



The Effect of Low Intensity Laser Irradiation on Breast Cancer Cells and Breast Cancer Stem Cells

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Abstract

The mechanism by which tumor proliferation, invasiveness and recurrence are sustained in malignant cancer has not been fully elucidated. Taking into account the findings of previous researches, one have strong reasons to believe that it might result from a small population of cells referred to as Cancer Stem Cells (CSCs). This study aimed to investigate and compare the photobiomodulative effect of Low Intensity Laser Irradiation (LILI) treatment on Breast Cancer Stem Cells (BCSCs) and Breast Cancer Cells (BCCs). BCSCs were isolated from the MCF-7 cell line based on their CD44⁺ phenotype using magnetic-activated cell sorting. CD44 antigen was detected in BCSCs using fluorescent microscopy. Cellular response to the treatment was evaluated based on their viability, proliferation and toxicity. Positive detection of CD44 confirmed the stemness of isolated BCSCs. Treated BCCs and BCSCs showed an increase in their proliferation and viability after being exposed to 5-40 J/cm² using wavelengths of 636, 825 and 1060 nm. Membrane integrity assay revealed a decrease in cytotoxicity in both BCCs and BCSCs after treatment with low fluences of LILI. This study revealed that LILI did not have a bioinhibitory effect on both cell types.

Keywords: Breast cancer cell; Breast cancer stem cells; Cancer stem cells; Low intensity laser irradiation

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Introduction

Cancer is a complex medical condition in which genetically or epigenetically altered cells, which have overcome crucial cellular processes, such as Deoxyribonucleic Acid (DNA) repair and apoptosis, undergo uncontrolled proliferation culminating in the formation of masses of tissue or lumps. Breast cancer is an increasing threat to the health and well-being of women worldwide. Although the rate of breast cancer incidence varies among different groups of people, it is classified as the second most frequently diagnosed cancer overall, with approximately 1.7 million new cases diagnosed in 2012. Globally, it represents one in four of all cancer in women worldwide [1].

Over the years, countless research projects have intended to find more responsive cancer treatments with improved outcome. Despite substantial therapeutic improvements, post-therapeutic loco regional and systemic recurrence remains a major issue encountered and new treatment strategies are urgently needed. As cancer recurrence would originate from residual therapy-resistant cells with the capacity to generate the original cancer phenotype, the lack of success of conventional therapeutic approaches to definitely cure the majority of solid cancers including breast cancer has re-ignited attention to the controversial Cancer Stem Cell (CSCs) theory of tumor initiation, therapeutic resistance and recurrence. The discovery of healthy stem cells and understanding of their properties has revived interest in the role that might play their cancerous counterparts in the repopulation of tumor sites after treatment. The ever-increasing advances in molecular biology have launched disruptive novel techniques such as molecular profiling (gene expression profiling) which have revolutionized our understanding of the heterogenic nature of breast cancer. As a result, CSCs have been identified as a minority of undifferentiated cell subtypes within a tumor mass, with stem-like properties and the ability of tumor regeneration. Healthy stem cell behaviour, also referred to as their stemness, is sustained by a group of signaling pathways, notably Notch, Hedgehog and Wnt, which turn out to play a similar role in CSCs behavior including self-renewal, differentiation, and fate determination [2]. Minor alterations in their regulation could induce drastic consequences and lead to malignant tumor formation [2,3]. It has been confirmed that these signaling pathways involved in the regulation of stemness of healthy stem cells happen to be altered in their cancerous counterpart [4]. In this regard, great understanding of their functioning will allow for a deeper understanding of the role that they could play in CSCs tumorigenic phenotype which could lead to the development of better therapeutic approaches [5].

With undeniable advances in science, it is now possible to identify and isolate CSCs from an entire tumor mass cell population. With methods such as magnetic-activated cell sorting (MACS), breast CSCs can be identified as a minority of cells expressing the hyaluronic receptor CD44 and subsequently be isolated. CD44 is a commonly expressed, multifunctional, cell-surface trans-membrane antigen and the main receptor of Hyaluronic Acid (HA) which is one of the principal components of the Extracellular Matrix (ECM). This trans-membrane antigen has always been associated with the cell to cell interaction,

cell adhesion, migration, stem cell homing and tumor metastasis [6]. It has also been identified as a cancer stem cell marker for several cancers including breast cancer [6]. The CD44⁺/CD24^{-low} phenotype supposedly differentiates tumorigenic (tumor initiating) from non-tumorigenic breast cancer cells within a malignant tumor mass [6,7]. Therefore, the possible involvement of this transmembrane glycoprotein in the carcinogenesis of various solid cancers has been intensively studied over the past years. Most published data have linked CD44 expression with CSCs phenotype and Epithelial-to-Mesenchymal Transition (EMT) [8]. Epithelial-to-mesenchymal transition is the basis of CSCs plasticity and has been observed in breast CSCs [9]. Studies have closely linked EMT to the metastasis and drug resistance observed in CSCs. In addition, EMT has been associated with the shifting of cancer cells from the differentiated to the undifferentiated state [9,10]. This supports other data that have associated high expression of CD44 in breast cancer with tumor initiation, growth, invasion, metastasis, therapeutic resistance and recurrence [11]. Hence, abnormal expression of CD44 has been highly linked to the increased efficiency of distant metastasis and the poor survival rate observed in patients with malignant breast cancer [6,12]. Interestingly, it has been revealed that blockage of CD44 expression seems to significantly diminish malignant phenotype in CSCs, slow their progression and reverse their resistance to conventional therapeutic approaches [8].

LILI, which involves the application of red or near infrared lasers irradiating between 600-1100 nm, was introduced as an alternative, non-invasive, therapeutic approach for various medical conditions such as osteoarthritis, rheumatoid arthritis, post-mastectomy lymphedema and chronic diabetic wound. The effectiveness of this phototherapy has been proven by the positive outcomes of findings and is no longer doubted [13,14]. Despite these positive findings, LILI remains a controversial treatment modality for some. The photobiomodulation effect of LILI relies on the sensitivity of specific cellular components (chromophores) to light photons applied. Studies have revealed that following photon absorption by photoacceptors of the mitochondrial respiratory chain, cellular activity could affect in one of the following ways: cell growth stimulation, production of anti-inflammatory response, enhanced cell regeneration, stimulation of long-term production of intracellular or extracellular reactive oxygen species (ROS overload), to name a few [15,16]. These photobiological responses to LILI don't induce any significant elevation in temperature and highly depends on factors such as the intensity and wavelength of light applied, and the type of cell. Hence, LILI can be defined as a non-thermal photo-biomodulation technique using optical waves that usually correspond to the visible red or near-infrared (NIR) light and low fluencies to induce photobiological process at the cellular level [17,18].

Studies have revealed the biphasic dose effect of LILI on different types of non-cancerous and cancerous cells [18,19]. This refers to the ability of LILI to either induce cell proliferation or death depending on the light density applied. As CSCs are responsible for the therapeutic resistance that occurs when dealing with malignant cancers, their response to LILI might be different from other cells. Higher doses of light might be required to induce apoptosis in CSCs as compare to other cells [16]. The main objective of this investigative research study is to explore the possible therapeutic virtue of laser in the suppression of malignant breast cancer growth. Since cancer stem cells are thought to be directly involved in the therapeutic resistance observed in malignant cancer, they were our main focus.

Materials and Methods

Cell culture

Isolated CSCs from commercially available adenocarcinoma MCF7 (ATCC: HTB 22) breast cancer cells were cultured in tissue flask (adherent culture) using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM F-12 Ham) as the base medium. To form a complete growth media DMEM F-12 Ham was enriched with 1% Penicillin-streptomycin and 1% Amphotericin B. No fetal bovine serum was added to avoid differentiation of BCSCs. The remaining differentiated breast cancer cells were cultured in tissue flask using Dulbecco's Modified Eagle Medium-high glucose (DMEM) as the base medium which was enriched with 10 % fetal bovine serum, 1% Penicillin-streptomycin and 1% Amphotericin B.

Magnetic-activated cell sorting

CSCs were isolated from commercial MCF-7 cell line based on their expression of CD44 antigenic surface marker using CD44 microbeads human. Prior the magnetic-activated cell sorting, a single cell suspension was prepared followed by trypan blue assay to quantitatively estimate the total number of viable cells. Working solution of the microbeads was prepared by performing a 1:10 dilution into Phosphate Bovine Saline (PBS). 5 μ L of pre-diluted Basic microBeads was added per 10^7 total cells followed by 15 minutes incubation at 4-8 °C afterward cells were washed by adding 2 mL of PBS per 10^7 total cells and resuspended (up to 10^8 cells in 500 μ L of PBS). For magnetic separation, cell suspension was applied onto the LS column. CD44 labeled cells (CD44+ cells or BCSCs) that stayed in the LS column and unlabeled ones (CD44- cells or BCCs) that flowed through were separately collected for further experiments. Note that for greater purity, magnetic separation was repeated twice using previously collected CD44+ cells.

CD44 cell surface marker detection

Fluorescent microscopy was used to detect specific antigen CD44 in isolated BCSCs and negative control BCCs after they have both been fluorescently labelled. Cells were first cultured on heat-sterilized glass coverslips placed in 3.5 cm petri dishes, at a seeding concentration of 2×10^5 cells in complete media. After a 24 hours incubation at 37°C in 5% CO₂ and 95% air atmosphere, cells were washed twice with 1% PBS and then incubated with 4% paraformaldehyde at room temperature for 10 minutes for fixation. After the fixation step, cells were washed twice with ice cold PBS and then incubated for 10 minutes with Triton X-100 (0.5 % Triton X-100 made in 1% PBS solution) as a permeabilization step (note that it is important to permeabilize to increase the chance of the antibody binding). The plates were then placed on a wet paper tower in a container to avoid drying out while staining slides, thereafter cells were rinsed with cold PBS and incubated with 2% BSA in PBS (blocking buffer) for 1 hour at room temperature as a blocking step. After that, cells were rinsed twice with ice-cold PBS and incubated for 1 hour with a pre-diluted antibody (0.8 μ L of CD44-FITC/400 μ L of 1% BSA in PBS) on the wet paper tower to minimize evaporation of the antibody. The last step before viewing cell using fluorescent microscope was the 4'-6-diamidino-2-phenylindole (DAPI) staining. Cells were washed three times with PBS before being stained with DAPI and left for 30 minutes incubation at room temperature followed by mounting on a glass slide. Slides were viewed using a fluorescent microscope (Carl Zeiss, Axio Observer Z1).

LILI treatment

Twenty four hours before irradiation, cells were seeded in 3.5 cm diameter petri dishes at a concentration of 2×10^5 cells in 3mL complete media. Prior to laser treatment, culture media was removed from each plate and cells were rinsed with Hanks' Balanced Salt Solution (HBSS) before adding 2 mL of 1% PBS. Culture media was replaced by 1% PBS for irradiation, in order to prevent possible interference of the phenol red, it contains with laser light. Cells were irradiated from the top with the culture dish lid off. To avoid any interference of external light with the laser effect, irradiation was performed in a dark room. Cell cultures were divided into two main groups, namely BCSCs and BCCs each divided into 5 study groups. Group 1 was an untreated control, group 2 was treated with 5 J/cm^2 , group 3 received 10 J/cm^2 , group 4 received irradiation at 20 J/cm^2 , and finally, group 5 received 40 J/cm^2 . All experimental groups were treated at respective wavelengths of 636, 825 or 1060 nm. Post-irradiation incubation times were 24 hours. Table 1 indicates different parameters of lasers used for this research study.

Parameters			
Laser type	Semiconductor (Diode)		
Wavelength (nm)	636	825	1060
Wave emission	Continuous	Continuous	Continuous
Power output (mW)	± 75	± 94	± 75
Intensity (mW/cm^2)	8.26	10.35	8.26
Fluence (J/cm^2) and corresponding exposure time	5: 10 min 48 sec 10: 20 min 9 sec 20: 40 min 21 sec 40: 1 h 20 min 30 sec	5: 8 min 18 sec 10: 16 min 4 sec 20: 32 min 10 sec 40: 1 h 4 min 18 sec	5: 10 min 48 sec 10: 20 min 9 sec 20: 40 min 21 sec 40: 1 h 20 min 30 sec

Table 1: Laser and irradiation parameters using 636, 825 and 1060 nm diode lasers.

Prior to LILI treatment, the exposure time which corresponds to the amount of energy that is given to the cells depending on the fluences/doses required ($5, 10, 20$ or 40 J/cm^2) were determined based on the laser power output, from which the intensity was calculated. The following formula was used to determine exposure time; Time (s) = Dose (J/cm^2) / Intensity (W/cm^2). Intensity (W/cm^2) = Power output (mW) / $[(\pi \times \text{Diameter}^2) / 4]$. Readings of different lasers (636, 825 and 1060 nm diode lasers) power outputs were taken at cell (bench) level using a power meter. During LILI treatment, culture dishes containing cells were placed directly into the laser light beam area which was previously set at 3.3 cm diameter for equal distribution of the light energy into the cells inside the 3.3 cm diameter petri dishes.

Viability

Trypan Blue dye exclusion test was conducted to quantitatively estimate the total number of viable and non-viable cells in terms of percentage. $10 \mu\text{L}$ of single cell suspension was added to an equivalent volume of trypan blue dye and mixed. During this assay, damaged membranes absorb the negatively charged chromophore dye in Trypan blue and appear blue whereas viable membranes do not absorb the dye, therefore remain translucent. $10 \mu\text{L}$ of the cell-dye mixture was transferred into both sides of a chamber slide which was subsequently inserted into an automated cell counter (Countess® Automated Cell Counter) that contains advanced autofocus and counting algorithms that have the capacity to accurately count cells and allow to instantly visualise the percentage of viable and non-viable cells.

Proliferation

The presence of metabolically active cells was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay. Adenosine Triphosphate (ATP) is an energy molecule involved in several metabolic processes in live cells. Overall, the principle behind this assay is to evaluate the mitochondrial activity by assessing their ATP production. The assay is based on the luciferase's requirement for ATP to generate luminescence signal. $50 \mu\text{L}$ of cell suspension was transferred into equal amount of CellTiter-Glo® reagent contained in an opaque-walled 96-well flat-bottom culture plate. The plate was first left for 2 minute on an orbital shaker to induce cell lysis followed by 10 minute incubation in the dark at room temperature. Detection and measure of luminescence signal produced by the luciferase were done by a Multilabel Counter (Perkin Elmer, VICTOR3™, 1420) in RLU.

Cytotoxicity

Cytotoxicity assay was conducted using the CytoTox 96® Non-Radioactive Cytotoxicity Assay to quantify damaged cells by measuring the Lactate Dehydrogenase (LDH) released from their cytosol. LDH is an oxidoreductase enzyme used as a biomarker for the plasma membrane damage and cellular cytotoxicity. The LDH present in the growth media converts the tetrazolium salt in the reagent into a red formazan. This chemical reaction was detected by colorimetric assay using a spectrophotometer (Perkin Elmer, VICTOR3™, 1420) at the absorbance of 490 nm. CytoTox96® nonradioactive cytotoxicity assay was used to quantitatively measure cytotoxicity which was proportional to the amount of damaged cells present in the growth media. $50 \mu\text{L}$ of cell suspension was added to equal amount of the reagent contained in a clear 96-well flat-bottom culture plate was cover with foil followed by 30 minutes incubation in the dark at room temperature.

Statistics

Accumulated results after three repeats (n=3) were statistically analysed using Sigma plot version 13. Statistical evaluation was done using student paired t-test and statistical significances were considered at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). All chemicals used were of research grade.

Results

CD44 cell surface marker detection

The stemness of isolated BCSCs was confirmed by the positive expression of CD44 cell surface marker using fluorescent microscopy. Prior to characterization, isolation of BCSCs was done by magnetic-activated cell sorting (MACS) using CD44 human microbeads as antibody. The CD44 antigens expressed in BCSCs have high affinities with these antibodies. Isolated CD44+ cells were stained with CD44-FITC antibody hence appeared as green on the fluorescent microscope. On the other hand, no green fluorescence was detected from the remaining BCCs which represented the majority of cells after isolation of BCSCs. BCCs were subsequently used as negative control during this study. CD44 cell surface marker detection results are shown in figure 1.

Post-irradiation cell proliferation

Changes in cellular proliferation 24 hours after treatment with LILI at 636, 825 and 1060 nm are demonstrated in figures 2, 3 and 4.

Twenty four hours post-irradiation at 5, 10, 20 and 40 J/cm², the production of Adenosine Triphosphate (ATP) was measured in both BCCs and BCSCs. All laser treated BCCs and BCSCs were compared to their respective untreated controls and subsequently compared to each other.

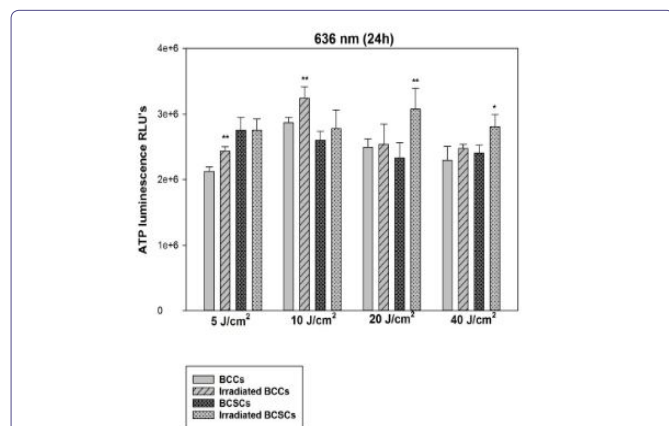
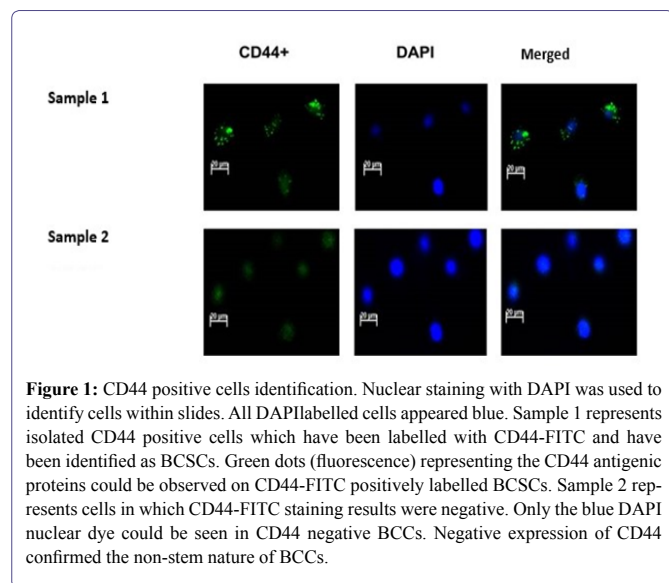


Figure 2: Changes in proliferation of BCCs and isolated BCSCs using 5, 10, 20 and 40 J/cm at 636 nm. In BCCs exposed to a 636nm diode laser statistically significant increases in ATP production were observed at 5 and 10 J/cm² with a p-value of 0.01 (**). However, in BCSCs, statistical significance was only observed at 20 J/cm² with a p value of 0.01 (**) and 40 J/cm² with a p value of 0.05 (*). In both cell types, the ATP production did not decrease at any fluence.

Post-irradiation percentage viability

Changes in the percentage of viable cells 24 hours after treatment with LILI at 636, 825 and 1060 nm are demonstrated in table 2. Twenty four hours following LILI treatment using 5, 10, 20 and 40 J/cm², the percentage of viable cells was assessed in both BCCs and BCSCs at 636, 825 and 1060 nm. LILI-treated groups were compared to their untreated control and then to each other. Statistically significant changes in the percentage of viable cells were assessed as follow; p<0.05(*), p<0.01(**) or p<0.001(***)

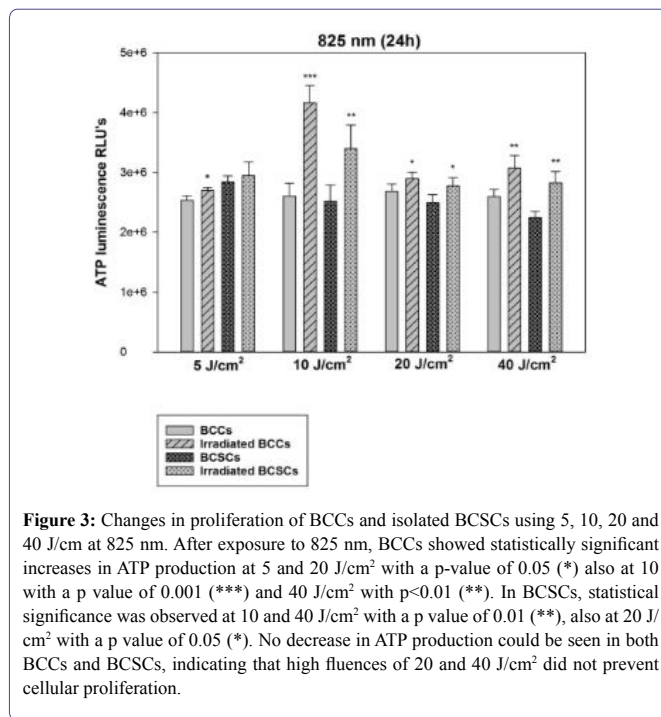


Figure 3: Changes in proliferation of BCCs and isolated BCSCs using 5, 10, 20 and 40 J/cm at 825 nm. After exposure to 825 nm, BCCs showed statistically significant increases in ATP production at 5 and 20 J/cm² with a p-value of 0.05 (*) also at 10 with a p value of 0.001 (***) and 40 J/cm² with p<0.01 (**). In BCSCs, statistical significance was observed at 10 and 40 J/cm² with a p value of 0.01 (**), also at 20 J/cm² with a p value of 0.05 (*). No decrease in ATP production could be seen in both BCCs and BCSCs, indicating that high fluences of 20 and 40 J/cm² did not prevent cellular proliferation.

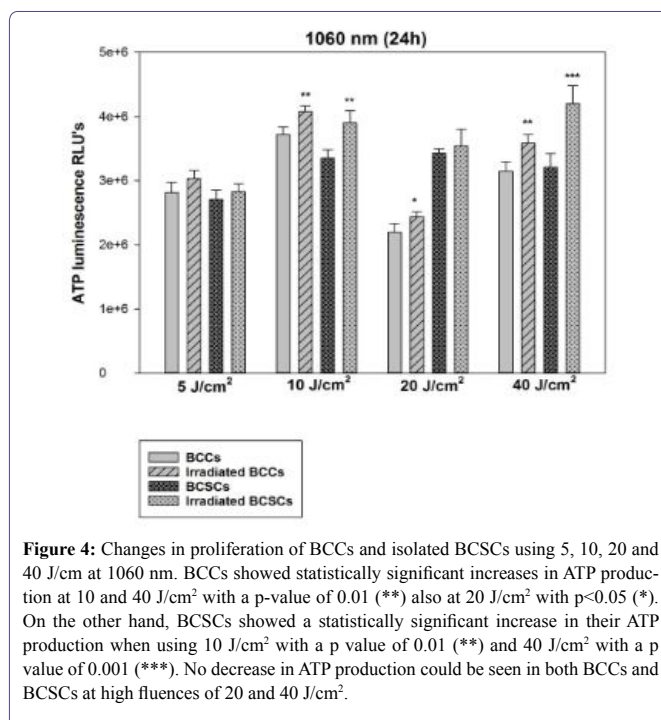


Figure 4: Changes in proliferation of BCCs and isolated BCSCs using 5, 10, 20 and 40 J/cm at 1060 nm. BCCs showed statistically significant increases in ATP production at 10 and 40 J/cm² with a p-value of 0.01 (**), also at 20 J/cm² with p<0.05 (*). On the other hand, BCSCs showed a statistically significant increase in their ATP production when using 10 J/cm² with a p value of 0.01 (**), and 40 J/cm² with a p value of 0.001 (***)). No decrease in ATP production could be seen in both BCCs and BCSCs at high fluences of 20 and 40 J/cm².

At 636 nm using all four fluences, a slight yet not significant increase in the percentage of viable BCCs and BCSCs was observed. The same thing could be seen at 825 and 1060 nm. No statistically significant decrease in the percentage of viable cells could be noticed in both cell types. Hence, one could say that LILI treatment even using high fluences of 20 and 40 J/cm² did not trigger cell death.

Post-irradiation cytotoxicity

Possible damage to the cell membrane was assessed by the level of LDH released in the culture media 24 hours after treatment with LILI using 5, 10, 20 and 40 J/cm at 636, 825 and 1060 nm. An increase in the LDH level in treated cells compared to their untreated controls would indicate a possible cell death. Cytotoxicity assay outcome after irradiation of BCCs and BCSCs are demonstrated in table 3. Statistically significant changes in the cellular level of LDH production were assessed as follow; $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)

At 636 nm, LILI treated BCCs portrayed a significant decrease in their LDH production with the highest changes with $p < 0.001$ (***) observed when using fluences of 5, 10 and 40 J/cm². Treatment with 20 J/cm² induced a drop in the LDH level with a p value of 0.05 (*). BCSCs on the other hand only had their LDH production dropped

when using 10 and 40 J/cm² with respective p values of 0.001 (***) and 0.01 (**). At 825 nm, a statistically significant decrease of LDH was observed in BCCs after treatment with 5, 10 and 40 J/cm² all with p values of 0.001 (***) . BCSCs had their LDH level lower following LILI treatment with 5, 10 and 40 J/cm² all with p values of 0.05 (*). At 1060 nm, BCCs had their lowest level of LDH when treated with 5, 10 and 20 J/cm² all with p values of 0.001 (***) . Treatment with 40 J/cm² induced a significant decrease in the level of LDH with $p < 0.05$ (*). BCSCs on the other hand showed the peak drop in the LDH production with a p value of 0.001 (***) after exposure to 20 and 40 J/cm². Treatment with 10 J/cm² induced a decrease in LDH with a p value of 0.05 (*). Note that no increase of LDH corresponding to the altered membrane integrity due to LILI treatment could be seen either in BCCs or in BCSCs irradiated samples.

		636 nm		825 nm		1060 nm	
5 J/cm ²	BCCs control	92	±1.581	96	±1.190	96	±0.408
	BCCs test	93	±1.601	95	±0.854	97	±0.408
	BCSCs control	95	±0.957	96	±0.750	94	±0.479
	BCSCs test	96	±0.323	97	±0.408	96	±0.854
10 J/cm ²	BCCs control	96	±479	96	±0.707	96	±0.479
	BCCs test	97	±408	96	±0.629	96	±0.408
	BCSCs control	96	±0.479	97	±0.479	96	±0.854
	BCSCs test	97	±707	98	±0.479	97	±0.707
20 J/cm ²	BCCs control	97	±0.866	96	±0.479	96	±0.479
	BCCs test	97	±0.629	97	±0.479	96	±0.289
	BCSCs control	97	±0.456	97	±0.408	96	±0.854
	BCSCs test	96	±0.479	98	±0.250	97	±0.750
40 J/cm ²	BCCs control	96	±0.479	97	±0.645	97	±0.645
	BCCs test	96	±0.479	95	±0.750	97	±0.629
	BCSCs control	97	±0.408	97	±0.289	96	±0.854
	BCSCs test	97	±0.408	97	±0.629	97	±0.816

Table 2: Changes in the percentage of viable cells after LILI treatment using 5, 10, 20 and 40 J/cm at 636, 825 and 1060 nm.

		636 nm		825 nm		1060 nm	
5 J/cm ²	BCCs control	0.662	±0.0041		±0.0039	0.682	±0.0329
	BCCs test	0.380	*** ±0.0067	0.396	*** ±0.0286	0.394	*** ±0.0095
	BCSCs control	0.598	±0.0337	0.573	±0.0296	0.525	±0.0147
	BCSCs test	0.521	±0.0142	0.488	* ±0.0054	0.518	±0.0025
10 J/cm ²	BCCs control	0.553	±0.0237	0.525	±0.0257	0.686	0.686
	BCCs test	0.362	*** ±0.0141	0.340	*** ±0.0143	0.357	*** ±0.0138
	BCSCs control	0.507	±0.0133	0.503	±0.0128	0.579	±0.0195
	BCSCs test	0.333	*** ±0.0125	0.451	* ±0.0068	0.470	* ±0.0116
20 J/cm ²	BCCs control	0.469	±0.0193	0.479	±0.0157	0.700	±0.0319
	BCCs test	0.412	* ±0.0117	0.449	±0.0123	0.417	*** ±0.0017
	BCSCs control	0.466	±0.0068	0.469	±0.00709	0.535	±0.0124
	BCSCs test		±0.0185	0.523	±0.0468	0.430	*** ±0.0096
40 J/cm ²	BCCs control	0.590	±0.0091	0.592	±0.0089	0.612	±0.0037
	BCCs test	0.442	*** ±0.0210	0.434	*** ±0.0130	0.527	* ±0.0177
	BCSCs control	0.518	±0.0108	0.515	±0.0119	0.654	±0.0103
	BCSCs test	0.459	** ±0.0067	0.463	* ±0.0107	0.539	*** ±0.0180

Table 3: Changes in LDH level 24 hours after LILI treatment using 5, 10, 20 and 40 J/cm² at 636 nm.

Discussion

The aim of this research study was to assess and compare the bi-phasic dose and wavelength related effects of low and high fluence LILI on both BCCs and BCSCs using light densities of 5, 10, 20 and 40 J/cm² at 636, 825 and 1060 nm. Furthermore, in this project we investigated the possible bioinhibitory effect that treatment with high fluences of 20 and 40 J/cm² could have on both BCCs and BCSCs using the same fluences that have already shown their effectiveness on previous studies on other malignancies such as lung cancer [18].

The presence of a minority of cells expressing the hyaluronic receptor CD44 CSC marker in malignant adenocarcinoma MCF-7 cell line has been confirmed in this study. The tumor initiation ability of these CD44positive cells has been confirmed in previous studies [6,11]. These highly tumorigenic cells that have previously been identified and isolated from breast cancer are thought to play a role in the poor prognosis and to be responsible for tumor malignancy and all the consequences thus arising [11].

Contrary to what is expected of an anti-cancer treatment, among other things the eradication of cancer cells, low fluence LILI treatment had a biostimulatory effect on the proliferation and viability of both BCCs and BCSCs. This finding is consistent with previous data from studies on lung CSCs [18]. Results deduced from the proliferation assay showed a statistically significant increase in the production of ATP in BCCs when applying light density of 5 and 10 J/cm² at wavelengths of 636, 5-40 J/cm² at 825 nm and 10-40 J/cm² at 1060 nm. However, in BCSCs, the proliferative effect of LILI could be seen after cells were exposed to 20 and 40 J/cm² at 636 nm, 10-40 J/cm² at 825 nm and finally 10 and 40 J/cm² at 1060 nm. Light density of 5 J/cm² did not induce any bio-stimulatory response in BCSCs. Trypan blue dye exclusion test that was conducted to determine the percentage of viable cells post treatment revealed no statistically significant changes in that matter. Results from the cytotoxicity assay that was carried to assess the membrane integrity following irradiation revealed that neither BCCs nor BCSCs had membrane damage post treatment with fluences ranging from 5 to 40 J/cm². Some of these data were similar to the ones found in a study done on lung cancer stem cells in which doses of light of 5, 10 and 20 J/cm² did not have a damaging effect on the cells membrane [18,20]. However, a statistically significant increase in the cell membrane damage was observed following exposure of lung cancer stem cells to 40 J/cm², which was not the case in the present study. Data showed a statistically significant decrease in the level of LDH production in BCCs when using fluences of 5 to 40 J/cm² at 636 and 1060 nm and 5, 10 and 40 J/cm² at 825 nm. In BCSCs, a statistically significant drop in the LDH production could be seen at 636 nm when applying fluences of 10 and 40 J/cm², at 825 nm when applying 5, 10 and 40 J/cm² and finally at 1060 nm after exposure to light densities of 10 to 40 J/cm².

Data of the present study demonstrated that even high fluences of 20 and 40 J/cm² were not sufficient to induce any bioinhibitory effect on both BCCs and BCSCs unlike human adipose derived stem cells (non-cancerous cells) and lung cancer in which high fluence of 40 J/cm² did incite a bioinhibitory response on the cellular level [18,19]. Results revealed that BCCs have different cellular response when compared to BCSCs after being exposed to the same treatment. This shows that different cells even from the same tumor bulk may react in different ways to the same treatment condition. Furthermore, breast

cancer, in general, might be more resistant to conventional therapeutic approaches as compared to other cancer such as lung cancer.

Conclusion

The theory stating that tumor initiation and sustain would be facilitated by a minority of cells possessing stem-like properties known as CSCs has always been the subject of contradictory discussions but still raise concern considering the role that these cells could play in tumor malignancy and resistance to conventional treatment modalities [21]. In this view, novel anti-tumor treatments should be evaluated not only based on their ability to shrink the affected cells but also to definitely eradicate CSCs in order to prevent post-therapeutic relapse.

Generally, the hypothesis supporting the possible curative effect of laser treatment has also been questioned. However, the beneficial effects of LILI using specific light parameters in the treatment of cancer and other medical conditions have been revealed [17]. The use of LILI alone with the aim to eradicate cancer cells has still not yield convincing results. On the other hand, LILI used in Photodynamic Therapy (PDT), which is the application of LILI in combination with a photoactive drug referred to as a photosensitizer, has shown better effectiveness in the treatment of cancer [22]. A metallophthalocyanine photosensitizer called Zinc phthalocyanine (ZnPcSmix) has shown effective stimulation and initiation of apoptosis in breast cancer [23]. Therefore, it is important to emphasise that PDT could be a potential therapeutic approach to take into consideration in cancer treatment.

Authors Contribution

N E Kiro conducted the laboratory work, wrote the manuscript and participated in conception and design. H Abrahamse edited the manuscript and provided final approval. M R Hamblin edited the manuscript. All authors agreed to the submission of this article. No data deposition has been done for this article, however is available upon request. All correspondence can be directed to H Abrahamse.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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