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INVESTIGATING THE BIOPHYSICAL IMPACT OF ELECTROMAGNETIC FIELDS ON CELLULAR STRUCTURES: A COMPARATIVE STUDY ON CANCER CELLS

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Reserve your right to think,

for even to think wrongly is better than not to think at all.

IPAZIA

To my dearest Grandad Ninotto,

who encouraged me to explore, to question,

and to never be content with the limits others set.

You always told me I was your little scientist.

Today, I can finally say I am a step closer.

Your beloved,

Tathata

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ABSTRACT

This research aimed to investigate the effects of two distinct light exposure systems, the Bioptron device (hyperpolarized light) and the Vielight NeuroPro device (InfraRed radiation), on cellular responses in three cancer cell lines: PC3, HeLa, and MCF7.

The study focused on elucidating the impact of different irradiation conditions on cell viability, morphological changes, ATP production, and metabolic shifts. Cells were cultured in standard conditions and exposed to light using the Bioptron device (40 mW/cm^2) or the Vielight NeuroPro device (60 mW/cm^2, 10 Hz frequency sweep). Exposure times ranged from 10 to 60 minutes, with some groups receiving intermittent breaks. Cell viability was assessed using the Alamar blue assay, while morphological changes were evaluated through immunofluorescence staining of actin, tubulin, and mitochondria. ATP production and metabolic shifts were analyzed using a Glycolysis/OXPHOS Assay kit.

Results revealed a complex, biphasic dose-response relationship across all cell lines. Initial exposure (10 minutes) consistently led to decreased cell viability, suggesting an initial stress response. However, longer exposure times yielded variable results, with some conditions promoting cell proliferation while others induced cell death. Notably, the Bioptron device, which generates "hyperpolarized light" through a unique polarization pattern, exhibited distinct cellular responses compared to the Vielight NeuroPro device.

Immunofluorescence analysis revealed cell line-specific morphological alterations, including cytoplasmic shrinkage, changes in actin distribution, and potential mitochondrial damage. These structural changes were more pronounced in cells exposed to the Bioptron device, particularly after 10 minutes of exposure.

Metabolic assessments indicated a shift in energy production pathways following irradiation. Some experimental sets showed increased glycolytic activity with reduced mitochondrial ATP production, while others demonstrated the opposite trend. This metabolic reprogramming appeared to be influenced by both the irradiation conditions and the specific cell line. Interestingly, experiments involving the irradiation of culture medium alone suggested that the medium plays a crucial role in mediating the effects of electromagnetic waves on cells. This finding highlights the complexity of PBM mechanisms and the potential involvement of extracellular factors in cellular responses to light exposure. The study also observed that the efficacy of PBM treatment appeared to follow the Arndt-Schultz law or hormesis principle, where low doses stimulated cellular processes while high doses exerted inhibitory effects. This biphasic response underscores the importance of optimizing treatment parameters to achieve desired therapeutic outcomes.

In conclusion, this comprehensive investigation into the effects of two distinct devices on multiple cancer cell lines reveals the complex interplay between light expo- sure parameters, cellular characteristics, and metabolic reprogramming. The findings highlight the potential of PBM and hyperpolarized light as a therapeutic modality while emphasizing the critical need for careful optimization of treatment protocols. Future re- search should focus on elucidating their underlying mechanisms, cellular responses and exploring the potential synergistic effects of combining PBM and hyperpolarized light with conventional cancer therapies.

CONTENTS

CHAPTER 1

INTRODUCTION

The present study, conducted in collaboration with the University of Alberta, delves into the intricate world of cancer research and the potential therapeutic applications of photobiomodulation.
Driven by the pivotal keywords "*cancer*" and "*photobiomodulation*," this investigation aims to unravel the intricate interplay between light-based therapies and the complex mechanisms underlying malignant neoplasms.

This introductory chapter sets the stage for the research endeavor, providing a comprehensive overview of the study's context, objectives, and significance. It outlines briefly the content of each chapter building up the project, describing shortly their main topic and their relevance in such study.

The intricate interplay between electromagnetic fields and the human body forms the mainspring of the first chapter, providing a comprehensive understanding of the factors that influence the fluctuations of these fields within each individual. The chapter is divided into two distinct subchapters, each exploring a specific aspect of this multifaceted phenomenon (endogenous and exogenous factors).

Through a comprehensive exploration of its various features, the third chapter is focused on describing the intricate cancer nature, with a particular emphasis on the electrochemical aspects. It aims to provide a solid foundation for the subsequent discussion of the specific cell lines tested in this study which will be discussed in the first subchapter; the second one investigates the Warburg effect, a metabolic adaptation that plays a pivotal role in fueling the cancer progression.

The fourth chapter reviews the cutting-edge field of photobiomodulation, a promising therapeutic modality that harnesses the power of light to modulate biological processes. Divided into three distinct subchapters, this section provides a

comprehensive overview of the current state-of-the-art, the methodological approach employed in this study, and the results obtained.

Building upon the findings presented in the previous chapter, the fifth section investigates comprehensively the results obtained from the photobiomodulation experiments. Through a meticulous and critical examination of the data and a thorough discussion of the implications, this chapter aims to provide a deeper understanding of the potential applications of photobiomodulation in cancer treatment.

The promising results obtained from the present study pave the way for further advancements and refinements in the application of photobiomodulation for cancer treatment. Indeed, the final chapter explores potential future developments and improvements to the experimental set-ups and methodologies employed, with the aim of enhancing the efficacy and translational potential of this innovative therapeutic approach.

CHAPTER 2

BACKGROUND ON ELECTROMAGNETIC FIELDS (EMFS)

Electromagnetic fields (EMFs) are a fundamental aspect of the physical world. This chapter explores the intricate nature of these fields, poring over their properties, sources, and potential implications for biological systems, with a particular emphasis on their relationship with the human body(Markov, 2015a; Vecchia P. et al., 2009). At the heart of EMFs lies a duality, a harmonious interplay between two distinct yet interrelated components: electric fields and magnetic fields. Indeed, unravelling the intricacies of this duality, it is crucial to shed light on such fundamental principles that govern their behavior.

Electric fields are born from the accumulation of stationary charges, exerting their influence on charged particles within their domain(Popović & Popović, 1999). These fields are characterized by their ability to store and transfer energy, shaping the trajectories of charged particles through the exertion of forces. Magnetic fields, on the other hand, arise from the motion of charges, or currents. These fields manifest as invisible lines of force, encircling and influencing the movement of charged particles within their sphere of influence(Rein, 2004).
While electric and magnetic fields can exist independently in static scenarios, their true nature is revealed in the dynamic dimension, where they are inextricably linked. Time-varying electric and magnetic fields are coupled, propagating through space as electromagnetic waves, governed by the fundamental laws of electromagnetism.

Here, a concise overview of these laws, highlighting their profound insights and unifying principles(Romano, 2011).

- The electrostatic force: Coulomb's law describes the force between two stationary charged particles, laying the foundation for our understanding of

electric fields and their interactions. It introduces the inverse square relationship and the principle of superposition, which simplify the analysis of complex electrostatic systems. Indeed:

$$F = k\frac{q_1 q_2}{r^2}$$

where k is the Coulomb constant, and the force is directed along the line joining the two charges.

- The magnetic force: Ampère's law unveils the intricate relationship between electricity and magnetism, describing the magnetic force exerted by electric currents. It encompasses the Biot-Savart law, which relates the magnetic field to the magnitude and direction of the current, and the Ampère circuital law, a powerful tool for analyzing magnetic fields in various geometries. Respectively, they are herein reported:

$$dB = \frac{\mu_0}{4\pi}\frac{(Idl \times \vec{r})}{r^3}$$

where μ_0 is the permeability of free space, I is the current, dl is a vector representing the current element, and r is the position vector from the current element to the point where the magnetic field is being calculated.

$$\oint_C \vec{B} \cdot \overrightarrow{dl} = \mu_0 I_{enc}$$

where B is the magnetic field, dl is an infinitesimal vector element of the closed loop C, μ_0 is the permeability of free space, and I_{enc} is the total current enclosed by the loop.

- Electromagnetic Induction: Faraday's law of electromagnetic induction reveals the dynamic interplay between electric and magnetic fields, stating that a changing magnetic flux through a closed loop induces an electromotive force (EMF) in that loop. Complemented by Lenz's law, which ensures the conservation of energy, this principle paved the way for the development of generators, transformers, and electrical machines. Hence:

$$\varepsilon = -\frac{d\Phi_B}{dt}$$

where ε is the induced EMF, and Φ_B is the magnetic flux through the loop.

- Electromagnetism: James Clerk Maxwell's groundbreaking equations unified the previously disparate fields of electricity, magnetism, and optics into a single, coherent theory. These four fundamental equations encapsulate the laws of Coulomb, Ampère, and Faraday, while also predicting the existence of electromagnetic waves. They include Gauss's law for electric and magnetic fields, the Faraday-Maxwell law incorporating the displacement current, and the Ampère-Maxwell law, a generalization of Ampère's circuital law.

    1. Gauss's Law for Electric Fields:

$$\oint_S \vec{E} \cdot d\vec{A} = \frac{Q_{enc}}{\varepsilon_0}$$

    2. Gauss's Law for Magnetic Fields:

$$\oint_S \vec{B} \cdot d\vec{A} = 0$$

    3. Faraday's Law (with Maxwell's Correction):

$$\oint_C \vec{E} \cdot d\vec{l} = -\frac{d\Phi_B}{dt}$$

    4. Ampère-Maxwell Law:

$$\oint_C \vec{B} \cdot d\vec{l} = \mu_0 I_{enc} + \mu_0 \varepsilon_0 \frac{d\Phi_E}{dt}$$

In these equations, E represents the electric field, B represents the magnetic field, Q is the electric charge, ε_0 is the permittivity of free space, and Φ_E is the electric flux.

These fundamental equations encapsulate the profound insights offered by the laws of electromagnetism, providing a quantitative framework for analyzing and predicting electromagnetic phenomena. They serve as the foundation for numerous applications in fields ranging from electrical engineering to optics and quantum physics, enabling us to harness the power of electromagnetism for the advancement of science and technology.

To this regard, electromagnetic fields are an intrinsic part of the human body's functioning(Markov, 2015a), arising from various physiological processes and activities (endogenous EMFs). Also, there is an external component (exogenous EMFs) which still influences significantly such fields. Both factors will be discussed in the following subchapters.

2.1 ENDOGENOUS EMFS

The human body is a remarkable electrochemical system, where numerous processes generate and modulate electromagnetic fields(Markov, 2015b; Raines, 1981a). These endogenous EMFs are not merely byproducts but play crucial roles in various physiological functions and cellular communication mechanisms(Funk, 2015a; Raines, 1981b; Zura et al., 2015a). At the cellular level, scientific studies have demonstrated that cells generate and respond to electromagnetic fields during their normal physiological activities(Markov, 2015b). Ion transport across cell membranes, regulated by their selective permeability and facilitated by ion channels and pumps, creates electrical gradients and currents known as membrane potentials that contribute to the overall electromagnetic environment within the body(Levin, 2014; Thar & Kühl, 2004a). These potentials are the result of the movement of such charged particles generating local electromagnetic fields, contributing to the overall endogenous EMF landscape within the body. Additionally, metabolic processes involving oxidation-reduction reactions and the transfer of electrons also generate localized electromagnetic fields. Hence, there is a metabolism-driven energy production, involving all the biosynthetic pathways(Funk, 2015b; Hammerschlag et al., 2015; Zura et al., 2015b).

Mitochondria, the powerhouses of cells, are significant sources of endogenous EMFs. The electron transport chain, a series of redox reactions within the mitochondrial inner membrane, generates electrochemical gradients that are harnessed for ATP production(Amaroli et al., 2021; Fantin et al., 2006; Thar & Kühl, 2004a). These gradients, along with the release of signaling molecules like reactive oxygen species, contribute to the regulation of electromagnetic fields and cellular signaling pathways. This electron transport within proteins enables them to act as antennas, emitting and receiving electromagnetic signals, adding another layer to the intricate tapestry of endogenous EMFs(Thar & Kühl, 2004b).

Beyond the cellular level, the human body exhibits intricate electromagnetic phenomena on a larger scale. The nervous system is a vast network of interconnected neurons that communicate through the propagation of electrical signals, known as action potentials. These action potentials are generated by the coordinated flow of ions across neuronal membranes, creating even in this case, localized EMFs that travel along the length of neurons(*DialoguesClinNeurosci-16-93*, n.d.; Wiginton et al., 2022). The intricate patterns of neuronal activity contribute significantly to the overall endogenous contribution landscape, forming the basis for techniques such as

electroencephalography (EEG) and magnetoencephalography (MEG), which measure the electrical and magnetic signals associated with the brain activity. The propagation of action potentials along neurons involves the coordinated flow of ions across neuronal membranes, creating localized electromagnetic fields that facilitate neuronal communication.

Similarly, the rhythmic contractions of the heart are governed by the coordinated depolarization and repolarization of cardiac muscle cells, generating powerful electromagnetic fields that can be detected externally(Belenkov & Ryff, 1981). The heart is a remarkable electromechanical pump, exhibiting a complex interplay of electrical and mechanical events that govern its rhythmic contractions. The coordinated depolarization and repolarization of cardiac muscle cells, facilitated by the conduction system, generate powerful electromagnetic fields that can be detected and measured externally. These cardiac EMFs not only reflect the heart's function but also, as for the case of neurons, build up diagnostic techniques such as electrocardiography (ECG) and magnetocardiography (MCG).

While the existence of endogenous EMFs is well-established, their precise roles and mechanisms in biological processes are the subject of ongoing research and exploration. One intriguing hypothesis suggests that endogenous EMFs may mediate long-range interactions between biomolecules, such as proteins, enabling rapid and efficient binding beyond the limitations of Brownian diffusion. This proposed mechanism could facilitate the coordination of complex biological processes. Also, such endogenous EMFs have been hypothesized to play a role in facilitating communication between cells and coordinating processes such as circadian rhythms. This proposed mechanism could provide a means for cells to synchronize their activities and respond to environmental cues, contributing to the overall homeostasis and regulation of biological systems. Patterns of resting membrane potentials and endogenous EMFs have been suggested to guide developmental processes and tissue regeneration. This hypothesis proposes that the intricate interplay of electromagnetic fields within the body may serve as a blueprint for the organization and differentiation of cells, contributing to the remarkable ability of organisms to grow, develop, and heal.
In a more speculative view, endogenous EMFs have been proposed as a means for non-chemical communication between organisms, facilitating interactions such as plant-plant and animal-plant communication. This concept, often referred to as "biofield interactions," suggests that living beings may possess the ability to exchange information and influence each other through the modulation of their electromagnetic fields, potentially opening new avenues for understanding the intricate web of life.
Through this comprehensive exploration of endogenous EMFs, we gain a deeper appreciation for the intricate tapestry of electromagnetic phenomena that permeates the human body. From the cellular electrochemistry that sustains life to the larger-scale phenomena of neuronal conduction and cardiac rhythms, these endogenous fields reveal themselves as an integral part of our existence. As we continue to

unravel the mysteries of life, the study of endogenous EMFs holds the promise of unveiling new insights into the fundamental mechanisms that govern biological processes, paving the way for potential therapeutic applications and a deeper understanding of the intricate workings of the natural world.

2.2 EXOGENOUS EMFS

Exogenous EMFs refer to electromagnetic fields that originate from external sources, as opposed to endogenous EMFs generated within biological organisms. These external fields can interact with and potentially influence biological processes and cellular functions. According to the review articles(Caliogna et al., 2021; Zhang et al., 2020), exogenous EMFs have been shown to have various effects on bone cells, which play a crucial role in bone remodeling, repair, and homeostasis. The specific effects and underlying mechanisms appear to depend on the frequency, intensity, and duration of exposure to the EMFs. Noteworthy, the specific effects of exogenous EMFs on bone cells can vary depending on the cell type, the stage of differentiation, and the experimental conditions. Additionally, the mechanisms underlying these effects are not fully understood and may involve complex interactions between various signaling pathways and cellular processes. Bone cells and mesenchymal cells are the most investigated ones in terms of exogenous EMFs, but in general, quite the same effects manifest in stem cells favoring their differentiation and other cell types like neurons and chondrocytes. Research focuses on three main categories of exogenous EMFs, here presented:

- Low-Frequency EMFs: static and extremely low-frequency (ELF) EMFs have been reported to influence bone cell proliferation, differentiation and mineralization; also, the potential mechanisms include modulation of intracellular signaling pathways, changes in gene expression, and alterations in calcium homeostasis (Costantini et al., 2022; Lei et al., 2017). Moreover, there is an effect in neural stem cells (NSCs), enhancing adult hippocampal neurogenesis by inducing proliferation and differentiation of neural stem/progenitor cells. In general, there is evidence of a trend in the effects consequential to ELF-EMFs such as favored proliferation and cellular interactions to activate signaling pathways determining cells' fate across different lineages like chondrogenesis(Ma et al., 2016; Tian et al., 2023a).
- Radiofrequency (RF) EMFs: some studies have reported increased osteoblast (bone-forming cells) proliferation and differentiation upon exposure to

specific RF-EMF frequencies and intensities; there are also proposed mechanisms involving the activation of specific signaling pathways, changing in protein expression, and the modulation of oxidative stress response (Atay et al., 2009; Gonul et al., 2016).

- Pulsed electromagnetic fields (PEMFs): they comprise short bursts of EMFs and they have been extensively studied for their potential therapeutic applications again in bone repair and regeneration. Indeed, they have been shown to enhance osteoblast functionality as well as promoting angiogenesis (formation of new blood vessels). The most supported mechanism involving PEMFs includes modulation of calcium signaling, upregulation of growth factors, and stimulation of bone morphogenic protein (BMP) pathways (Ceccarelli et al., 2013).

However, when referring to EMFs from external sources, noteworthy are the geomagnetic fields and cosmic rays which contribute to the human EMF baseline, even if they are partially screened by the atmosphere. Hence, considering the Earth's natural electromagnetic environment, including the global atmospheric electrical circuit, Schumann resonances and geomagnetic field(Füllekrug & Fraser-Smith, 2011; Vainio et al., 2009), it is believed to play a non-negligible role in regulating various physiological processes in living organisms, including the human circadian rhythm. Additionally, there could be disturbances in such environment due to cosmic rays and geomagnetic storms that can potentially disrupt and alter associated biological processes. Notwithstanding all the aforementioned factors, further research is needed to fully understand the underlying mechanisms and the extent of these effects on human health and disease development.

CHAPTER 3

BACKGROUND ON CANCER

Cancer is the second leading cause of mortality worldwide, accounting for millions of deaths annually. Its impact exerts a substantial economic burden on healthcare systems but even more on humans who find themselves forced to deal with it. Despite significant advances in the understanding of cancer biology and the development of novel therapeutic strategies, the complexity and heterogeneity of this disease continue to pose uphill challenges.
Cancer is characterized by uncontrolled cell growth and the ability to invade and metastasize to other parts of the body. In their crucial work, Hanahan and Weinberg proposed a framework for understanding the fundamental characteristics of cancer, known as the “Hallmarks of Cancer”. Such hallmarks involve the acquisition of sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, enabling of replicative immortality, induction of angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism, evasion of immune destruction, tumor-promoting inflammation, and genomic instability and mutation.
At the heart of cancer lies a complex interplay of genetic and epigenetic alterations that disrupt the intricate regulatory mechanisms governing cellular processes. These alterations can arise from various factors, including inherited genetic predispositions, environmental exposures, and lifestyle choices.

3.1 CELL-LINES CHARACTERISTICS

The present study aimed to investigate the potential effects of photobiomodulation (PBM) and hyperpolarized light on various cell lines by exposing them to two distinct electromagnetic field (EMF) spectrum windows. In particular, three cancer cell lines have been tested. Herein, some details about the principal characteristics of each one:

- PC3 Cell Line: it is derived from a human prostatic adenocarcinoma, and it is one of the most aggressive forms of prostate cancer. Established from a bone metastasis of a grade IV prostate cancer, the PC3 cell line exhibits several distinctive features such as the absence of functional androgen receptors (AR) and the lack of prostate-specific antigen (PSA) expression. Also, they express neuroendocrine markers and the stem cell marker CD44 (Tai et al., 2011);
- MCF7 Cell Line: it is derived from a human breast adenocarcinoma, and it represents a suitable model system for investigating hormone receptor-positive breast cancers. Established from a pleural effusion of a 69-year-old Caucasian woman with metastatic breast cancer, the MCF7 cell line exhibits a unique molecular profile that closely resembles the luminal A subtype of breast cancer. One of the most defining characteristics of the MCF7 cell line is its expression of estrogen receptor (ER) and progesterone receptor (PR), while being negative for the human epidermal growth factor receptor 2 (HER2). Its well-established molecular profile and responsiveness to hormonal stimuli provide a reliable platform for evaluating the efficacy and mechanisms of action of potential therapeutic agents (Baxter et al., 2017a; Welsh, 2013);
- HeLa Cell Line: it is derived from a cervical adenocarcinoma. Established in 1951 from the tumor cells of Henrietta Lacks, a patient diagnosed with cervical cancer, the HeLa cell line has played a pivotal role in numerous scientific discoveries and advancements. One of the most defining features of the HeLa cell line is its immortality and ability to continuously divide and proliferate indefinitely in culture. This remarkable property is attributed to the reactivation of the telomerase enzyme, which maintains the length of telomeres, the protective caps at the ends of chromosomes. It has a high susceptibility to viral infections, making it a valuable model for studying viral replication and pathogenesis(Masters, 2002).

3.2 WARBURG EFFECT

Cancer, characterized by uncontrolled cell proliferation and metastasis, exhibits profound alterations in cellular metabolism(Ward & Thompson, 2012a). The Warburg effect, named after its discoverer Otto Warburg, refers to the unique metabolic phenotype observed in cancer cells(Otto, 2016a). The discovery process, spearheaded by Warburg in the 1920s, unfolded as follows: in 1926, Warburg postulated that due to their rapid proliferation, cancer cells would exhibit elevated oxygen consumption rates compared to normal cells. To test this hypothesis, he designed a series of experiments utilizing rat liver tissue slices, comparing the metabolic profiles of normal and cancerous tissues. Contrary to his initial expectations, Warburg's experiments revealed that oxygen consumption rates in cancer cells did not significantly differ from those of normal cells. However, he observed a striking increase in lactate production - approximately 70-fold higher in cancer cells compared to their normal counterparts, even under aerobic conditions (Liberti & Locasale, 2016a). Based on these unexpected results, Warburg formulated his groundbreaking hypothesis: while cancer cells retain the capacity for respiration, they preferentially utilize glycolytic pathways for energy production, even in the presence of oxygen. This metabolic phenotype, which normal cells typically only employ under anaerobic conditions, became the defining feature of what we now know as the Warburg effect (Otto, 2016a). Warburg published his findings in a series of seminal papers between 1923 and 1924, detailing the unique metabolic characteristics of cancer cells. The scientific community quickly recognized the significance of these discoveries, with the Nobel committee even considering Warburg for the 1927 Nobel Prize in Medicine for his work on cancer metabolism. These metabolic reprogramming events are now recognized as crucial for supporting the rapid growth and survival of cancer cells. Among these alterations, the Warburg effect stands out as a fundamental metabolic hallmark of cancer, shaping our understanding of tumor biology and opening new avenues for therapeutic interventions.

This phenomenon is characterized by the preferential utilization of aerobic glycolysis for energy production, even in the presence of sufficient oxygen and it has profound implications for the metabolic reprogramming of cancer cells and their adaptation to the tumor microenvironment(Hardie, 2022; Pascale et al., 2020). Specifically, cancer cells exhibit (Vander Heiden et al., 2009a):

- ◊ Enhanced glucose uptake: To sustain high rates of glycolysis, cancer cells exhibit increased glucose uptake. This is often facilitated by the overexpression of glucose transporters, such as GLUT1, on the cell membrane. The enhanced glucose uptake ensures a continuous supply of glucose to fuel the glycolytic pathway, providing the necessary energy and biosynthetic precursors for rapid cell growth and division.
- ◊ Increased glycolytic flux: the reliance on glycolysis allows cancer cells to rapidly produce ATP, albeit less efficiently than oxidative phosphorylation, supporting their high energy demands for proliferation and survival.
- ◊ Reduced oxidative phosphorylation: the Warburg effect helps maintain redox balance by producing NADPH through the pentose phosphate pathway, which is crucial for counteracting oxidative stress and supporting anabolic reactions. NADPH is essential for maintaining the cellular redox state and for the biosynthesis of fatty acids and nucleotides, further supporting cancer cell growth and survival.
- ◊ Elevated lactate production and secretion: lactate can act as a signaling molecule, influencing the behavior of surrounding stromal cells, endothelial cells, and immune cells, thereby creating a supportive niche for tumor growth and dissemination. The by-product of glycolysis in cancer cells is lactate, which is produced in large quantities and secreted into the tumor microenvironment. This lactate production contributes to the acidification of the surrounding tissue, promoting invasion and metastasis. The acidic microenvironment can also suppress immune responses and enhance the degradation of the extracellular matrix, facilitating tumor progression.

This metabolic shift contrasts sharply with the metabolism of normal differentiated cells, which primarily rely on mitochondrial oxidative phosphorylation for ATP generation under aerobic conditions. Additionally, the glycolytic pathway provides intermediates for various biosynthetic processes, supporting the synthesis of nucleotides, amino acids, and lipids necessary for cell growth and division. Moreover, the reliance on glycolysis allows cancer cells to survive and proliferate in hypoxic conditions commonly found within tumors.
Finally, hypoxia-inducible factors (HIFs) play a crucial role in this adaptation by upregulating the expression of glycolytic enzymes and glucose transporters, further enhancing the glycolytic capacity of cancer cells (Liberti & Locasale, 2016a; Otto, 2016a; Vander Heiden et al., 2009a; Ward & Thompson, 2012a).

The Warburg effect's significance lies not only in its near-universal presence across diverse cancer types but also in its potential as a target for diagnostic and therapeutic strategies. The elucidation of the Warburg effect represents a seminal moment in cancer research, fundamentally altering our perception of tumor biology.

CHAPTER 4

PHOTOBIOMODULATION (PBM)

Photobiomodulation (PBM), a therapeutic approach utilizing non-ionizing light sources to elicit biological effects in living tissue, has its roots in a fortunate discovery made in the late 1960s. This chapter examines the historical context of PBM's discovery, its subsequent development, and the evolution of scientific understanding surrounding this phenomenon.

4.1 STATE-OF-THE-ART

In 1967, Hungarian physician and scientist Endre Mester conducted an experiment aimed at investigating the potential anti-tumor effects of laser irradiation(Hamblin, 2016). Mester's experimental design involved the use of a low-powered ruby laser on mice with implanted tumors. Mester observed that the laser irradiation led to accelerated hair growth and enhanced wound healing in the shaved areas surrounding the tumors. This finding was particularly noteworthy given the low power output of the ruby laser employed, which was not expected to produce such significant biological effects. Following these observations, the phenomenon was initially termed "Low Level Light Therapy" (LLLT), reflecting the low-power nature of the light sources used in these early experiments(Anders et al., 2019).
However, subsequent research demonstrated that both lasers and light-emitting diodes (LEDs) could be effective in PBM applications, broadening the range of available light sources. Hence, as understanding of the underlying mechanisms improved and applications expanded, the scientific community gradually adopted the

more precise term "Photobiomodulation" in place of LLLT(Heiskanen & Hamblin, 2018).

PBM primarily acts on cellular mitochondria(Hamblin, 2018a; Ravera et al., 2019, 2021), specifically on Cytochrome C Oxidase (COX), which absorbs light in the red (600-700 nm) and near-infrared (700-1100 nm) spectrum(Karu, 2014a). Further studies identified several key mechanisms underlying the biological effects of PBM (de Freitas & Hamblin, 2016):

1. Increased ATP production: enhanced ATP production boosts cellular metabolism and energy availability, which is critical for cell repair and regeneration(Hamblin, 2016).
2. Modulation of reactive oxygen species (ROS) and nitric oxide (NO): the production of ROS and NO leads to improved cellular signaling, reduced inflammation, and enhanced tissue repair(Moriyama et al., 2009).
3. Activation of transcription factors: PBM activates various transcription factors that regulate gene expression related to cell proliferation, migration, and anti-inflammatory responses(H. P. Kim, 2014; R Hamblin, 2017a).

The efficacy of this new light therapy is dependent on several parameters that can significantly influence treatment outcomes(Hamblin et al., 2018). This therapeutic approach is governed by the Arndt-Schultz law, which describes a biphasic dose response curve in biological systems (Calabrese, 2001a, 2001b, 2002, 2004, 2013). Such law postulates that low levels of light have a stimulating effect on biological systems, while higher levels have an inhibitory effect. This principle underscores the importance of identifying an optimal therapeutic window for PBM treatments(Bensadoun et al., 2020a; Jo et al., 2023). Within this window, the beneficial effects of the therapy can be maximized while minimizing potential adverse effects (Robijns et al., 2022a; Zein et al., 2018a). In particular:

- ◊ Wavelength: different wavelengths penetrate tissue to varying depths and interact with specific cellular chromophores.
- ◊ Power density: the amount of energy delivered per unit area affects the depth of penetration and cellular response.
- ◊ Treatment duration: the length of exposure time influences the total energy delivered to the target tissue.
- ◊ Pulsing vs. continuous wave: some studies suggest that pulsed light may have different biological effects compared to continuous wave light.

PBM has shown promise in various clinical applications, including:

- Skin rejuvenation: A controlled trial demonstrated significant improvements in skin feeling, complexion, and collagen density following treatment with red and near-infrared light(Glass, 2023; Robijns et al., 2021a).
- Mood disorders: Light therapy has been found effective in treating seasonal affective disorder (SAD) and non-seasonal depression (Ji et al., 2024).

Meta-analyses have revealed significant reductions in depression symptom severity associated with bright light treatment in both SAD and non-seasonal depression. For SAD, bright light treatment showed an effect size of 0.84 (95% CI: 0.60 to 1.08), while dawn simulation demonstrated an effect size of 0.73 (95% CI: 0.37 to 1.08). In non-seasonal depression, bright light treatment yielded an effect size of 0.53 (95% CI: 0.18 to 0.89)(Gaggi et al., 2024; Montazeri et al., 2022).

Recently, PBM has been explored as a potential cancer treatment, yielding mixed results. While PBM appears promising in mitigating the side effects of conventional cancer therapies (R Hamblin, 2017b)emerging evidence suggests it may also serve as a viable alternative to these treatments. Indeed, In vivo studies have demonstrated that PBM can inhibit cancer cell proliferation and tumor progression. For instance, research conducted by Hamblin et al. (Hamblin, 2018b) showed that specific wavelengths of light could reduce the growth rate of certain cancer cell lines in animal models. These findings suggest that PBM may have a direct anti-cancer effect, potentially offering a non-invasive alternative to traditional therapies. Despite these promising results, other studies have reported conflicting outcomes. Some evidence indicates that PBM may not only be ineffective but could also exacerbate cancer progression. For example, a study by Karu (Karu, 2014b; Karu et al., 2005)found that under certain conditions, PBM could promote more aggressive tumor growth. These discrepancies highlight the need for further research to elucidate the conditions under which PBM is beneficial versus detrimental. For this reason, it is essential to address the current research in terms of:

1. Methodological heterogeneity: Many studies suffer from small sample sizes and varying treatment protocols.
2. Placebo control: Designing appropriate placebo conditions for light therapy studies remains challenging.
3. Long-term efficacy: More research is needed to assess the long-term effects of PBM therapy.

Future research should focus on standardizing treatment protocols, conducting larger randomized controlled trials, and exploring the potential synergistic effects of combining PBM with other therapeutic modalities.

4.2 VIELIGHT DEVICE

The device used in the experiments is the Vielight NeuroPro. It is a wearable brain photobiomodulation system developed by Vielight Inc. that delivers transcranial-intranasal photobiomodulation via a headset and intranasal applicator.
For the experiments it was used just the Module A without any other application device.

The NeuroPro has the ability of experimenting different exposure time, a parameter that has been changed in order to investigate different outcomes.
The Neuro Pro comes with the Neuro Pro app installed in a pre-synced smartphone provided with the Neuro Pro from Vielight Inc.
From the Neuro Pro app, it is possible to customize certain parameters such as power density and pulse rate as showed in the table.

PARAMETER	SPECIFICATION
LIGHT SOURCE	LED (810 NM)
FREQUENCY PULSE	10 HZ
POWER SETTING	80%
POWER DENSITY	60 MW/CM^2
RUN TIME	TUNABLE
DUTY CYCLE	50%

Table 1. VieLight NeuroPro setting for the Module A

Figure 1. VieLight NeuroPro modules and controller. The first on the right is the Module A which was used for this experimental study

CHAPTER 5

HYPERPOLARIZED LIGHT

The wave-particle duality of light is a fundamental concept in quantum mechanics that has revolutionized our understanding of the nature of light and matter. The debate over the nature of light dates back to ancient times(Atkins & De Paula, 2006; Hecht, 2016). In the 17th century, two competing theories emerged:

1. Corpuscular theory: Isaac Newton proposed the corpuscular theory of light in 1704, suggesting that light consisted of tiny particles or corpuscles. This theory successfully explained phenomena such as reflection and refraction but struggled to account for diffraction and interference.
2. Wave theory: Christiaan Huygens proposed the wave theory of light in 1690. This theory gained support in the early 19th century when Thomas Young conducted his famous double-slit experiment, demonstrating the interference of light waves(*May 1801: Thomas Young and the Nature of Light*, n.d.).

Focusing on the latter one in particular, the British scientist Thomas Young provided compelling evidence on how to perceive light. The experiment set-up consisted of:

- A single light source (sunlight)
- A screen with a small aperture to create a coherent light source
- A second screen with two closely spaced parallel slits
- A detection screen to observe the resulting pattern

When light passed through the two slits, Young observed a series of alternating bright and dark bands on the detection screen. This interference pattern could only be explained by the wave nature of light, as it resulted from the constructive and destructive interference of light waves emanating from the two slits. Young conducted additional experiments to support his wave theory:

- He demonstrated that light waves could cancel each other out, a phenomenon impossible with particles.

- He showed that different colors of light had different wavelengths, explaining the dispersion of white light into a spectrum.

These experiments, along with the double-slit experiment, provided a comprehensive body of evidence for the wave nature of light, revolutionizing our understanding of optics and paving the way for modern physics(“I. The Bakerian Lecture. Experiments and Calculations Relative to Physical Optics,” 1804).

In 1905, Albert Einstein proposed a revolutionary explanation for the photoelectric effect, suggesting that light was composed of discrete quanta of energy, later termed photons(Einstein, 1905a, 1905b). Einstein's work, which earned him the Nobel Prize in Physics in 1921, revived the particle aspect of light and laid the foundation for the concept of wave-particle duality. In 1924, Louis de Broglie extended the concept of wave-particle duality to matter (De Broglie, n.d.). He proposed that all particles, including electrons, could exhibit wave-like properties. De Broglie's hypothesis was experimentally confirmed by Clinton Davisson and Lester Germer in 1927, demonstrating electron diffraction (Davisson & Germer, 1927). Niels Bohr and Werner Heisenberg developed the Copenhagen interpretation of quantum mechanics in the late 1920s. This interpretation embraced the wave-particle duality, stating that the nature of light (and matter) as either a wave or a particle depends on the experimental setup and measurement process (Bohren & Huffman, 1940; Born & Wolf, 2019).

Light, as an electromagnetic wave, consists of oscillating electric and magnetic fields perpendicular to each other and to the direction of propagation. In unpolarized light, these oscillations occur in multiple planes. Polarization refers to the process by which the oscillations of the electric field are confined to a single plane or a specific pattern. Light polarization is a fundamental property of electromagnetic waves that has significant implications in various scientific and technological fields, including optics, telecommunications, and medical therapies(Born & Wolf, 2019; Goldstein, 2017).

There are three types of polarization: linear, circular and elliptical.

Linear polarization occurs when the electric field of light oscillates in only one plane. This can be achieved using polarizing filters, which block all light waves except those oscillating in the desired plane. Reflection and scattering are natural methods of achieving linear polarization, as seen with polarized sunglasses reducing glare. The mathematical representation of linearly polarized light can be expressed as:

$$\mathrm{E(t)} = E_0 \cos(\omega \mathrm{t} + \varphi)$$

where E_0 is the amplitude, ω is the angular frequency, and φ is the phase.

Circular polarization occurs when the electric field vector rotates in a circular motion as the light wave propagates. The direction of rotation distinguishes between right-handed and left-handed circular polarization. Elliptical polarization is a more general form, where the electric field describes an ellipse. These forms can be produced

using quarter-wave and half-wave plates, which alter the phase of the light waves. The mathematical representations for circular and elliptical polarization are reported respectively:

$$E(t) = E_0(\cos(\omega t + \varphi)\hat{x} + \sin(\omega t + \varphi)\hat{y})$$

$$E(t) = E_x\cos(\omega t + \delta_x)\hat{x} + E_y\sin(\omega t + \delta_y)\hat{y}$$

Where ω is the angular frequency of the light, δ_x and δ_y are the phase angles of the electric field components and $\hat{x}$ and $\hat{y}$ are unit vectors in the x and y directions, respectively.

5.1 STATE-OF-THE-ART

Polarized light is generally used in a variety of applications, including scientific tools for stress analysis and chemical analysis or microscopy techniques to enhance contrast and detail. Lately, the attention of the scientific community has been driven to a new application of such polarized light: indeed, the hyperlight therapy is a new approach to pain management, with a strong potential in alleviating chronic pain without the side effects associated with traditional treatments.
The name ‘hyperlight’ refers to the hyperpolarization of light: its core component is a nanophotonic light generator incorporating fullerene C60 molecules(Koruga, 2018; Willemse et al., n.d.).
Fullerene C60 is a molecule composed of 60 carbon atoms arranged in a spherical structure resembling a soccer ball. This unique geometry plays a crucial role in the light polarization process. In this case, C60 molecules are incorporated into a polymer material, creating an icosahedral twisting structure within the material matrix. When light passes through the C60-polymer structure, it undergoes a Fibonacci-sequential effect. Such process involves the rotation and twisting of the plane of photon polarization in all directions in pentagons. In particular, there is the creation of a 2D energy membrane described by the equation

$$[\Phi 2 + \varphi 2 = 3]$$

which "filters" propagating photons according to the Fibonacci law [Φ, φ].
The output is characterized as a Fibonacci twisting torus, resulting from the dynamics of three energy forms: left helix φ, right helix Φ, and attractive-repulsive interactions.
The output of this process is hyperpolarized light with both circular (left and right) and linear (vertical and horizontal) polarization components. The unique polarization pattern generated is described as a "sunflower seeds photons pattern," reflecting its

geometric arrangement, which lead to the final product of such process which is termed “Quantum Hyperlight”(Willemse et al., n.d.).

Hyperlight therapy utilizes specific wavelengths of light to penetrate tissues and promote healing at the cellular level. In particular, it consists of a polychromatic light beam (350-3400 nm), incoherent and 95% polarized.
According to Dr. Whitten, this method can enhance cellular repair, reduce inflammation, and improve blood circulation by stimulating mitochondrial function and activating photoacceptors in our cells. Recent studies have corroborated these findings, demonstrating the therapy's effectiveness in various medical conditions(*BIOPTRON Hyperlight*, n.d.). For instance, a meta-analysis by Chen et al. (Tian et al., 2023b) found that hyperlight therapy significantly reduced pain intensity and improved functional outcomes in patients with chronic neck pain. The authors attributed these benefits to the therapy's ability to modulate inflammatory pathways and enhance tissue regeneration. Indeed, the exposure to such hyperpolarized light leads to an increase in nitric oxide production, followed by an increase in blood flow and an improvement in circulation. Also, there is a positive response from the immune system and a downregulation of inflammation. In addition, cellular healing is stimulated due to the enhanced production of both collagen and fibroblasts (Raeissadat et al., 2014).

5.2 BIOPTRON PRO1 DEVICE

The BIOPTRON Pro 1 is an advanced light therapy device that employs polarized light technology for medical and therapeutic applications. It generates light within a wavelength range of 480-3400 nm, with a high degree of polarization (>95% between 590-1550nm). The device delivers a specific power density of approximately 40 mW/cm² and an energy output of about 2.4 J/cm² per minute. Featuring a 11 cm diameter filter and weighing 3.4 kg, the Pro 1 is designed with a 360° rotatable head for precise light application. It operates on a universal 100-240 V AC power supply, consuming 90 VA, and utilizes a 50 W halogen lamp. The device incorporates user-friendly features such as a timer function, adjustable height and inclination, and an integrated distance rod to ensure optimal treatment positioning. Certified to CE standards (CE0124), the BIOPTRON Pro 1 includes safety features like overheating protection and is operable within a temperature range of +10°C to +40°C, making it suitable for both clinical and home environments.

PARAMETER	SPECIFICATION
LIGHT SOURCE	HALOGEN LAMP
WAVELENGTH RANGE	590-1550 nM
POLARIZATION	95%
POWER SETTING	100%
POWER DENSITY	40 mW/Cm^2
RUN TIME	TUNABLE
DUTY CYCLE	50%

Table 2. Summary of setting used for Bioptron Pro1 experiments

Figure 2. Bioptron Pro1 device used in this investigation

CHAPTER 6

PROJECT OVERVIEW

6.1 Materials and Methods

6.1.1 Cell Culture

The PC3 prostate cancer cell line was sourced from Dr. John Lewis's research facility at the Katz Group Center. The HeLa and MCF7 cell lines were procured from Dr. Godbout's laboratory at the Cross Cancer Institute. These cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) enhanced with 10% (V/V) Fetal Bovine Serum (FBS) and 1% antimicrobial compounds (100 U/ml penicillin and 100 µg/ml streptomycin). The cellular cultures were maintained in a regulated environment at 37°C with a 5% CO_2 atmosphere.

6.1.2 Sample Preparation and Irradiation

Cellular cultures were maintained in T75 flasks until reaching approximately 80% confluence before being transferred to µ-Dish 35 mm high-wall glass-bottomed vessels (ibidi Inc, Gräfelfing, Germany). Upon achieving the desired density, the depleted medium was extracted, and the culture was rinsed with Phosphate-Buffered Saline (PBS). Following PBS removal, 2 mL of TrypLETM Express Enxyme 1X (Glibco, ThermoFisher Scientific, Burlington, Ontario, Canada) was introduced to detach cells. To accelerate this process, the flask was placed in a 5% CO_2 incubator for a brief period. Once cell

detachment was complete, 8 mL of fresh culture medium (DMEM) was introduced.

Subsequently, cell enumeration was performed using a hemocytometer, targeting a cellular density of 25×10^4 cells per dish. A cellular suspension of 10 µL was formulated in a 96-well plate with 40 µL of 0.4% Trypan Blue (Sigma Aldrich, St. Louis, Missouri, USA), resulting in a final dilution factor of 5.

Following cell seeding and overnight incubation, the medium was substituted with fresh DMEM supplemented solely with 1% antibiotics to synchronize the cells in the G0/G1 phase. This synchronization process required a 24-hour incubation period.

Two distinct light exposure systems were utilized in this investigation. The Bioptron apparatus operates at a fixed power density of 40 mW/cm^2, with adjustable exposure duration. In contrast, only module A of the Vielight device NeuroPro was employed, featuring a power density of 60 mW/cm^2 and a frequency sweep of 10 Hz, while the exposure time remained variable.

For the PC3 cell line, samples were categorized into four groups: (I) 10 minutes exposure, (II) 15 minutes exposure, (III) 20 minutes exposure, (IV) 20 minutes exposure with a 10-minute intermission. Maintaining these irradiation intervals, fresh growth medium was also irradiated in a 1.5 ml Eppendorf tube, then directly transferred to the dishes containing the pre-seeded cells.

Regarding HeLa and MCF7 cells, the irradiation intervals for Bioptron-treated samples were kept consistent with the PC3 protocol. However, for NeuroPro-treated samples, the selected exposure times were: (I) 15 minutes, (II) 33 minutes, (III) 60 minutes, (IV) 30 minutes with a 15-minute intermission.

Across all experimental groups and devices, the cell samples were positioned at a constant distance of 125 mm from the beam source during irradiation. This uniform distance ensured consistent exposure conditions and facilitated comparative analyses between the various treatment groups. The irradiation was conducted at ambient temperature.

6.1.3 Cell Viability

The Alamar blue assay was employed to evaluate cellular viability 24 hours post-irradiation. This test relies on a reduction reaction in which viable cells convert resazurin to resorufin, accompanied by a corresponding change in the

medium's color. Specifically, 100 µL of resazurin was introduced to 1 mL of pre-warmed medium and incubated for 2 hours. Cell viability was quantified using a fluorescence-based Fluostar Omega plate reader (BMG LABTECH, Ortenberg, Germany) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Experiments were conducted in triplicate in a 96-well plate, utilizing fresh medium as a blank control.

6.1.4 IMMUNOFLUORESCENCE

For this analysis, a gelatin-coated coverslip was introduced into each dish and allowed to dry. Cell fixation was achieved using 3.7% formaldehyde in Phosphate Buffer Solution (PBS) for 15 minutes at room temperature, followed by two PBS washes. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, Missouri, USA) in PBS for 15 minutes at room temperature, followed by two PBS rinses. For actin staining, 1.4 µL of Alexa-Fluor 488 Phalloidin (Invitrogen, ThermoFisher Scientific, Burlington, Ontario, Canada) in 500 µL of 1% Working Buffer Solution (WBS) composed of MACS Bovine Serum Album (BSA) (Miltenyi Biotec, Bergisch Gladbach, Germany) in PBS was applied, incubated for 30 minutes at room temperature in darkness, then rinsed with PBS and mounted on microscope slides using ProLong Gold Antifade Mountant with DAPI (Invitrogen, ThermoFisher Scientific, Burlington, Ontario, Canada).

For tubulin staining, 5 µL of Primary antibody ATN02 Anti-AlphaBeta Tubulin Sheep Polyclonal (Cytoskeleton Inc, Denver, Colorado, USA) in 1 mL of 1% WBS in PBS was applied to cells, incubated overnight, and rinsed with PBS the following day. Subsequently, 2.5 µL of Donkey anti-Sheep IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 647 (Invitrogen, ThermoFisher Scientific, Burlington, Ontario, Canada) in 500 µL of WBS was introduced and incubated for 30 minutes at 4°C on a tilting plate in a dark environment. Samples were rinsed twice with PBS and then mounted following the aforementioned procedure for Phalloidin.

Immunofluorescence was also utilized to assess mitochondrial activity: 250 nM of MitoTracker™ Orange CM™Ros (Invitrogen, ThermoFisher Scientific, Burlington, Ontario, Canada) in 1mL of pre-warmed DMEM was introduced to the samples immediately post-irradiation and incubated at 37°C for 45 minutes. The samples were then rinsed thrice with fresh medium before immunofluorescence analysis. Samples were examined using the Zeiss 710 Confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

6.1.5 GLYCOLYSIS-OXPHOSPHORYLATION

The Glycolysis/OXPHOS Assay kit (Dojindo Molecular Technologies, Minato City, Tokyo, Japan) was employed to quantify total ATP, glycolytic ATP, mitochondrial ATP, and lactate production in cells.

The ATP quantification kit provided a 5.5 mL ATP Buffer solution, to which 10 µL of lyophilized luciferase enzyme was added to create the ATP working solution (ATP WS). The luciferase enzyme catalyzes the oxidation of luciferin, a process coupled to ATP consumption and light emission. A 96-well microplate was used for the assay setup. Each well received 100 µL of the prepared ATP WS, followed by 100 µL of cell supernatant from the respective samples. The microplate was then incubated at 25°C for 10 minutes in darkness. The Fluostar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) was used to measure the luminescence signal from each well.

To assess metabolic shifts due to mitochondrial activity, samples were treated with Oligomycin. Post-irradiation, a 10 mmol/l Oligomycin stock solution was diluted 1,000-fold with culture medium to obtain a 2-fold solution. Each experiment included one dish treated with Oligomycin (Oligomycin +) and one with fresh medium only (Oligomycin -) for both control and treated samples. Wells were incubated for 3 hours (37°C, 5% CO_2). The ATP assay was then performed following the aforementioned protocol, and luminescence was measured using the Fluostar Omega microplate reader.

For lactate production quantification, the assay compared absorbance values between the Lactate Standard, the sample, and the blank. The 10 mmol/l Lactate Standard provided in the kit was diluted to 1 mmol/l with double-deionized H_2O (ddH_2O). Cell culture supernatants were diluted 10-fold with ddH2O, and ddH2O served as the blank.

Subsequently, 20 µL of each solution was added in triplicate to a 96-well plate, followed by 80 µL of Lactate Working Solution. The plate was incubated at 37°C for 30 minutes. Absorbance was measured at 450nm using the Fluostar Omega microplate reader.

6.2 Results

6.2.1 Bioptron

6.2.1.1 Cellular Viability Assessment

The experimental findings depicted in Figure 3.a reveal a notable reduction in cellular viability across all experimental cohorts following a 10-minute exposure to the radiation source. This initial decline in viability suggests that the applied irradiation parameters exerted a detrimental effect on the cellular population during the early stages of exposure. The fourth experimental group, subjected to 20 minutes of exposure with a 10-minute intermission, yielded a significant increase in cellular mortality, a phenomenon not observed in samples irradiated for 15 minutes continuously. Interestingly, the 20-minute uninterrupted exposure did not produce a noteworthy outcome, with the proportion of viable cells remaining nearly equivalent in both control and treated samples. The observed trend in cellular viability indicates a potential manifestation of adaptive mechanisms or intrinsic cellular characteristics that modulate the response to irradiation.

Furthermore, experiments involving the exclusive irradiation of culture media corroborated the aforementioned trend, potentially indicating a crucial role of the medium in mediating the effects of electromagnetic waves on cellular populations.

The research also investigated the potential effect of re-irradiation after six days from the initial treatment (10 minutes of exposure). A significant decrease in cell viability was observed, possibly suggesting a potential treatment strategy. However, the experiment conducted is reductive to draw a conclusion that can be applied as a general protocol, but it nonetheless suggests a possible exploration in this direction to confirm or disprove the hypothesized trend.

For what concerns the same settings but applied to the HeLa cell line, the initial viability decrease following 10 minutes of exposure aligned with the observations in the PC3 cell line. However, while a non-significant reduction was noted after 15 minutes, a significant enhancement in viability was

observed following 20 minutes of exposure, deviating from the anticipated trend. Similarly, a non-significant change was observed for the fourth group (20 minutes with a 10-minute intermission).

Regarding the MCF7 cell line, the sole confirmed trend was a significant diminution in viability following 10 minutes of exposure. Notably, while a non-significant decrease in viability was observed after 15 minutes, both the third and fourth experimental groups exhibited a significant augmentation in cellular viability, further underscoring the cell line-specific responses to irradiation.

6.2.1.2 Alterations in Cellular Structures

As illustrated in Figure 12.a, a distinct contraction of the cytoplasm towards the nuclei was observed, a phenomenon frequently associated with the response of neoplastic cells to irradiation (10 minutes). This morphological modification manifested as a more spherical cellular shape and a diminution in cellular spreading on the substrate, aligning with previous reports in the scientific literature (reference). The 20 minutes exposure confirmed the same trend.

Regarding tubulin, no substantial alterations were apparent when comparing control and treated samples for the 10 minutes exposure. In the case of 20 minutes uninterrupted exposure, instead, there seems to be favored the disassembly of the microtubules as it is possible to observe that cells are rounder in shape and less spread.

Conversely, mitochondria, as depicted in Figure 16.a-b, exhibited a notable reduction in fluorescence signal intensity within treated cells, suggesting potential toxicity affecting mitochondrial integrity. This finding raises the possibility of mitochondrial impairment induced by the specific irradiation parameters employed (10 min, 40 mW/cm^2, 2.4 J/cm^2), which appears to have deleterious consequences for cellular metabolism and energy production.

6.2.1.3 ATP Production Analysis

To further elucidate the observed effects of irradiation on mitochondrial integrity, as indicated by the immunofluorescence analysis, a comprehensive evaluation of mitochondrial activity and cellular metabolic dynamics was conducted. The investigation was performed in triplicate and repeated thrice to ensure reproducibility and statistical robustness. Cellular ATP production from both glycolytic and mitochondrial pathways was quantified using the biochemical assay by Dojindo (Dojindo Molecular Technologies, Minato City, Tokyo, Japan) as detailed in the Materials and Methods section, providing insights into the metabolic reprogramming induced by the irradiation conditions.

Notably, the experimental data revealed distinct trends in mitochondrial functionality and cellular energy metabolism across the three sets of experiments. The initial set (Figure 19.b) demonstrated a metabolic shift towards enhanced glycolytic activity, accompanied by relatively low mitochondrial ATP production. The second set of experiments (Figure 19.c) yielded contrasting results, with diminished glycolytic activity compared to the control, but significantly elevated mitochondrial ATP production. Intriguingly, the third set of experiments (Figure 19.d) highlighted a slightly increased glycolytic ATP production compared to the control, coupled with a remarkable tenfold enhancement in mitochondrial ATP production relative to non-irradiated cells. The first set of experiments is confirmed for what concerns a negative drop in glycolytic activity, since in Figure 20.b, it is possible to observe a strong significat increase in the lactate production.

6.2.2 VieLight

6.2.2.1 Cellular Viability Assessment

The observed cellular viability trends in PC3 cells varied considerably depending on the exposure duration. Notably, the 15-minute irradiation protocol resulted in a statistically significant enhancement in viability compared to non-irradiated controls.

In contrast, the 10-minute exposure elicited a non-significant reduction in viability, indicating a relatively neutral effect on cellular processes at this duration. However, extending the irradiation time to 20 minutes led to a smaller but statistically significant diminution in viable cells, suggesting that

prolonged light exposure may exert detrimental effects on cell health or survival.

Following the experimental protocol established for the Biopropton device, cell culture media were irradiated according to the four designated time exposure groups. Notably, a statistically significant increase in cellular viability was observed in samples irradiated for 10 minutes compared to non-irradiated controls. A similar trend of enhanced viability, although not statistically significant, was also observed in the group exposed for 20 minutes with a 10-minute intermittent break. Conversely, the remaining two groups, comprising 15 minutes of continuous irradiation and 20 minutes of continuous irradiation, exhibited a non-significant decrease in cell viability relative to control samples.

These divergent responses underscore the complex interplay between irradiation parameters, such as dose, duration, and fractionation, and the resulting cellular outcomes.

Even in this case, we investigated the impact of repeated irradiation on the viability of cell cultures. Interestingly, the sample that was irradiated on day 1 and subsequently re-irradiated on day 7 exhibited a decrease in cell viability, in contrast to the sample that was exposed to a single 10-minute irradiation. This observation suggests that the growth state of the cells, as determined by the number of subpassages during cell culture, may play a role in the cellular response to repeated irradiation. Specifically, the sample that was re-irradiated after six days from the initial 10-minute exposure showed a significant decrease in cell viability. This finding was surprising, as the single exposure sample did not exhibit the same level of reduced viability. This result has led us to hypothesize that the growth state of the cells, influenced by the number of subpassages performed during the cell culture process, may be a contributing factor to the observed differences in cellular response to repeated irradiation. Further investigation is needed to elucidate the underlying mechanisms responsible for this phenomenon.

Regarding HeLa cells, they were subjected to irradiation for 15 minutes, 33 minutes, 60 minutes, and 30 minutes with a 15-minute intermittent break, respectively, maintaining a constant energy fluence of 2.4 J/cm^2. The initial three exposure durations (15 minutes, 33 minutes, and 60 minutes) exhibited a non-statistically significant increase in cellular viability compared to control samples. This trend suggests that low-dose or moderate light exposure may potentially stimulate cellular proliferation or metabolic activity in HeLa cells, although the observed effects were not statistically robust. However, the final exposure time point failed to demonstrate a significant difference in viability between treated and untreated cells, indicating a potential threshold beyond

which the beneficial effects of light irradiation may be diminished or counteracted by detrimental processes.

For the MCF7 cell line, the viability trends across different exposure intervals mirrored those observed in HeLa cells. Notably, three out of the four exposure groups exhibited a significant decrease in viability upon irradiation treatment, except for the 15-minute exposure samples, which showed a significant increase in viability. This outcome suggests a possible indication for optimizing exposure duration.

6.2.2.2 Alterations in Cellular Structures

Actin staining revealed structural modifications in the cytoplasm of irradiated cells compared to non-irradiated controls (10 minutes exposure). Specifically, the cytoplasm appeared more condensed towards the nucleus. The 20 minutes exposure, instead, showed an opposite trend with respect to the 10 minutes irradiation, seemingly favoring the spreading of cells.

Regarding tubulin staining, no visible alterations were observed in the treated cell population subjected to the same exposure time for both 10- and 20-minutes exposure. However, the signal in the control group appeared slightly dimmer compared to cells subjected to NIR-radiation when referring to 10-minute samples.

Conversely, mitochondria, as depicted in Figure 16.c-d, the signal is quite dimmer in both control and treated samples. However, we can observe a stronger signal in the 10-minutes irradiated samples, confirming the ATP analysis suggesting an enhanced activity of mitochondria indeed.

6.2.2.3 ATP production Analysis

Although not statistically significant, an increase in total ATP levels was observed in irradiated cells compared to controls (Figure 19.a) . This trend was maintained for mitochondrial ATP; however, glycolytic ATP exhibited a decrease in irradiated samples relative to non-irradiated controls. This reduction was further corroborated by a lactate assay (Figure 20.a)

These findings indicate a potential metabolic shift induced by light irradiation, favoring mitochondrial respiration over glycolytic pathways for energy production.

6.3 DATA FIGURES

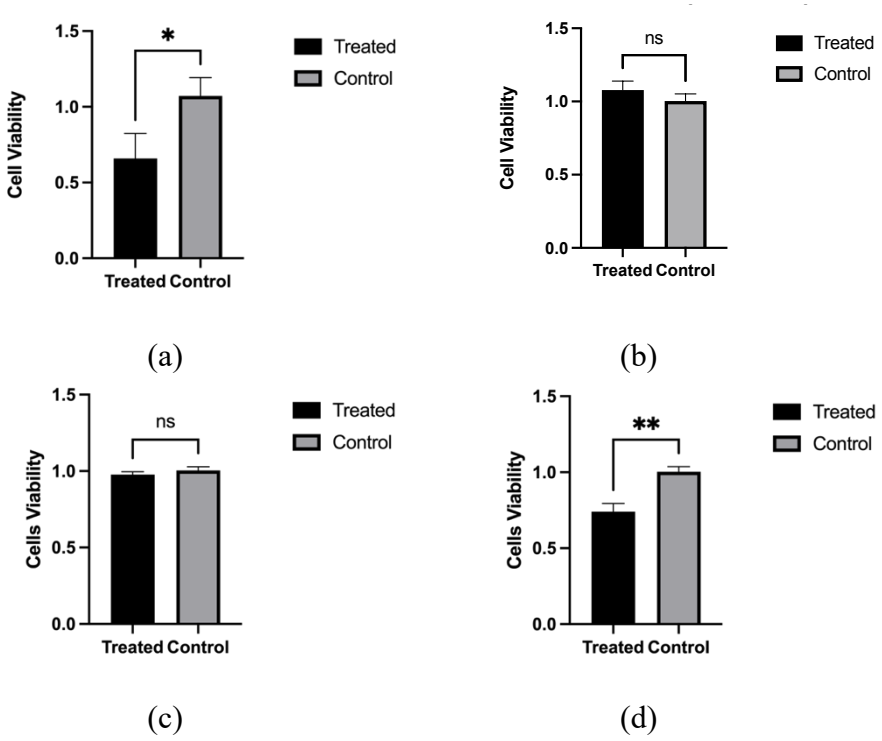


Figure 3. Bioptron PC3 exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

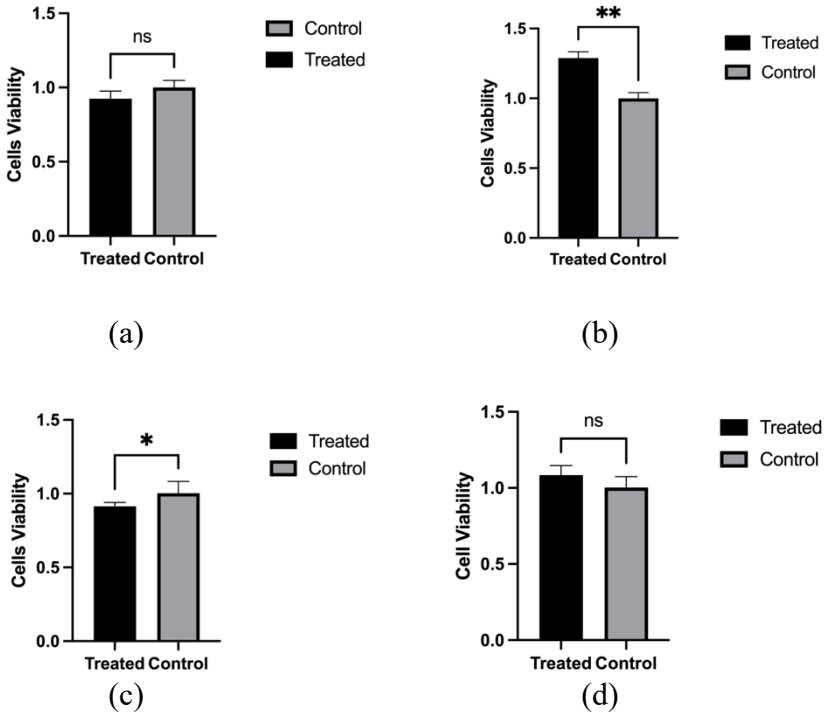


Figure 4. Vielight PC3 exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

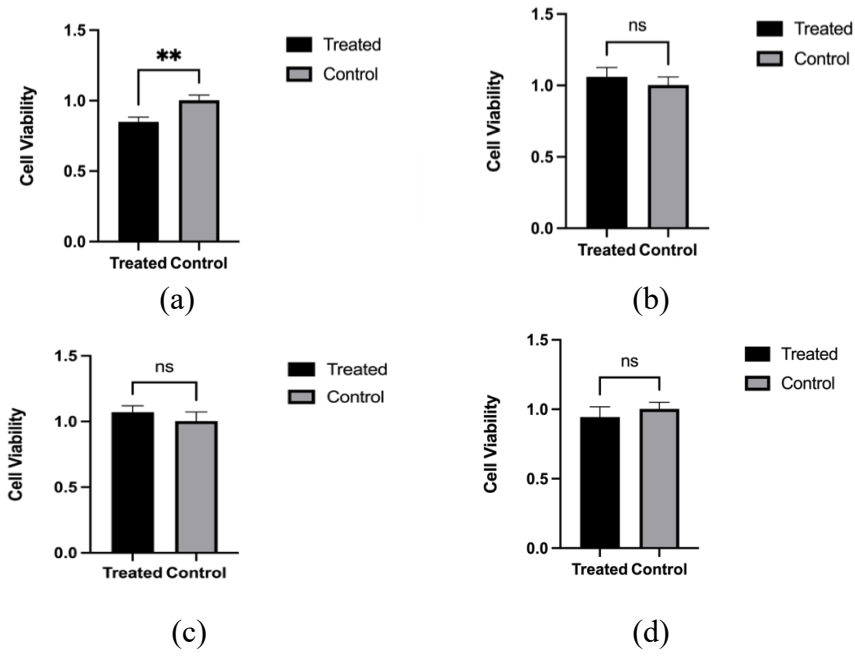


Figure 5. Bioptron PC3 medium exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

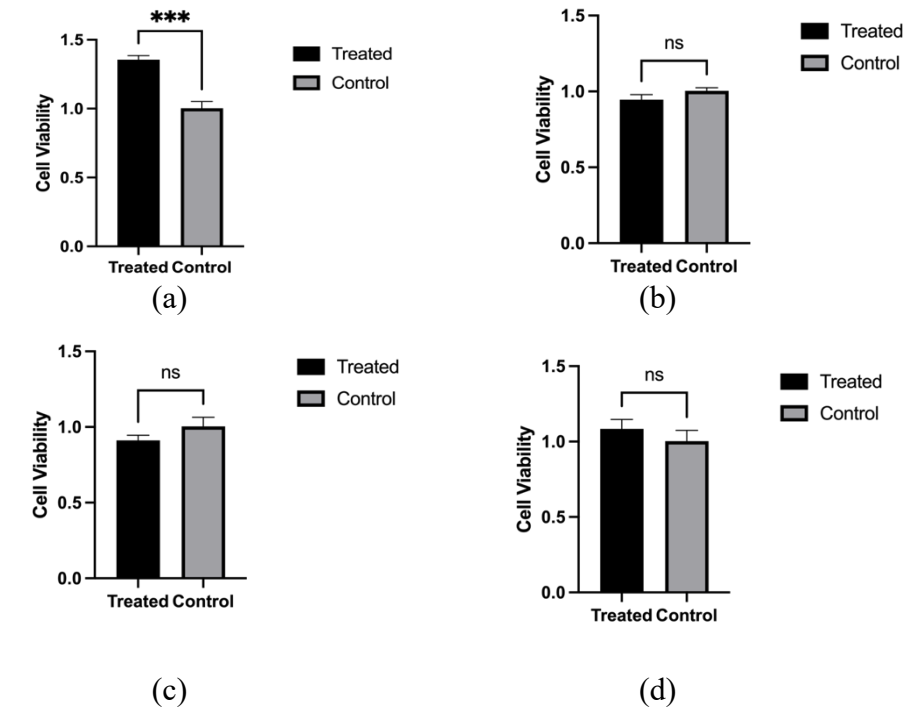


Figure 6. Vielight PC3 medium exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

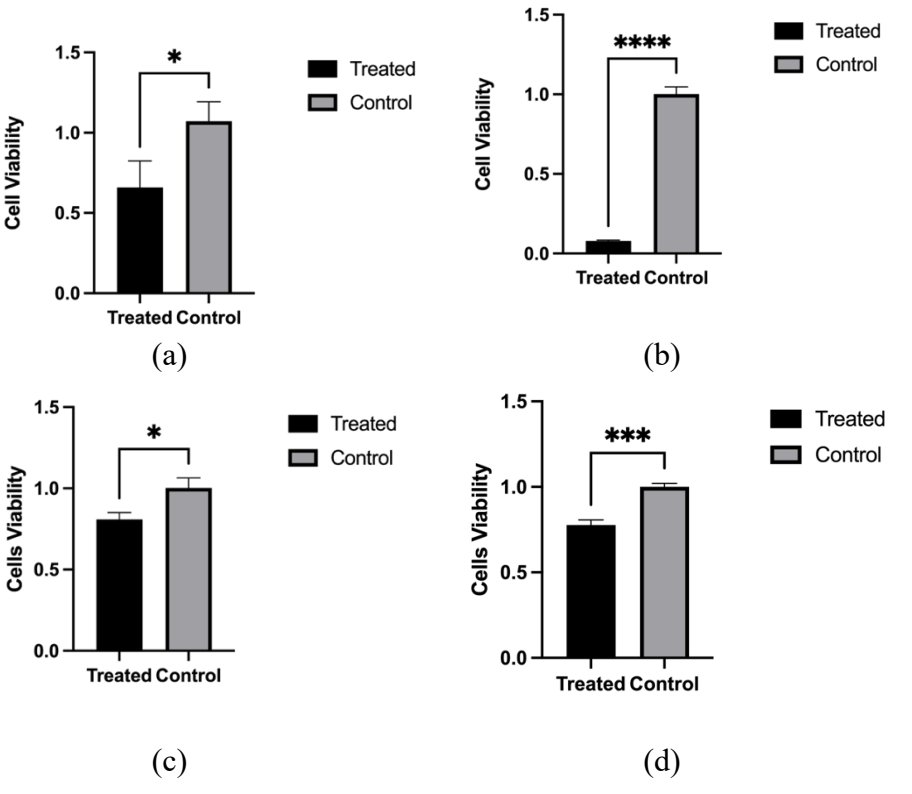


Figure 7. PC3 day 1 and day 7 exposure: (a) Bioptron - 10 minutes exposure, day 1; (b) Bioptron - 10 minutes exposure, day 7; (c) Vielight - 10 minutes exposure, day 1, 125 mm from the spot; (d) Vielight - 10 minutes exposure, day 7, 125 mm from the spot.

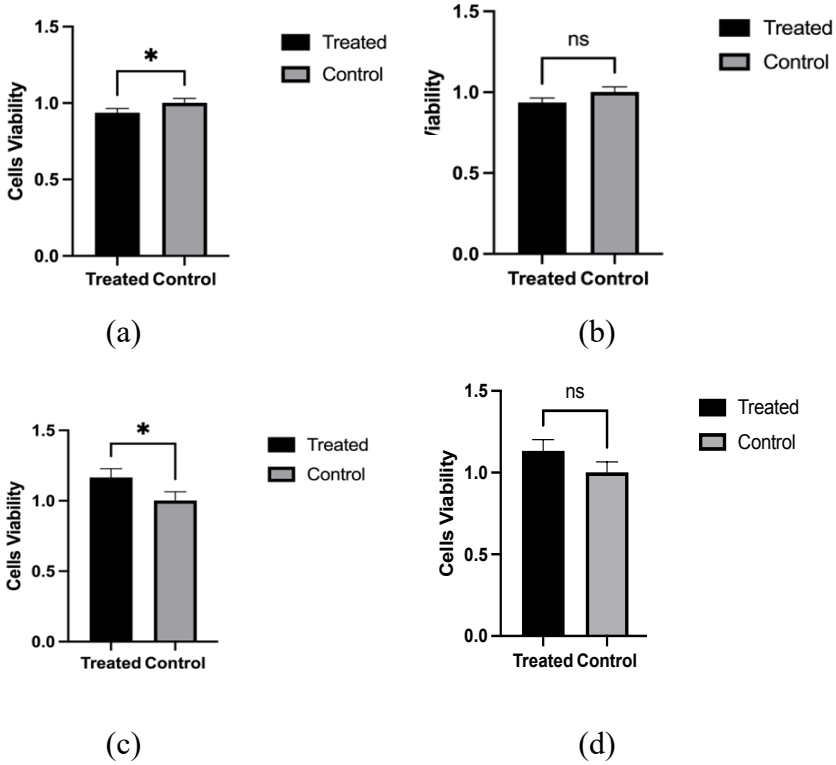


Figure 8. Bioptron HeLa exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

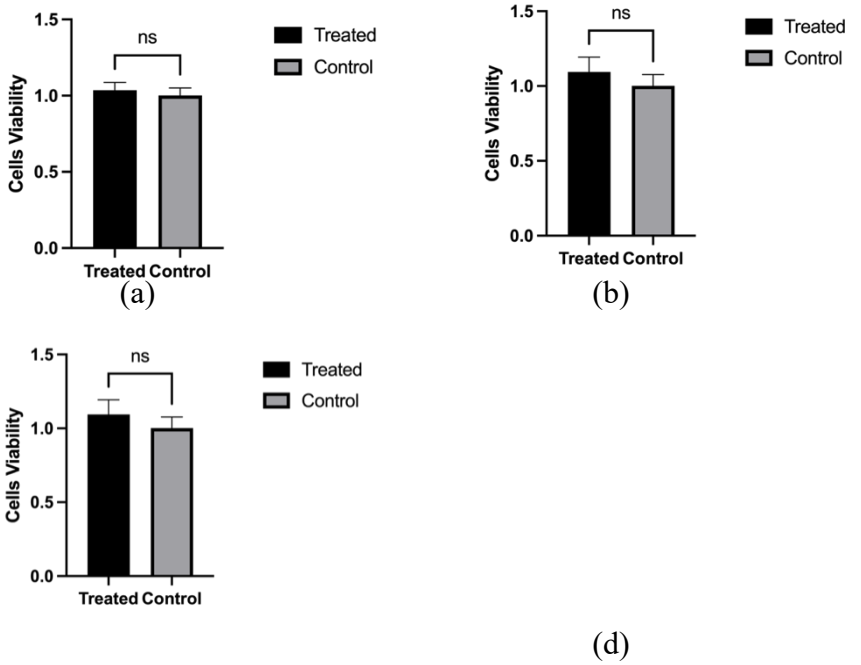


Figure 9. Vielight HeLa exposure: (a) 15 minutes exposure; (b) 33 minutes exposure; (c) 60 minutes exposure; (d) 30 minutes exposure with 15 minutes of intermission

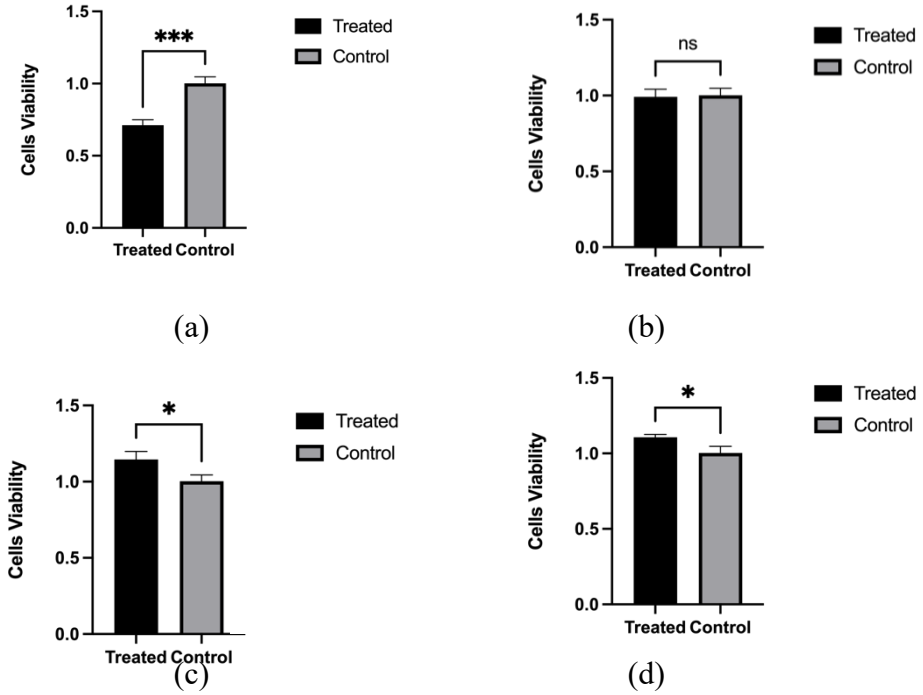


Figure 10. Bioptron MCF7 exposure: (a) 10 minutes exposure; (b) 15 minutes exposure; (c) 20 minutes exposure; (d) 20 minutes exposure with 10 minutes of intermission

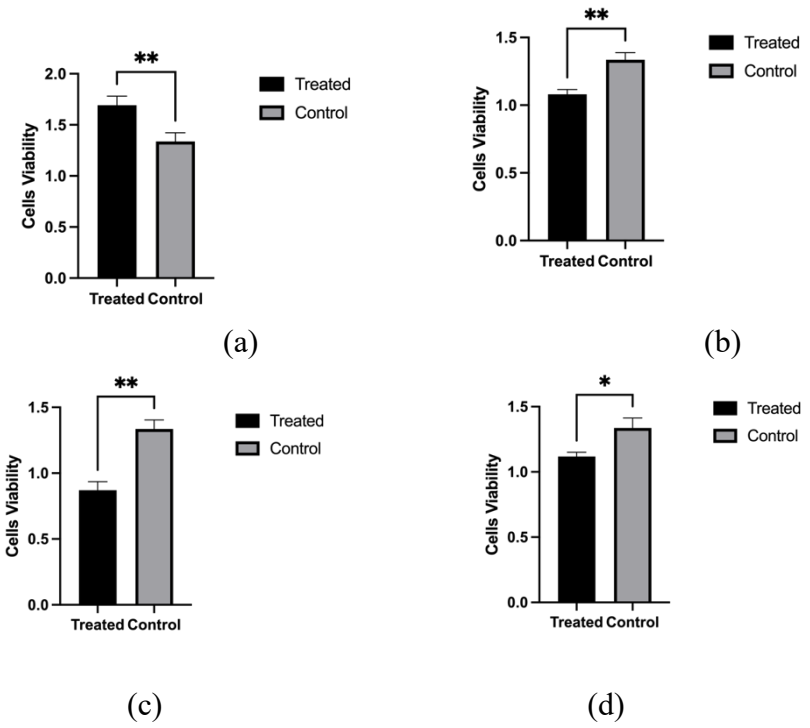


Figure 11. Vielight MCF7 exposure: (a) 15 minutes exposure; (b) 33 minutes exposure; (c) 60 minutes exposure; (d) 30 minutes exposure with 15 minutes of intermission

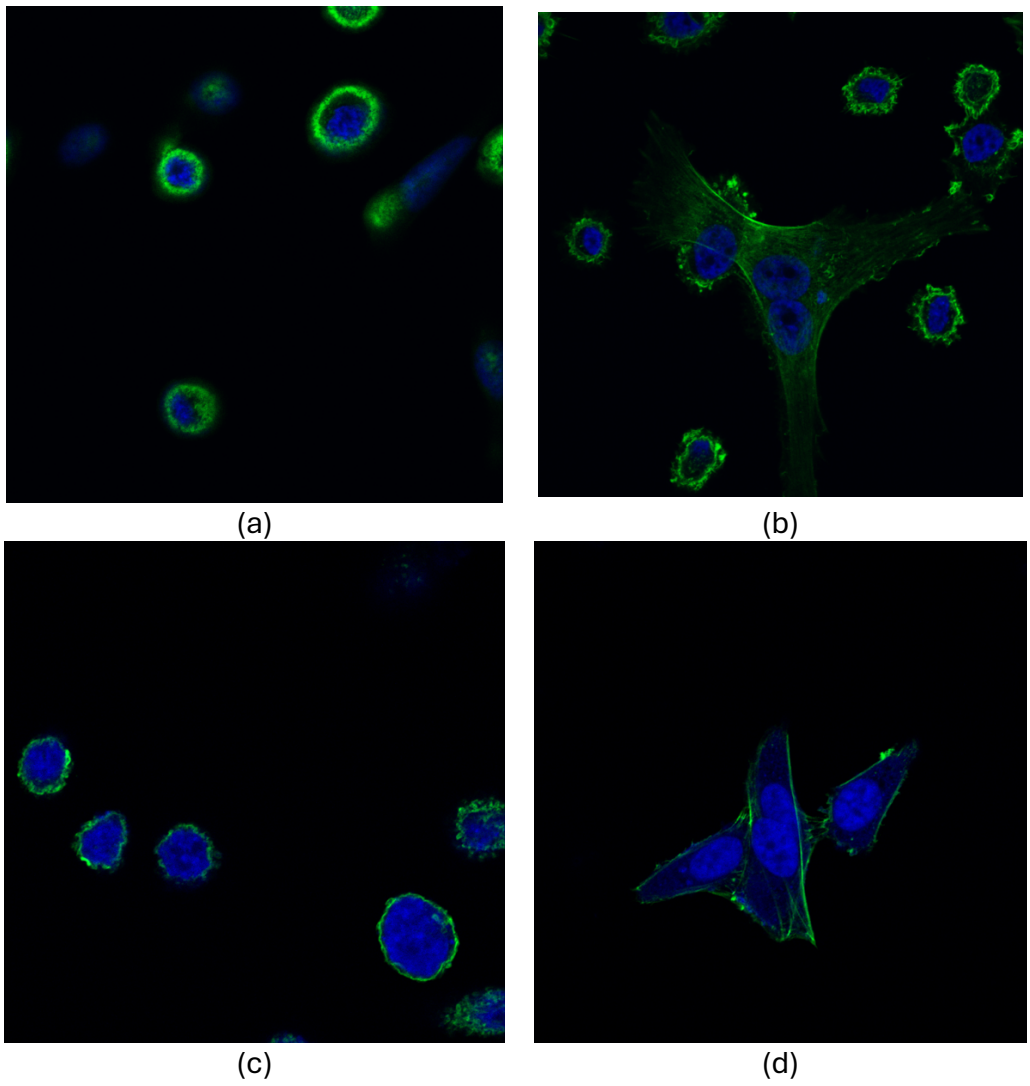


Figure 12. PC3 Actin staining. (a) Bioptron, 10-minutes exposed, treated; (b) Bioptron, 10-minutes exposed, control; (c) Vielight, 15-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 15-minutes exposed at a distance of 125 mm, control.

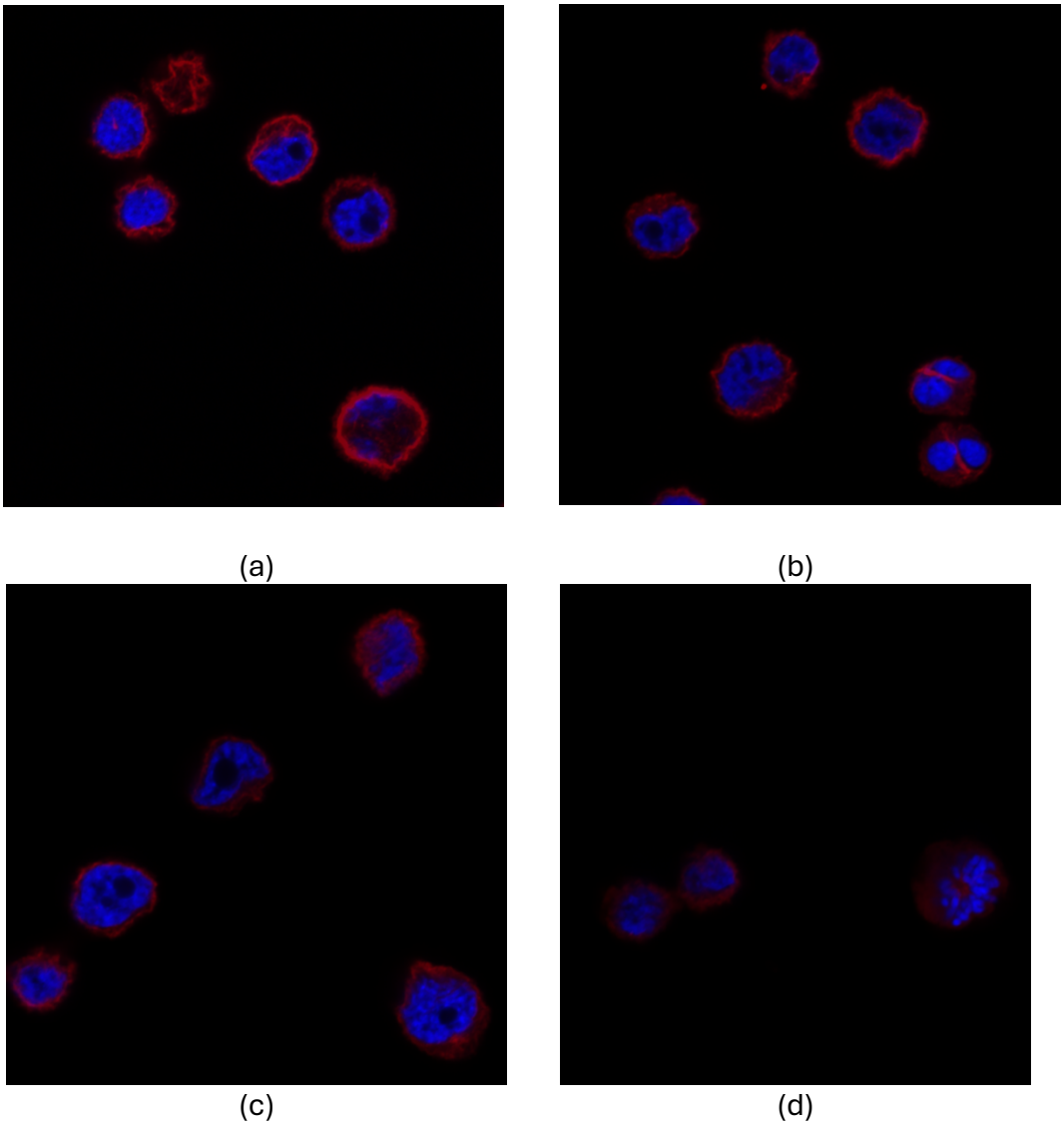


Figure 13. PC3 Tubulin staining. (a) Bioptron, 10-minutes exposed, treated; (b) Bioptron, 10-minutes exposed, control; (c) Vielight, 15-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 15-minutes exposed at a distance of 125 mm, control.

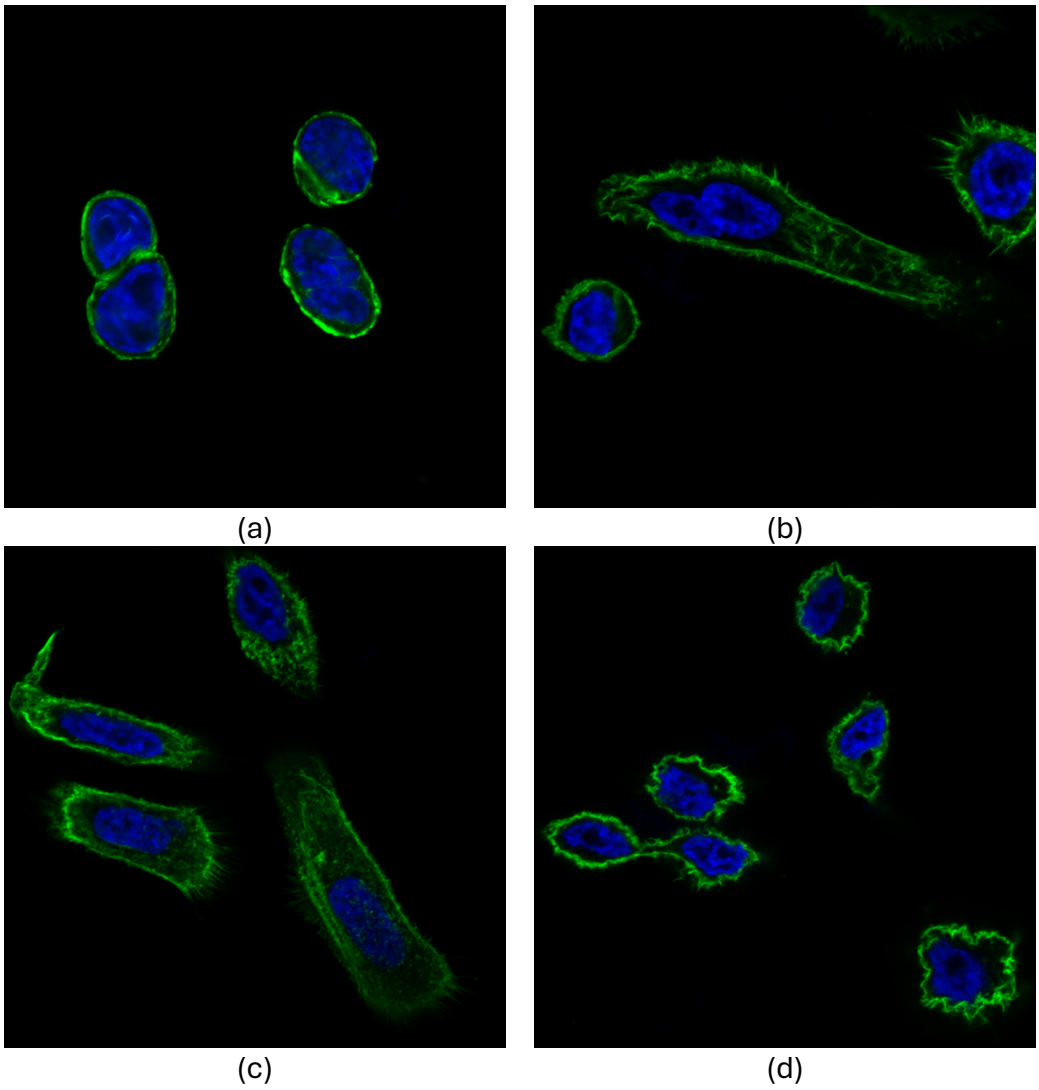


Figure 14. PC3 Actin staining. (a) Bioptron, 20-minutes exposed, treated; (b) Bioptron, 20-minutes exposed, control; (c) Vielight, 20-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 20-minutes exposed at a distance of 125 mm, control.

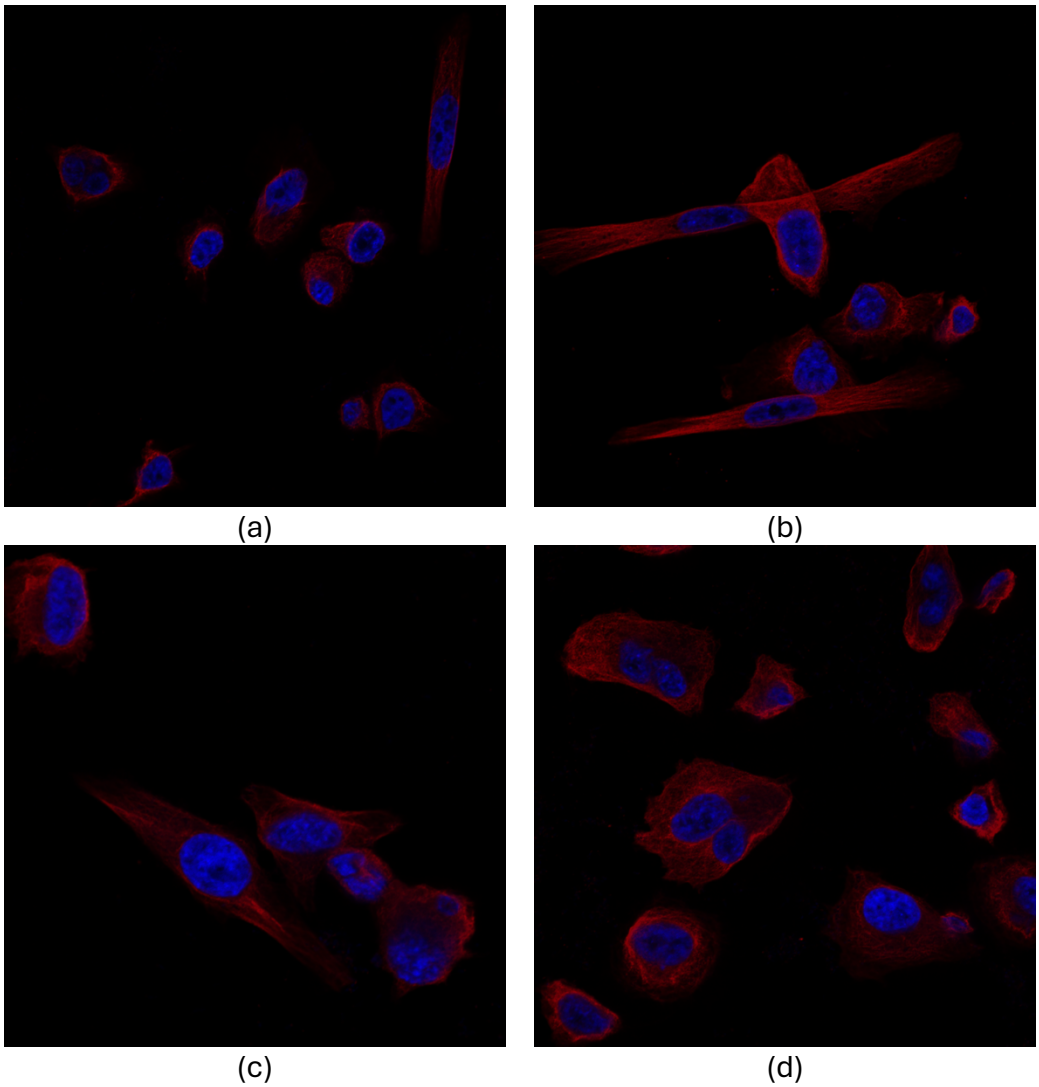


Figure 15. PC3 Tubulin staining. (a) Bioptron, 20-minutes exposed, treated; (b) Bioptron, 20-minutes exposed, control; (c) Vielight, 20-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 20-minutes exposed at a distance of 125 mm, control.

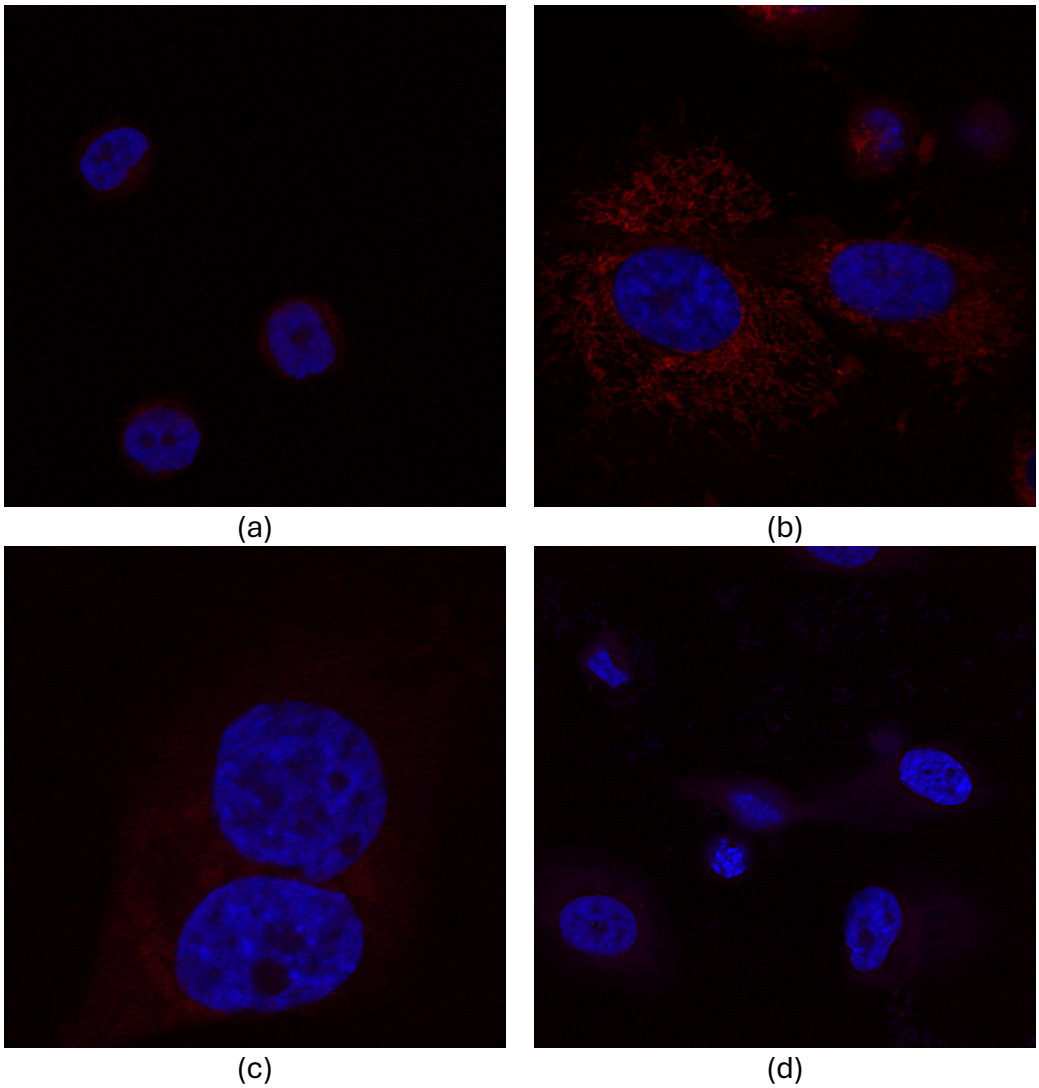


Figure 16. PC3 Mitochondria staining. (a) Bioptron, 10-minutes exposed, treated; (b) Bioptron, 10-minutes exposed, control; (c) Vielight, 15-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 15-minutes exposed at a distance of 125 mm, control.

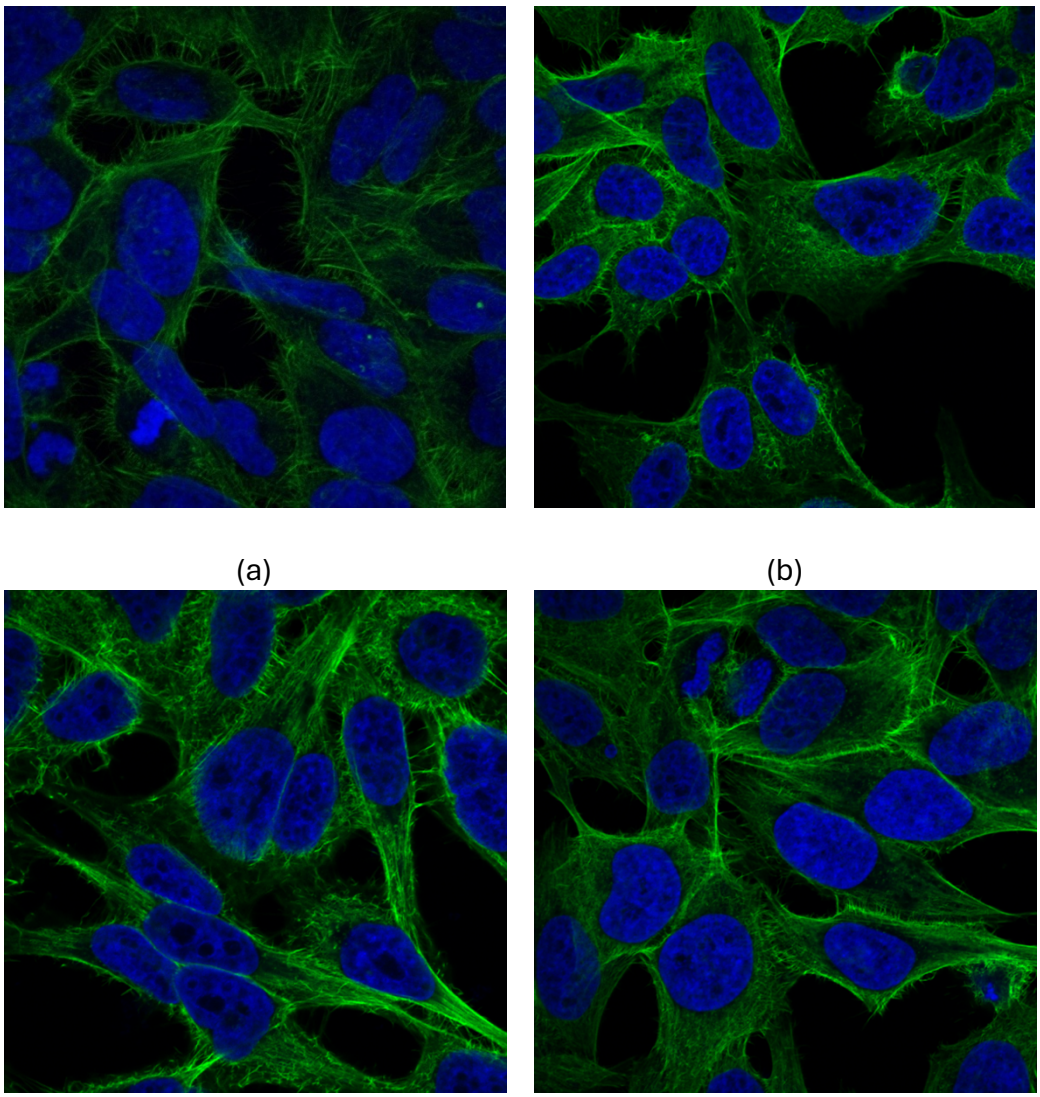


Figure 17. HeLa Actin staining. (a) Bioptron, 10-minutes exposed, treated; (b) Bioptron, 10-minutes exposed, control; (c) Vielight, 15-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 15-minutes exposed at a distance of 125 mm, control.

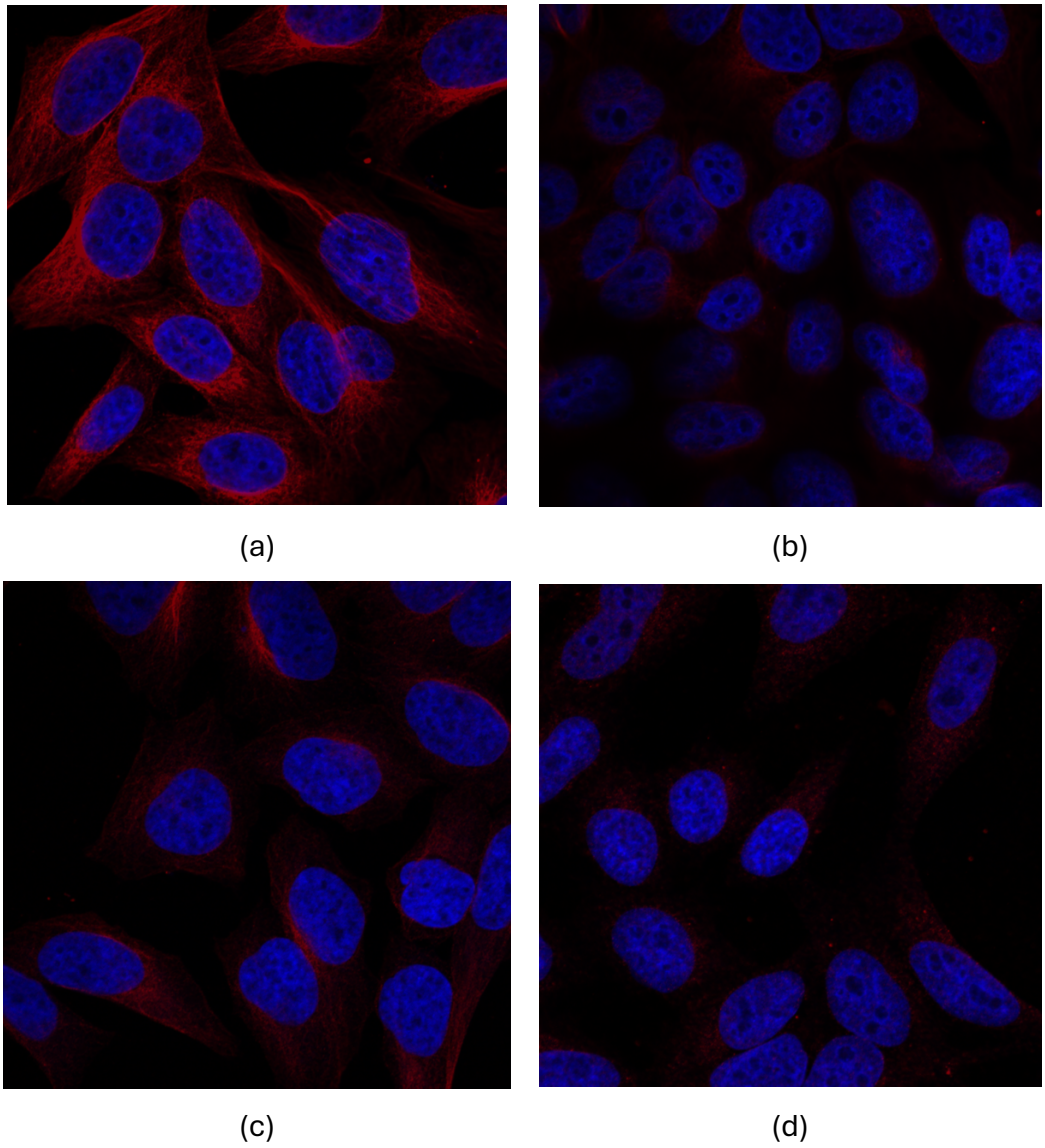


Figure 18. HeLa Tubulin staining. (a) Bioptron, 10-minutes exposed, treated; (b) Bioptron, 10-minutes exposed, control; (c) Vielight, 15-minutes exposed at a distance of 125 mm, treated; (d) Vielight, 15-minutes exposed at a distance of 125 mm, control.

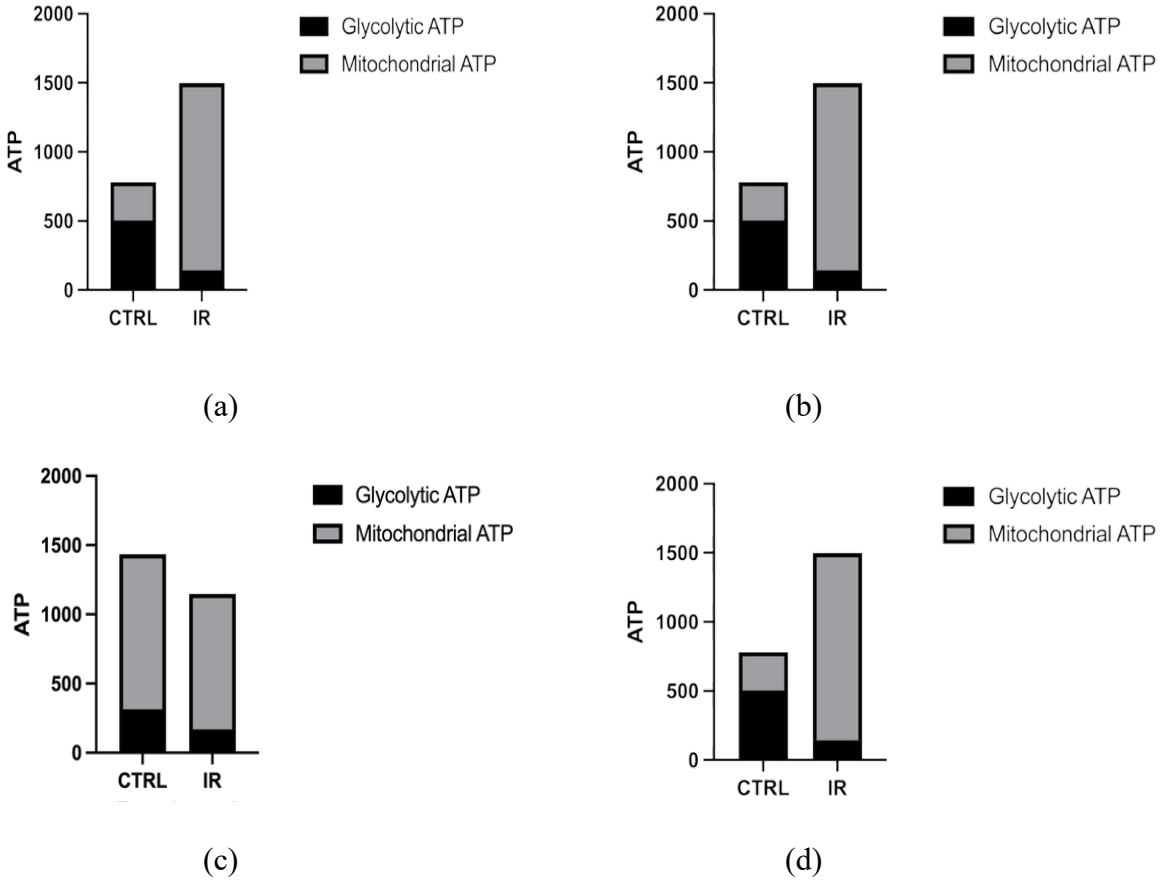
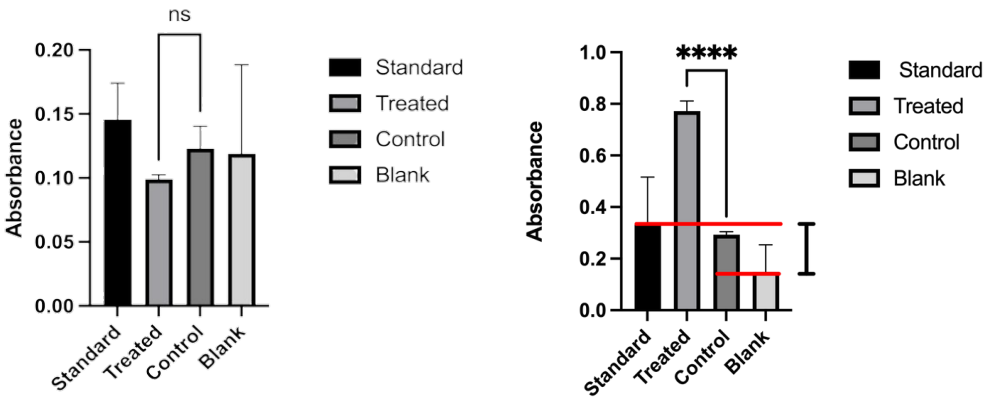


Figure 19. PC3 ATP assay: (a) Vielight, 15-minutes exposed at a distance of 125 mm; (b) Bioptron, 10-minutes exposed, experiment 1; (c) Bioptron, 10-minutes exposed, experiment 2; (d) Bioptron, 10-minutes exposed, experiment 3



(a) (b)

Figure 20. PC3 Lactate assay: (a) Vielight, 15-minutes exposed at a distance of 125 mm; (b) Bioptron, 10-minutes exposed.

6.4 DISCUSSION

The possible therapeutic benefits of photobiomodulation (PBM) therapy on cellular mechanisms have sparked an enduring debate regarding its effectiveness in cancer treatment(Hamblin, 2018b; Tam et al., 2020). This debate is fueled by inconsistent findings across a plethora of in vitro and in vivo studies. Research indicates that the therapeutic outcomes of PBM are intricately linked to several pivotal factors, such as the wavelength of the applied light, dosage, target cell line, and specific exposure conditions(Bensadoun et al., 2020b; Robijns et al., 2021b, 2022b; Zein et al., 2018b). The absence of consensus emphasizes the complexity of PBM's action mechanisms and their interaction with the diverse nature of cancer cells. While some studies report promising anti-tumor effects, others have observed negligible impacts or even potential tumor-promoting effects under certain circumstances(Ando et al., 2011; Baxter et al., 2017b; Robijns et al., 2021a). This dichotomy underscores the necessity for more systematic investigations to decode the complex interplay between PBM and the molecular landscapes of different cancer types(Jo et al., 2023; Kara et al., 2018). To elucidate the observed variations in cellular viability and explore the influence of cell line-specific characteristics and irradiation parameters, our study adopted a structured approach. We assessed the viability responses of the PC3 cell line under varying irradiation conditions, including different exposure durations and wavelengths. Specifically, PBM was applied to cells using the standard procedure within the Infra-Red (IR) window (810 nm) with the NeuroPro Device Module A (Vielight, Toronto, Canada) and with hyperpolarized light using the Bioptron Pro 1 device (Zepter, Zurich, Switzerland) which has a spectrum range of 350-3400 nm and a polarization degree exceeding 95% (590-1550 nm).

The rationale for this comparative study originates from the literature: PBM's potential beneficial effects on cells have been widely discussed, and recent findings highlight the promising aspects of using hyperpolarized light, similar to PBM in its effects.

In this particular study, the experimental setup ensured a consistent beam spot distance of 125 mm across all irradiation conditions. To systematically examine the effects of varied irradiation parameters, the experiments were categorized into four groups with different exposure times, detailed in the Materials and Methods section

(10, 15, 20, and 20 minutes with a 10-minute break in between). A power density of 60 mW/cm^2 was chosen for NeuroPro-exposed samples, in line with previous studies (references), facilitating comparisons with the Bioptron device's fixed power output of 40 mW/cm^2. The results indicated that hyperpolarized light was effective for shorter exposure intervals, while NeuroPro-irradiated cells showed significant reduction in cell viability only after 20 minutes of exposure. This suggests that prolonged exposure increases cell death. Interestingly, cell proliferation was notable after 15 minutes of direct exposure, followed by a significant viability drop when extended to 20 minutes. This trend was also observed with the Bioptron device, where both the initial and final group showed significant viability reduction.

Additionally, the role of the medium was investigated to determine its influence on the results regarding the beneficial effects of these light therapies. As expected, the medium exhibited a direct involvement when exposed to the NIR spectrum, potentially limiting the therapy's efficacy due to bound molecules in aqueous solutions absorbing the radiation. Confirming this, the viability of the exposed medium in the first group (10-minute exposure) was significantly higher than direct cell irradiation; with 15-minute and 20-minute exposures, a time-dependent viability decrease was noted. This suggests a screening effect of the medium over approximately 10 minutes when exposed to NIR, prompting the need for longer exposure to avoid this effect and enhance therapeutic efficiency (Serhan et al., 2019; Szymborska-Małek et al., 2018).

This screening phenomenon was absent with hyperpolarized light, explaining the unchanged exposure intervals in experiments. To further validate the window optimization for both Bioptron and Vielight devices, the energy fluence (dose) was set at 2.4 J/cm^2 for testing two distinct cell lines, HeLa and MCF7. Notably, the decrease in viability with longer exposure was significant in MCF7 cells but not in HeLa cells under NeuroPro irradiation, consistent with literature showing HeLa cells' response to NIR only at higher doses, around 5 J/cm^2 (H. B. Kim et al., 2021). These observations underscore the importance of cell line-specific characteristics in cellular response to light irradiation. The viability trend in Bioptron-irradiated samples was confirmed for HeLa cells and the first group (10 minutes) of MCF7 cells. However, for the 20-minute group with a 10-minute break, the trend was opposite, supporting the hypothesis of dependence on specific cell line features.

It is worth noting that for both HeLa and MCF7 cells, 15-minute exposure led to an increase in dead cells, though not significantly, suggesting some flexibility in the exposure window up to 20 minutes, beyond which significant proliferation was observed.

Tracking the effect of a 2.4 J/cm^2 dose, we investigated potential structural changes post-irradiation in PC3 and HeLa cell lines, noting similar trends for 10-minute Bioptron and 15-minute NeuroPro irradiations. Confirming cell-line dependency, both actin and tubulin staining in PC3 and HeLa cells showed different behaviors.

Specifically, PC3 cells displayed cytoplasmic shrinkage toward the nucleus favored by actin filaments after Bioptron and Vielight irradiation, while tubulin filaments showed no significant difference between treated and control samples considering 10 minutes exposure, contrary to what was expected considering previous experiments done with the same Vielight device on pure tubulin protein(Staelens et al., 2022). Instead, when considering the 20-minutes exposure, the Bioptron irradiated samples show no significant change in the cytoplasm (actin staining) but a possible disassembly in microtubules; the 20-minutes VieLight irradiated samples show a favored spreading of cells when actin-stained but no significant change in microtubules dynamics. This makes it challenging to determine light therapy's impact on both actin and tubulin dynamics. In contrast, HeLa cells showed no significant cytoplasmic changes but indicated a tendency for microtubule assembly and reorganization under experimental conditions.

In alignment with this, ATP levels were measured for Bioptron and NeuroPro irradiation samples after 10 and 15 minutes of exposure. NIR irradiation resulted in decreased glycolytic ATP and lactate levels, suggesting a modulation of cellular metabolism, potentially reversing the Warburg effect and promoting oxidative metabolism. The Warburg effect, identified by Otto Warburg in the 1920s, describes the preference for glycolysis over oxidative phosphorylation even when oxygen is available. Mitochondria appeared activated, producing about five times more ATP than glycolysis(Liberti & Locasale, 2016b; Otto, 2016b; Vander Heiden et al., 2009b; Ward & Thompson, 2012b). Hyperpolarized light treatment yielded three outcomes: an initial switch to glycolytic activity with evident mitochondrial damage, increased mitochondrial activity with glycolytic ATP production, and a decrease in glycolytic ATP followed by a tenfold increase in mitochondrial ATP, similar to NeuroPro samples.

Moreover, to investigate more the role of mitochondria, they have been stained after the 10 minutes exposure to have confirmation of what deducted from ATP analysis. Indeed, for the Vielight irradiated samples, mitochondria seem to be activated even if the signal does not appear as strong as expected. For the Bioptron case, the MitoTracker was used just once, immediately after having done the first ATP analysis: the result was confirmed, showing a much dimmer signal in the treated samples. However, it is not possible to speculate on this result due to the changing in mitochondrial ATP deducted in the experiments done with Oligomycin (see Results section).

This indicates a correlation between cancer cell aggressiveness and growth in culture over time, explaining Bioptron irradiated samples' contrasting results and a potential influence of hyperpolarized light. There might be a synergistic upregulation of glycolytic and mitochondrial pathways, reflecting metabolic adaptation to meet increased energy demands for cellular repair and recovery.

To understand these outcomes better, further studies targeting mitochondria and genetic investigations are needed to improve PBM and hyperpolarized light application characterization in cancer cells and develop standardized protocols.

CHAPTER 7

CONCLUSION AND FUTURE WORK

The findings of this comprehensive study shed light on the intricate interplay between photobiomodulation (PBM) therapy, hyperpolarized light therapy, and cellular responses in the context of cancer. The research highlights the importance of carefully optimizing irradiation parameters and considering cell line-specific characteristics when employing these therapeutic modalities. The observed dichotomy in viability patterns across different exposure durations, wavelengths, and cell lines underscores the complexity of their mechanisms of action and their interaction with the heterogeneous nature of cancer cells. The study revealed that shorter exposure intervals with hyperpolarized light (Bioptron) appeared more effective in reducing cell viability, while longer durations with near-infrared (NeuroPro) irradiation yielded significant anti-proliferative effects. The screening effect of the culture medium on NIR irradiation and the lack of such an effect with hyperpolarized light further highlight the nuances in cellular responses. Moreover, the study provides evidence that the composition of the medium, particularly the percentage of FBS, can affect cell proliferation in vitro cultures after exposure to laser therapy. It is crucial to deeply explore how the medium affects cell response during and after irradiation to better understand the underlying mechanisms. The observed structural changes, including cytoskeletal reorganization and alterations in microtubule dynamics, suggest that both PBM and hyperpolarized light may modulate cellular processes beyond viability. Furthermore, the modulation of cellular metabolism, with shifts towards oxidative phosphorylation and potential reversal of the Warburg effect, indicates their potential to reprogram cancer cell metabolism. Future research should systematically investigate the interplay between irradiation parameters (wavelength, dose, exposure time) and cell line-specific characteristics, including genetic profiles and metabolic phenotypes. This knowledge can lead to the development of tailored irradiation strategies based on the unique characteristics of tailored irradiation strategies based on the unique characteristics of different cell types or disease contexts. Exploring potential synergies with other therapies, elucidating molecular pathways modulated by these two different light therapies (mitochondrial function, cytoskeletal dynamics, metabolic reprogramming), evaluating effects on cancer stem cells and tumor

microenvironment, conducting preclinical in vivo studies, and optimizing light delivery systems are crucial for enhancing PBM's specificity and efficacy across different cancer types. Additionally, as a future work, tracking oxygen consumption could provide valuable insights into the metabolic changes induced by PBM and hyperpolarized light therapies. By monitoring oxygen consumption rates, researchers can gain a better understanding of the alterations in cellular respiration and energy production pathways, which may contribute to the observed effects on cell viability and proliferation. Incorporating oxygen consumption measurements into future studies could shed light on the bioenergetic shifts and mitochondrial function modulated by these light-based therapies. Addressing these areas will deepen our understanding of PBM's mechanisms and facilitate the development of more effective and personalized cancer treatment strategies. The findings of this study pave the way for further exploration and optimization of photobiomodulation and hyperpolarized light therapies in the fight against cancer.

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