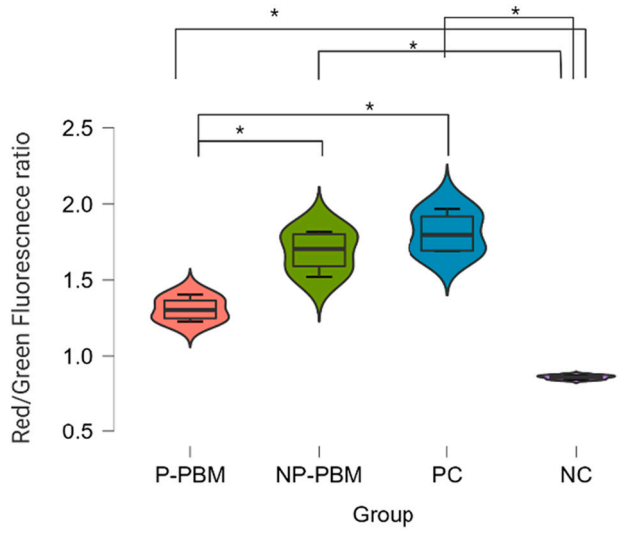

Fig. 7. (a) Red/green fluorescence intensity ratio of HFFF2 cells. P-PBM: Polarized photobiomodulation, NP-PBM: Non-Polarized photobiomodulation, PC: Positive control (0.5 mM H_2O_2, No PBM), NC: Negative control (Untreated). * demarcates $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

physiological mechanisms, and the optimum irradiation parameters to best deliver its effects [23]. Hence, the aim of this study was to determine the effect of both polarized and non-polarized PBM on the viability, proliferation, ΔΨ and magnitude of apoptosis of fibroblasts undergoing oxidative stress. Overall, when compared to non-irradiated controls, P-PBM appeared to promote an increased proliferative, metabolic and protective effect when compared to NP-PBM and its matched controls.

Fibroblasts play a key role in mammalian wound healing, making them an attractive point of investigation for PBM research. Fibroblasts exist in a quiescent state throughout the body, until they are activated by chemoattractants and growth factors resulting from tissue damage [44]. At this point they strongly interplay with the ECM through increased tensional forces contributing to the remodelling of the extracellular matrix, allowing increased cell replication, migration and differentiation [45]. Oxidative stress can be a driver of chronic dermal wounds, of which above optimal H_2O_2 levels can contribute to [46,47]. Namely H_2O_2, can have a negative effect on fibroblast proliferation and migration, therefore negatively affecting wound healing [46,48]. Interestingly, this work demonstrated that PBM, particularly when polarized, can negate some of the proliferative functional impairments that H_2O_2 can have on these cells, in the process highlighting potential advancements for this therapy. Furthermore, our results show an S-Phase block with the addition H_2O_2, with PBM appearing to attenuate this effect, particularly when polarized. This suggests that P-PBM may better help preserve cell metabolism and DNA structure so that more cells can bypass the G1 cell cycle checkpoint. Additionally, the NC group demonstrated a significant decrease in the percentage of cells in the G2 phase, which at first seems counterintuitive, but is likely due to the samples in this group undergoing further replication given they were uninhibited by the addition of H_2O_2.

Polarization of light is one of many PBM variables which remains

under investigated [38]. Currently, there is evidence that suggests that compared to otherwise matched non-polarized light, polarized light may exert additional positive biological effects [38]. The results from this study appear to support this, demonstrating enhanced metabolic, proliferative and cytoprotective effects compared to both non-polarized and non-irradiated controls. When light interacts with biological tissue, it can be absorbed, reflected or transmitted, with absorption most prevalent in biological tissues [9]. Light absorption is also influenced by the total amount of light scattering, which is high in most biological tissues, particularly the dermis, due to the density and specific three-dimensional structure of collagen [49]. Previous research noted that in the superficial layers of the skin, polarized light can penetrate these tissues with minimal depolarization [50], but in denser biological tissues linear polarized light is maintained better than circularly polarized light [51]. Hence, it is thought that at the light-tissue interface, polarization may penetrate biological tissues more effectively, hence the enhanced biological effects. It has also been shown that when polarized light is aligned parallel to the orientation of biological tissue, it penetrates with more energy, than when perpendicular [42]. Despite this, and given the two-dimensional nature of this project, in addition to decreased light attenuation observed with polarization, there may be other biophotonic interactions which require further investigation responsible for the observed effects in this study.

Not only can PBM increase proliferation, it can also have a cytoprotective effect on cells under oxidative stress [33]. The present results demonstrate a higher proportion of healthy, non-apoptotic cells in the P-PBM group compared to NP-PBM, and a significant decrease in late-stage apoptotic cells when comparing the P-PBM to the PC group, highlighting the potential benefits of polarization. There is limited research discerning the effects of PBM on cellular apoptosis, but these findings align with other studies that demonstrate changes to CASP enzyme signalling pathways when cells are exposed to H_2O_2 [29,35]. $\Delta\Psi$ on the other hand, is more extensively investigated, with multiple studies demonstrating PBMs ability to enhance cellular $\Delta\Psi$ [11]. Following a similar pattern across all analyses in this paper, P-PBM again demonstrated an increased $\Delta\Psi$ compared to the NP-PBM and PC groups. Given the relationship between cellular apoptosis, $\Delta\Psi$ and cytochrome *c*, future research should explore these pathways in greater detail.

Cellular viability assays in PBM *in vitro* research are controversial due to them often being quoted as proliferation measures when they do not directly measure proliferation and due to the large variability in reported results [11]. The results of this study appear to exemplify this, although capable capturing large differences, such as that between the NC and PC groups, at this level, the effects of PBM may be more subtle, and the viability assay as appeared to miss important changes in cellular metabolism, which the EdU assay later detected. Although cellular viability assays can be used as a cheap and easy 'screening' assay at the outset of *in vitro* PBM research, they may have limited utility in the present setting.

Despite this study being designed to model a chronic wound environment using fibroblasts exposed to oxidative stress, *in vivo* wounds are much more intricate and dynamic than the *in vitro* setting, and hence the difference between settings should be taken into account when interpreting our findings. Additionally, when wounds are treated in the clinical setting, they are often treated multiple times weekly, over a number of weeks [52]. In the present study the cells were irradiated only once, due to the rapid pace at which dermal fibroblasts can reach confluency in culture. We suspect that the single dose was responsible for the small, but significant effects seen, which when extrapolated into the clinical setting, demonstrate promise. Furthermore, there was only one set of irradiation parameters used in this work, additional fluences may be beneficial in determining the optimum dose-response parameters of both polarized and non-polarized PBM. Several exciting further avenues could be explored from this research. Although we have demonstrated several mechanisms by which polarization may exert its effects, more specific genetic and metabolic pathways should be explored to further elucidate these. Ongoing translation into 3D *in vitro*, animal, and clinical studies are required to understand the full spectrum of the effects of polarization on PBM therapy. Although previous research has indicated that PBM only undergoes a small amount of depolarization in the early layers of skin [38], the amount of depolarization occurring and the cellular monolayer in this study is unknown, and could be a topic of future investigations. Finally, further photonic investigations need to be performed to better understand the fundamental light-tissue interactions of polarized and non-polarized PBM alike.

5. Conclusion

PBM is a therapy that has a wide range of clinical applications, however research outlining its fundamental biological effects is lacking. Additionally, there remain a host of possible application variables that remain under investigated. This study demonstrated that despite having no effect of cellular viability, polarized PBM demonstrated increases in cellular proliferation and $\Delta\Psi$ compared to non-polarized and non-irradiated otherwise matched controls. Additionally, polarized PBM decreased the magnitude of cellular apoptosis brought about by oxidative stress. Taken together, these findings indicate that polarization may be a way to further augment the biological effects of PBM. Further research is needed to understand the full spectrum of effects brought on by PBM and polarization at both a biological and photonic level.

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Author statement

The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: potential applications to wound healing.

CRediT authorship contribution statement

Nicholas Tripodi: Validation, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration. **Fotios Sidiroglou:** Formal analysis, Supervision, Writing – review & editing. **Sarah Fraser:** Formal analysis, Supervision, Writing – review & editing. **Maja Husaric:** Formal analysis, Supervision, Writing – review & editing. **Dimitrios Kiatos:** Formal analysis, Supervision, Writing – review & editing. **Vasso Apostolopoulos:** Formal analysis, Writing – review & editing, Resources, Supervision, Project administration. **Jack Feehan:** Validation, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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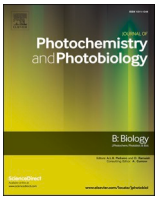

Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts

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ABSTRACT

Background: Photobiomodulation (PBM), the therapeutic use of light, is used to treat a myriad of conditions, including the management of acute and chronic wounds. Despite the presence of clinical evidence surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear. There are several properties of light that can be altered in the application of PBM, of these, polarization—the filtering of light into specified plane (s)—is an attractive variable to investigate.

Aims: To evaluate transcriptomic changes in human dermal fibroblasts in response to polarized PBM.

Results: A total of 71 Differentially Expressed Genes (DEGs) are described. All DEGs were found in the polarized PBM group (P-PBM), relative to the control group (PC). Of the 71 DEGs, 10 genes were upregulated and 61 were downregulated. Most DEGs were either mitochondrial or extracellular matrix (ECM)-related. Gene Ontology (GO) analysis was then performed using the DEGs from the P-PBM vs. PC group. Within biological processes there were 95 terms found ($p < 0.05$); in the molecular function there were 18 terms found ($p < 0.05$); while in the cellular component there were 32 terms enriched ($p < 0.05$). A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p < 0.05$). Finally, there were 24 significantly enriched reactome pathways when comparing the DEGs of the P-PBM vs. PC groups ($p < 0.05$).

Discussion and conclusions: The P-PBM DEGs were almost always down regulated compared to the comparator groups. This may be explained by the P-PBM treatment conditions decreasing the amount of cellular stress, hence causing a decreased mitochondria and ECM protective response. Alternatively, it could point to an alternate mechanism, outside the mitochondria, by which PBM exerts its effects. Additionally, PBM appears to have a more widespread effect on the mitochondria than previously thought, opening up many new avenues of investigation in the process.

1. Introduction

Chronic wounds are a major burden on the health systems globally, with an estimated prevalence of 2.21 persons in every 1000 [1]. There are a number of treatments for chronic wounds including, debridement, standard and bioengineered dressings, and anti-microbial agents [2,3]. Given the high prevalence, there have been several investigations into new, low-cost and minimally invasive therapies to aid in the management of these conditions, one of these being phototherapies [4]. There are numerous clinical applications of light therapy in use today, none more so than that of photobiomodulation (PBM). PBM is used to treat numerous of conditions in clinical practice—from wound healing to sports injuries [5]. Despite a significant body of clinical knowledge surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear [5,6]. Currently the leading mechanistic model centres on mitochondrial Cytochrome c Oxidase (CcO), oxygen and nitric oxide (NO) [7]. In this model the red and infrared photons emitted during PBM interact with the chromophore CcO, in the process dislodging NO molecules, leaving oxygen to bind with CcO in their absence. This is thought to lead to an increase in overall ATP production by the

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mitochondria, subsequently responsible for the clinical effects seen with PBM [7,8]. However, there has been no direct photonic interactions observed between CcO and PBM, and recent evidence suggests that PBM can exert its effect in the absence of CcO [9,10], raising questions surrounding the fundamental mechanisms of PBM.

Regardless of the fundamental mechanisms underpinning PBM, there have been cellular effects resulting from its use in in vitro studies across a number of settings [4], however one area of significant study is the treatment of wounds. When examining the specific effects of PBM on wound healing, many PBM studies have investigated the effect of PBM on fibroblasts, due to their critical role in the process. Fibroblast survival and proliferation are crucial in the process of wound closure [11], and so have been widely investigated in the PBM field. While a range of fluences (Joules/cm^2) have been shown to increase these metabolic parameters, there are many conflicting findings, particularly when it comes to viability, highlighting the need for more stringent experimental parameters [4]. For example, PBM can influence multiple genes related to cell proliferation and wound healing such as vascular endothetial growth factor (VEGF) and genes related to collagen production (COL1AI, COL4A1, COL5A1) [4]. However, similar studies have also shown PBM to have no effect on these genes [12,13], while some even decrease their expression [14]. Additionally, in vitro proliferation assays have been further established via work showing increased cellular migration brought on by PBM [4]. Given ATP production in the mitochondria is at the heart of the proposed mechanisms of PBM [6,15], how it affects functional measures of mitochondrial substrate and energy production have been reported. Again, these studies demonstrate that a range of fluence are able to increase both ATP production and mitochondrial membrane potential [4]. In addition, genes related to mitochondrial energy metabolism, have shown that PBM contribute to genes influencing the function of complexes I, IV and V, and hence energy production [16]. However, there remains debate around the illumination dose needed to illicit the maximum amount of mitochondrial function [17].

Beyond the fundamental mechanisms of PBM, there is conjecture surrounding the optimal method of delivering PBM both in vitro and clinically [10]. There are many variables that can be altered during the application of PBM, and include: beam area, irradiation time, fluence, power, polarization, wavelength, pulse parameters and treatment number, all which may modulate treatment outcomes [10]. Of these, polarization—the filtering of light waves whose electric field vectors move in a specific plane or planes—presents as an interesting variable to investigate [5,18]. There is a small but growing body of research demonstrating polarization of light may provide additional biological efficacy in PBM [5,19,20]. This is thought to occur due to the polarized light having a greater level of tissue penetration, compared to equivalent non-polarized PBM [18]; however, further research is required to determine the therapeutic mechanisms of polarized light. Hence, the aim of this work was to profile the transcriptome of human dermal fibroblasts using RNA-seq to provide novel insights into how polarization of PBM affects gene expression.

2. Methods

2.1. Setting

All experiments were undertaken in standard laboratory conditions, in a PC2 facility at a public university in Victoria, Australia.

2.2. HFF2 Fibroblast Cell Culture and Experimental Treatments

The human caucasian foetal foreskin fibroblast (HFFF2) (Cell Bank Australia NSW, Australia) cell line was used for all experiments. The cells were cultured according to the manufacturers recommended protocol, documented in previous works [21]. Due to the scattering of light that occurs in standard, clear-walled plates, cells were plated at 4×10^4 cells per well in 500 μL of growth media in black-walled, 24-well plates (Eppendorf, Germany) [22]. To induce oxidative stress, the cells were treated with 0.5 μM of hydrogen peroxide (H_2O_2), twenty-four hours after seeding [23]. Immediately after peroxide treatment, the cells were exposed to PBM at a fluence of 1 J/cm^2 (A full description of the light parameters used is presented in Table 1). Three treatments were used to compare effects, with four technical replicates used in each. The treatments were polarized PBM (P-PBM); non-polarized PBM (NP-PBM); and a no-light control (positive control - PC), with all exposed to the (H_2O_2) stressor. 24 h post irradiation, the RNA extraction was performed as described below.

2.3. Light Source

The light source used for experimental treatment was a fiber coupled 670 ± 5 nm BWF laser diode (B&W Tek, Delaware, USA) (Table 1). The fluence dose used in the treatments was calculated as described previously [21]. A linear, 25 mm glass filter (Edmund Optics, New Jersey, USA) was used to polarize the laser diode (Supplementary Fig. 2). The laser output power was appropriately adjusted in both polarized and non-polarized treatment setting to ensure consistent light treatment parameters across all experimental wells. [18,21].

2.4. RNA Sequencing

RNA was extracted with an RNeasy mini-kit according to the manufacturer's instructions (Qiagen, USA), and immediately stored at −80 °C until sequencing. RNA sequencing was performed by the Micromon genetics facility (Monash University, Melbourne, Australia). RNA quality was assessed via Agilent Bioanalyzer electrophoresis and Qubit fluorometer (Invitrogen, USA). A minimum of 2 μg of total RNA underwent library preparation and sequencing. Secondary quality control pf the RNA was performed using the AATI fragment analyzer prior to sequencing to asses for possible degradation of the samples during transport and/or preparation (Invitrogen, USA).

2.5. Statistical Analysis

Raw files were analyzed using the RNAsik pipeline [24] utilising STAR aligner [25] with the Genome Reference Consortium Human Build 38 (GRCh38; *Homo sapiens*) genome reference. Feature Counts was employed to quantify the reads [26] producing the raw genes count matrix and various other quality control metrics. Raw counts were then analyzed with Degust [27], which performed the normalisation using trimmed mean of M values [28], and differential expression analysis using limma/voom [29]. Differentially expressed genes (DEGs) were obtained using a False Discovery Rate (FDR) $\leq$ 0.05. Functional enrichment analysis (GO, KEGG and reactome pathways) was performed using STRING-db [30], where the data were exported and plotted using either SR plot and ggplot packages. Enrichment groups were considered significant at $p < 0.05$.

Table 1
Laser system and fluence parameters.

Manufacturer	B&W Tek
Model	BWF1
Emitter	Laser Diode
Class	III B
Pulse Mode	Continuous wave
Wavelength	670 nm
Distance from target	80 mm
Target spot size	1.9 cm^2
Power at target site (mW)	11.2
Exposure Duration (sec)	169
Total Fluence per site (J/cm^2)	1

3. Results

3.1. RNA Quality Control

The RNA integrity number of all samples was ≥9.9, representing high sample quality (Supplementary Fig. 1). The mean Phred score was 36 across the samples, indicating >99.9% accuracy across sequencing reads (Supplementary Fig. 1). Additionally, the size of each RNA library, distribution of *p*-values and normalized expression were all within acceptable limits across all samples (Supplementary Fig. 1). The fourth NP-PBM was excluded as it was an outlier in the MDS analysis.

3.2. Screening Analysis of DEGs

There were a total of 71 (from 16,280) DEGs when each experimental group was compared only to the control group (FDR <0.05). All these DEGs were found in the PPBM group, relative to the PC group (Fig. 1). Of the 71 DEGs, 10 were upregulated and 61 were downregulated (Table 2).

3.3. Network Pathway Analysis

There were two main gene association clusters found on network pathway analysis using the DEGs from above. The first involved mitochondrial genes associated with energy production, whilst the *sec*ond involved genes associated with the ECM and collagen production (Fig. 2).

3.4. Functional Enrichment Analysis

Gene Ontology (GO) analysis was performed using the DEGs from the P-PBM vs. PC group. In the biological process ontology there were 95 significant terms found ($p < 0.05$); in molecular function there were 18 terms ($p < 0.05$); and in the cellular component ontology there were 32 terms found ($p < 0.05$) (Fig. 3).

A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways

Table 2
Full list of both upregulated and downregulated DEGs.

Group Comparison	Upregulated DEGs	Downregulated DEGs	
P-PBM vs. PC	AC048341.2	ACTC1	MT-CYB
	AKR1B1	AMOTL2	MT-ND1
	AKR1C1	C1orf198	MT-ND2
	CLU	CLDN1	MTND2P28
	LAMB3	COL1A1	MT-ND3
	MIR199A1	COL4A1	MT-ND4
	PCNA	COL4A2	MT-ND4L
	PHLDA3	COL5A1	MT-ND5
	S100A4	CTGF	MT-ND6
	Z74021.1	CYR61	MT-RNR1
		DCLK2	MT-RNR2
		DDAH1	MT-TC
		DIO2	MT-TE
		FZD7	MT-TH
		GOPC	MT-TI
		IGFBP3	MT-TS2
		LAMA4	MT-TV
		LDLR	MT-TW
		LMO7	MT-TY
		LMOD1	NRBP2
		MARCKS	P3H2
		MIR100HG	PCNA
		MRVI1	SMAD3
		MSRB3	SSBP4
		MT-ATP6	SULF1
		MTATP6P1	TAF10
		MT-ATP8	THBS1
		MT-CO1	THBS2
		MTCO1P12	TPM1
		MT-CO2	TXNDC5
		MT-CO3	

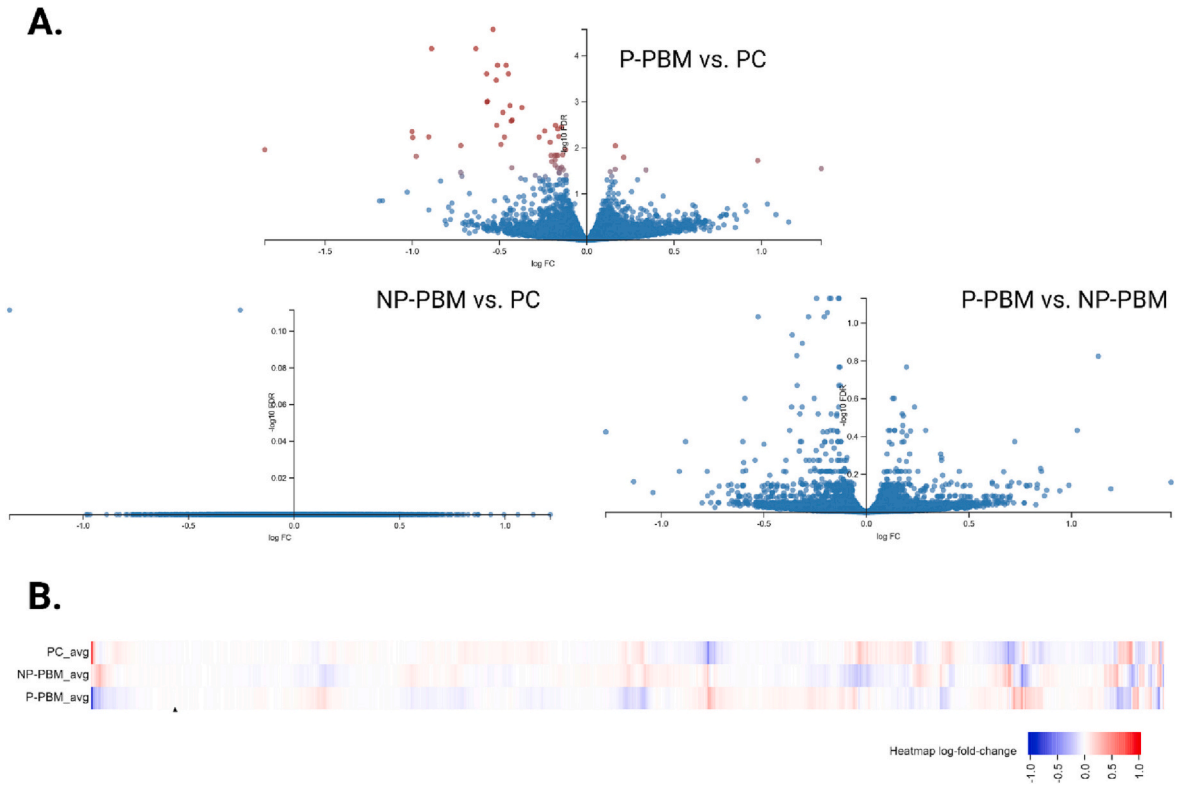

Fig. 1. A. Volcano plot analysis of all genes analyzed across all groups. B. Heat map analysis of all genes analyzed across all groups. Figure sourced from the Degust bioinformatics platform.

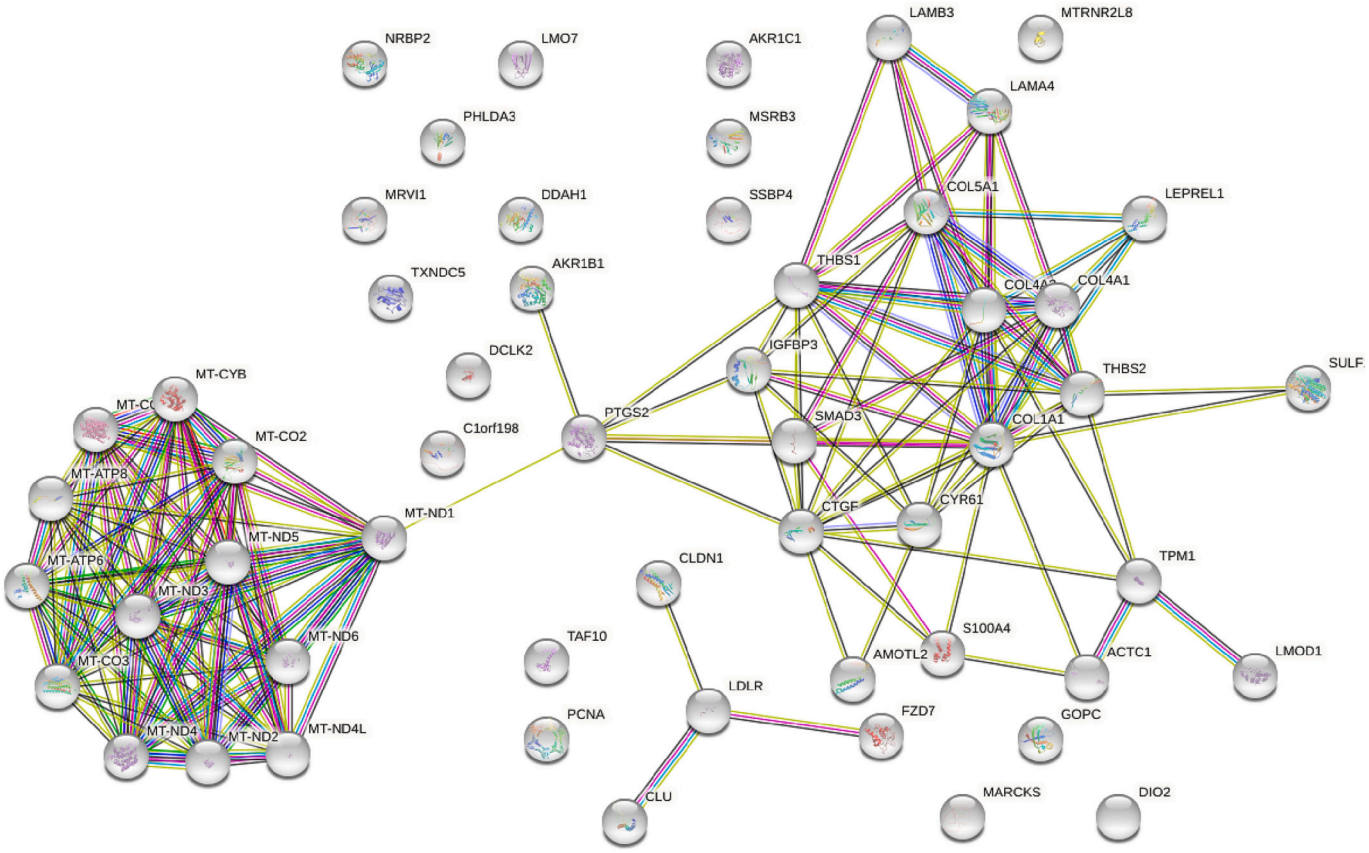

Fig. 2. StringDB Network Analysis using DEGs. Light blue lines indicate known interactions from curated databases; Pink lines indicate experimentally determined known interactions; Dark green lines indicate gene neighbourhood predicted interactions; Red lines indicate predicted interaction from gene fusions; Dark blue lines indicate gene co-occurrence predicted interactions; Light green lines indicate text mining interactions; Black lines indicate co-expression interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($p < 0.05$) (Fig. 4).

Finally, there were 24 significantly enriched reactome pathways found when comparing the DEGs of the P-PBM vs. PC groups ($p < 0.05$) (Fig. 5).

4. Discussion

PBM is a commonly employed intervention across multiple areas of clinical practice, often producing tangible clinical benefits. Despite this widespread use, there remains conjecture around the fundamental biological mechanisms responsible for the clinical effects observed [10]. As such, the transcriptome of human dermal fibroblasts were profiled following their exposure to oxidative stress, in response to both polarized and non-polarized PBM. The overarching results demonstrated that, P-PBM can influence the expression of multiple genes, mostly associated with the mitochondria and ECM, which relate to a number of important ontological and functional pathways.

The current leading mechanistic model of PBM centres on the mitochondria. To our knowledge, this is the first study which has investigated the mitochondrial transcriptome of human dermal fibroblasts in response to PBM. Interestingly, all the mitochondrial DEGs were downregulated when exposed to P-PBM. Previous research demonstrated that in healthy cells, and cells grown in ischaemic and diabetic models, PBM produces an upregulation in genes encoding for enzymes involved in ATP synthase and complexes I and IV [16]. That said, the previous works analyzed nuclear mitochondrial-related genes, as opposed to the specific mitochondrial genes analyzed in this study. Given how susceptible the mitochondrial genome is to oxidative damage [31], in addition to the known cellular protective effects of PBM [21,32], we propose that the downregulation of mitochondrial DEGs may have been caused by PBM ameliorating some of the effects brought on by the addition of an oxidative stress-inducing agent—H_2O_2. Recent findings have also cast doubt on the CcO-NO-ATP model of PBM as the sole mechanism underpinning its effect, demonstrating that PBM increased cellular proliferation and other metabolic parameters similarly in cells both with and without CcO [9]. Taken together with our findings, it appears that PBM fundamentally influences mitochondrial function, but it may be that it influences other areas of the mitochondria equally, or more so than CcO.

Currently, much research has focused on the efficacy of PBM in the treatment of dermal wounds [33–35]. Fibroblasts play a key role in this, by being stimulated from a mostly dormant state, in response to factors released in response to tissue damage [36]. They play an integral part in the integrity of the ECM in healing tissue by increased tensional forces brought about by their contractile capacity [37]. Importantly, one of fibroblasts chief functions is to produce the collagen matrix—the main structural component of connective tissue, which ultimately helps form focal adhesion complexes, which have important regulatory and structural functions [38]. The collagen-related and other ECM-related DEGs in this study, were universally downregulated, which conflicts with some, but not all of the findings relating to ECM-related gene expression in PBM exposed fibroblasts [4]. Several studies within the field, have demonstrated that collagen, and other ECM-related genes can either be upregulated, unchanged, or downregulated by PBM within acceptable fluence levels [4,13,22]. This is likely due to experimental inconsistencies, chiefly being, irradiation timings and cellular growth conditions [4,10,39]. It appears that the timing of PBM in response to cellular stress or damage is important, as the known protective effects of

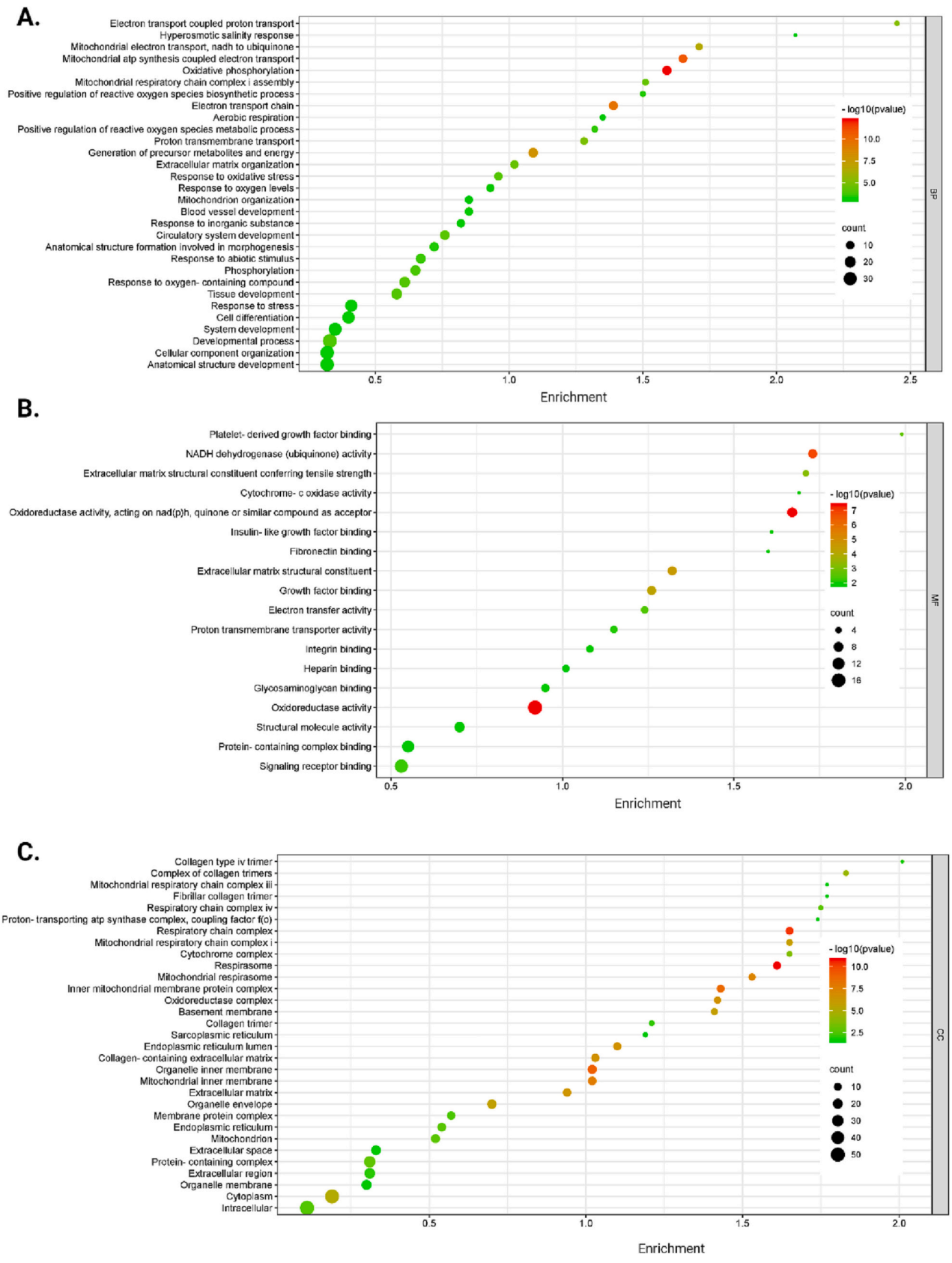

Fig. 3. A. Top 30 Biological Process (BP) GO terms. B. Significantly enriched Molecular Function (MF) GO terms. C. Top 30 Cellular Component (CC) GO terms. Figure created with https://www.bioinformatics.com.cn/en

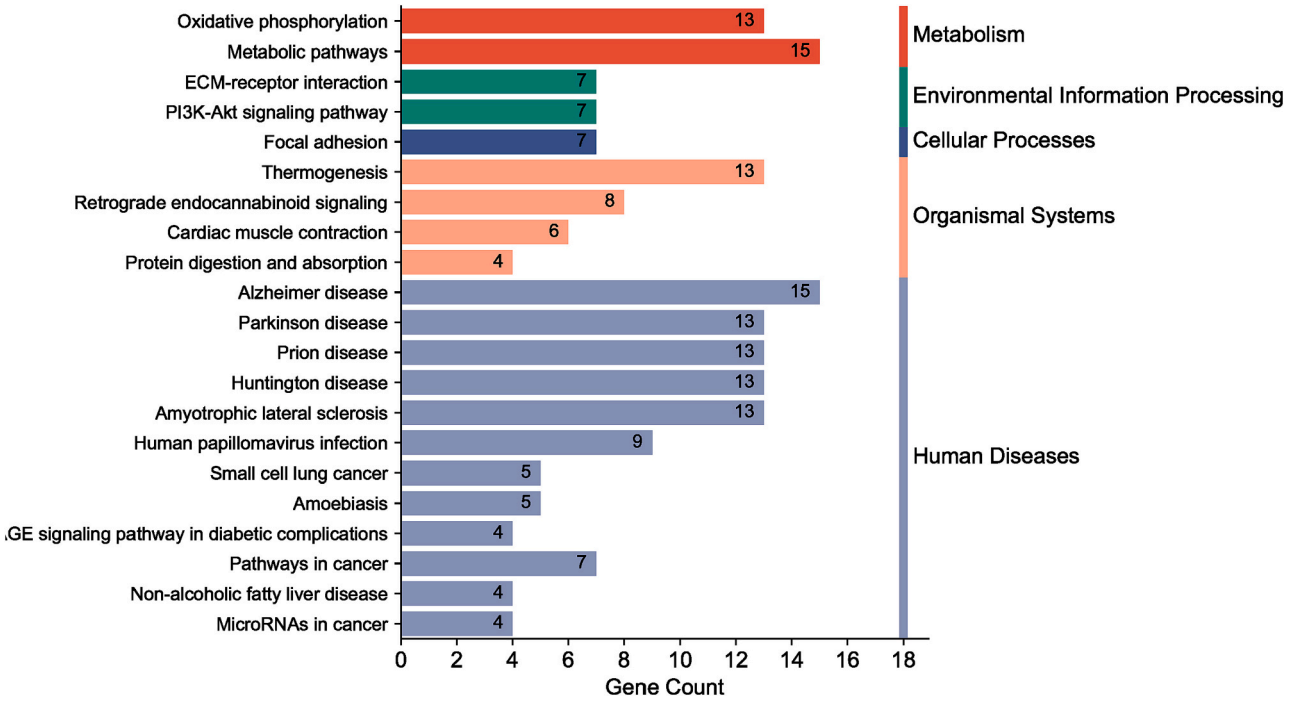

Fig. 4. KEGG pathway analysis using DEG count. Figure created with https://www.bioinformatics.com.cn/en

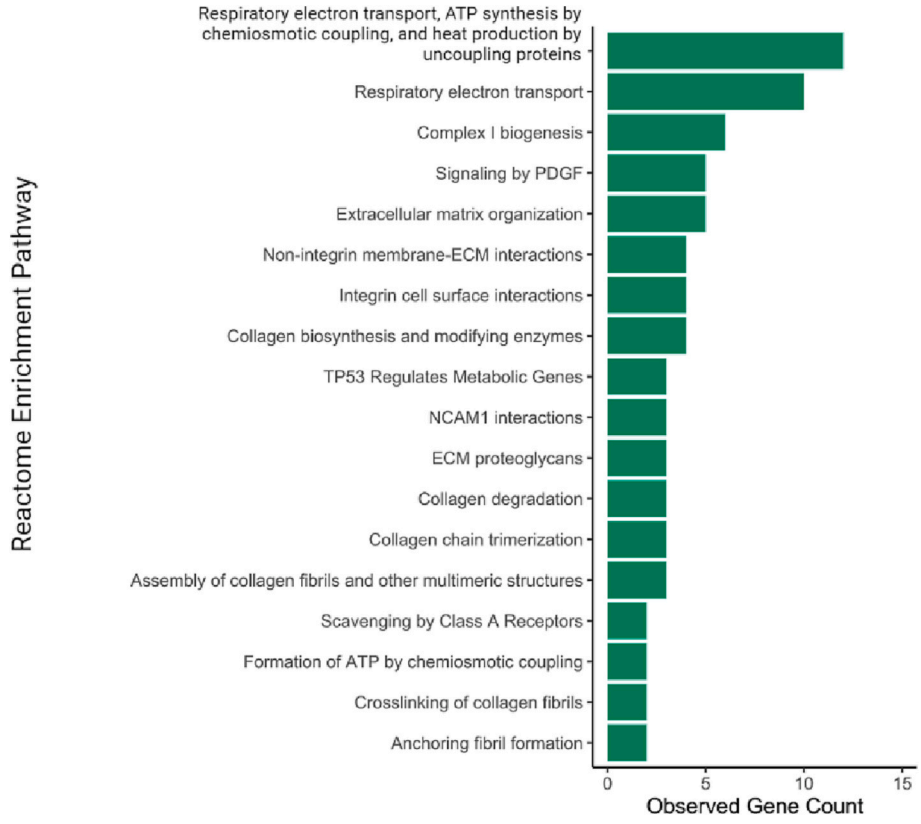

Fig. 5. Reactome pathway analysis using DEG count. Figure created using ggPlot.

PBM, such as apoptosis inhibition [21,40,41], may more effectively inhibit cellular damage when applied closer to the initiating cellular stressor. This raises important clinical implications for the treatment of both acute and chronic dermal wounds, with timing of PBM application in relation to these conditions remaining underexplored.

This work has identified several functional ontological pathways which are influenced by PBM and relate to both cellular metabolism and wound healing. All the mitochondrial DEGs which were downregulated contribute to the ontological processes and pathways concerned with oxidative phosphorylation, ATP synthesis and the electron transport

chain. More specifically, the Mitochondrial respiratory chains I, III, and IV cellular component ontological pathways, as well as the Reactome pathway Complex I biogenesis were significantly enriched with the downregulated DEGs, further supporting the notion that PBM can influence multiple parts of the mitochondria, not only CcO [9]. Furthermore, there were multiple significantly enriched pathways associated with the ECM and wound healing processes including ECM organisation, structure, and interactions, collagen formation and biosynthesis, and integrin binding and interactions. Taken together these pathway analyses demonstrate that PBM has a strong influence on multiple areas of mitochondrial energy production, and pathways associated with wound healing, revealing many avenues for further research.

Interesting among the findings of this study, was the superiority of polarized light over equivalent non-polarized PBM. Work by ourselves and others has demonstrated that when compared to non-polarized, otherwise matched PBM, P-PBM can increase cellular viability and proliferation, decrease apoptosis, increase mitochondrial membrane potential and increase functional outcomes post-spinal injury in mice [5,18,21,42]. The present results follow this trend, with P-PBM demonstrating the most profound influence on gene expression. The mechanisms underpinning these changes in PBM efficacy that polarization can affect are not fully understood, but currently it is thought that polarized light may present a way to better penetrate biological tissue through minimizing light attenuation, possibly through reduced light scattering, and therefore, be able to exert its effects more efficiently [5,18]. This effect may be further enhanced when the plane of polarization is aligned to the tissue histological orientation [18]. Despite these findings, further research is required to determine the exact biophotonic interactions at play.

4.1. Limitations and Future Research

Although the methodological processes of this project being stringently controlled, there are some limitations we would like to acknowledge. One of the NP-PBM replicates was excluded from the analysis due to it being an outlier. This may have influenced the magnitude of gene expression in the NP-PBM group, however, the results in this study reflect our previous work demonstrating that P-PBM has a greater effect on cellular metabolic and regenerative function compared to NP-PBM and experimental controls [21]. Furthermore, the in vitro model of wound healing that was employed in this study, may not fully reflect the clinical treatment of wounds, with them often undergoing multiple exposures to PBM. These findings open many exciting avenues for future research. Firstly, the numerous significantly enriched ontological pathways found could be further explored to confirm if they translate to functional cellular changes. Secondly, these experiments could be replicated with other wavelengths and intensities, as well as being translated to 3D in vitro cell cultures, animal studies and clinical translation studies to determine the full scale of effects that P-PBM and NP-PBM can have on wound healing. Finally, it is important to note that changes in gene expression doesn't necessarily reflect changes in downstream protein expression, hence, these could be further explored in future research.

5. Conclusion

PBM is a widely used therapy for a number of clinical conditions, including wounds, however, both the exact fundamental mechanisms underpinning its effects, as well as the optimum irradiation conditions remain unclear. The leading mechanistic theory of PBM is centred on increasing the efficiency of mitochondrial CcO. This study has shown that PBM, specifically when polarized, can have a more generalzsed effect on mitochondrial energy production, affecting multiple mitochondrial complexes, not only complex IV, which aligns with more contemporary PBM research. Additionally, this work supports other fundamental and clinical literature by identifying that PBM can strongly influence the pathways that influence the ECM and therefore wound healing. Further research should explore the cellular and molecular pathways identified herein, to continue to build a better understanding of the fundamental mechanisms of PBM.

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CRediT authorship contribution statement

Nicholas Tripodi: Validation, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration. **Fotios Sidiroglou:** Formal analysis, Supervision, Writing – review & editing. **Vasso Apostolopoulos:** Formal analysis, Writing – review & editing, Resources, Supervision, Project administration. **Jack Feehan:** Validation, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2023.112696.

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Appendix II: Abstract for Australian Physiological Society Annual Meeting 2022

Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts

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[2] First Year College, Victoria University, Melbourne, Australia

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Introduction and Aims: Photobiomodulation (PBM), the therapeutic use of light, is used to treat a myriad of conditions in clinical practice—from wound healing to neonatal jaundice. Despite the presence of clinical evidence surrounding PBM, the fundamental mechanisms underpinning its efficacy remain unclear. There are many variables that can be altered in the application of PBM, including: wavelength, power, irradiation time, beam area, fluence, polarization, pulse parameters and treatment cycles, all of which influence treatment outcomes. Of these, polarization—the filtering of light into specified plane(s)—is an attractive variable to investigate. Therefore, the aim of this work is to evaluate transcriptomic changes in human dermal fibroblasts in response to polarized PBM, to uncover key mechanisms driving its clinical outcomes.

Methods: All experiments were completed using the human caucasian foetal foreskin fibroblast cell line. 24 hours after plating, the cells were exposed to 0.5 μM of H_2O_2 to induce oxidative stress. Immediately after H_2O_2 exposure, cells were irradiated by PBM at a fluence of 1 J/cm^2. There were three experimental groups, all conducted in quadruplicate: 1: linearly polarized light + H_2O_2 (P-PBM); 2: non-polarized light + H_2O_2 (NP-PBM); 3. no-light + H_2O_2 (positive control - PC). RNA was subsequently extracted, and underwent RNA-sequencing. The resulting data underwent analysis for differentially expressed genes (DEGs), ontological enrichment, and pathway analysis through STRING-db and SR plot. DEGs were obtained with a False Discovery Rate (FDR) ≤ 0.05 and enrichment groups were considered significant at $p<0.05$.

Results: There were a total of 71 (from a total of 16280) DEGs when each experimental group was compared only to the control group (FDR <0.05). All of these DEGs were found in the PPBM group, relative to the PC group (Fig x). Of the 71 DEGs, 10 genes were upregulated and 61 one were downregulated. Most DEGs were either mitochondrial or extracellular matrix (ECM)-related. Gene Ontology (GO) analysis was then performed using the DEGs from the P-PBM vs. PC group. Within biological processes there were 95 terms found ($p <0.05$); in the molecular function there were 18 terms found ($p<0.05$); while in the cellular component there were 32 terms enriched ($p<0.05$). A KEGG pathways analysis was performed for the DEGs found in the P-PBM vs. PC group. This revealed 21 significantly enriched pathways ($p<0.05$). Finally, there were 24 significantly enriched reactome pathways found when comparing the DEGs of the P-PBM vs. PC groups ($p<0.05$).

Discussion and Conclusions: The P-PBM DEGs were almost always down regulated compared to the comparator groups, conflicting with analogous research. This may be explained by the P-PBM treatment conditions decreasing the amount of cellular stress, hence causing a decreased mitochondria and ECM protective response. Alternatively,

it could point to an alternate mechanism, outside the mitochondria, by which PBM exerts its effects. Overall, further research is needed to elucidate the fundamental mechanisms of PBM.

Appendix III: List of published works, conference presentations and academic prizes by the applicant during their candidature

Published Works

Corcoran, D., McNamara, T., Feehan, J., & **Tripodi, N.** (2023). Adductor magnus: Extending the knowledge–A short review of structure and function. *International Journal of Osteopathic Medicine*, 100671.

Tripodi, N., Sidiroglou, F., Apostolopoulos, V., & Feehan, J. (2023). Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts. *Journal of photochemistry and photobiology B: Biology*, *242*, 112696.

Tripodi, N., Dagiandis, T., Hameed, A., Heilberg, L., Olbinski, E., Reid, C., ... & McLaughlin, P. (2023). Inter-rater reliability between osteopaths of differing clinical experience on sagittal plane running gait analysis: A pilot study. *International Journal of Osteopathic Medicine*, *47*, 100653.

Harkin, K., Apostolopoulos, V., Tangalakis, K., Irvine, S., **Tripodi, N**., & Feehan, J. (2023). The impact of motivational interviewing on behavioural change and health outcomes in cancer patients and survivors. A systematic review and meta-analysis. *Maturitas*.

Tripodi, N., Cordina, J., Jaffre, D., Mason, K., McMahon, G., Xeureb-Graham, B., ... & Wospil, R. (2022). Diagnosis and management of headache disorders in osteopathic practice: A qualitative study. International Journal of Osteopathic Medicine.

Tripodi, N., Sidiroglou, F., Fraser, S., Husaric, M., Kiatos, D., Apostolopoulos, V., & Feehan, J. (2022). The effects of polarized photobiomodulation on cellular viability, proliferation, mitochondrial membrane potential and apoptosis in human fibroblasts: Potential applications to wound healing. Journal of Photochemistry and Photobiology B: Biology, 236, 112574.

Tripodi, N., Wright, B., Lawton, A., Zanker, J., & Feehan, J. (2022). A clinician's guide to the management of geriatric musculoskeletal disease: Part 2–Sarcopenia. International Journal of Osteopathic Medicine.

Feehan, J., **Tripodi, N**., & Apostolopoulos, V. (2022). Exercise to Prevent and Manage Chronic Disease Across the Lifespan. Academic Press.

Feehan, J., **Tripodi, N**., Fleischmann, M., Zanker, J., & Duque, G. (2022). A clinician's guide to the management of geriatric musculoskeletal disease: Part 1-Osteoporosis. International Journal of Osteopathic Medicine, 43, 53-62.

Tripodi, N., Cossar, S., Davidson, J., Farmer, R., Gorbonos, J., McDonald, A., & Pierlot, M. (2021). Patient perceptions of allied health student communication and interpersonal skills: A cross-sectional study. Focus on Health Professional Education: A Multi-Professional Journal, 22(3), 18-32.

Feehan, J., Smith, C., **Tripodi, N**., Degabrielle, E., Al Saedi, A., Vogrin, S., ... & Levinger, I. (2021). Higher Levels of Circulating Osteoprogenitor Cells Are Associated With Higher Bone Mineral Density and Lean Mass in Older Adults: A Cross-Sectional Study. JBMR plus, 5(11), e10561.

Tripodi, N., Feehan, J., Husaric, M., Sidiroglou, F., & Apostolopoulos, V (2021). The effect of low-level red and near-infrared photobiomodulation on pain and function in tendinopathy: a systematic review and meta-analysis of randomized control trials. BMC Sports Sci Med Rehabil 13, 91

Tripodi N, Vaughan B, Wospil R, 2021, 'Peer feedback as a strategy to foster feedback literacy in first-year allied health students ', MedEdPublish, 10, [1], 115

Feehan, J., Degabrielle, E., **Tripodi, N**., Al Saedi, A., Vogrin, S., & Duque, G. (2021). The effect of vitamin D supplementation on circulating osteoprogenitor cells, a pilot randomized controlled trial. Experimental Gerontology, 150, 111399.

Feehan, J., **Tripodi, N**., & Apostolopoulos, V. (2021). The twilight of the immune system: The impact of immunosenescence in aging. Maturitas.

Bibby, L., & **Tripodi, N**. (2021). Non-surgical management and return to play of an anterior cruciate ligament rupture: A case report. International Journal of Osteopathic Medicine.

Tripodi, N., Feehan, J., & Apostolopoulos, V. (2021). Polarized Photobiomodulation: Biological Characteristics and Acceleration of Dermatological and Musculoskeletal Healing. In A. Terry (Ed.), *The Fundamentals of Polarized Light* (pp. 77-118). New York City, New York: Nova Publishers.

Tripodi, N., Corcoran, D., Antonello, P., Balic, N., Caddy, D., Knight, A., ... & Feehan, J. (2020). The effects of photobiomodulation on human dermal fibroblasts in vitro: A systematic review. Journal of Photochemistry and Photobiology B: Biology, 112100.

Tripodi, N., Feehan, J., Wospil, R., & Vaughan, B. (2020). Twelve tips for developing feedback literacy in health professions learners. Medical Teacher, 1-6.

Tripodi, N., Garrett, A., Savic, D., Sadrani, K., Robertson, L., Volarich, S., & Sirgiovanni, T. (2020). Patient expectations of manual and non-manual therapy within an osteopathic consultation: A cross sectional study. International Journal of Osteopathic Medicine.

Tripodi, N., Kelly, K., Husaric, M., Wospil, R., Fleischmann, M., Johnston, S., & Harkin, K. (2020). The Impact of Three-Dimensional Printed Anatomical Models on First-Year Student Engagement in a Block Mode Delivery. Anatomical sciences education.

Tripodi, N., Feehan, J., Husaric, M., Kiatos, D., Sidiroglou, F., Fraser, S., & Apostolopoulos, V. (2020). Good, better, best? The effects of polarization on photobiomodulation therapy. Journal of Biophotonics, 13(5), e201960230.

Conference Presentations

Tripodi, N., Apostolopoulos, V., Feehan, J. (2022). Transcriptome analysis of the effects of polarized photobiomodulation on human dermal fibroblasts. Australian Physiological Society Annual Meeting 2022. Hobart, Australia.

Tripodi, N., Vaughan, B., Wospil, R. (2020). Peer feedback as a strategy to foster feedback literacy in first-year allied health students. ANZAHPE 2020: Vision for learning cultures. Melbourne, Australia.

Tripodi, N., Wospil, R., Husaric, M. (2019). An Engaging Classroom: Anatomy with 3D Printed Models.

International Conference on Information Communication Technologies in Education, Crete, Greece.

Academic Prizes

Victoria University Block Model Professional Learning Program Working Group Appointee: 2022

Secomb Conference and Travel Fund Grant Winner. Victoria University, 2022.

Research Fellow, UTS: International Osteopathy Research Leadership and Capacity Building Program 2020-Present

Literacy Research Program Leader: Victoria University, First Year College Scholarship and Education Research Committee

Victoria University Doctorate of Philosophy Research Training Program Stipend Scholarship 2019-2022

Victoria University Vice-Chancellor's Citation for Excellence in Learning and Teaching 2020

Victoria University Capital Expenditure Grant 2022: $163,000

Osteopathy Australia, Seed Funding Grant 2021: $5,000

Defence Science Institute Research Collaboration Grant 2020: $50,000

COVID-19 RTP Allowance Application 2020: $1,200