Biochemical Responses of Isolated Lung CSCs After Application of Low Intensity Laser Irradiation

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ABSTRACT

Studies have shown that using high fluences of Low Intensity Laser Irradiation (HF-LILI) produce apoptotic effects on normal and neoplastic cells. This study aimed to determine whether HF-LILI induce cell death in lung CSCs. Lung CSCs were isolated using the stem cell marker CD 133, characterized using flow cytometry, and applied in experiments which included treatment with LILI at wavelengths of 636, 825 and 1060 nm with fluences ranging from 5 J/cm^2 to 40 J/cm^2. Viability and proliferation studies, using Alamar blue assay and adenosine triphosphate luminescence (ATP), indicated an increase when treating lung CSCs with low fluences of 5 – 20 J/cm^2 and a decrease in viability and proliferation as well as an increase in apoptosis when applying a fluence of 40 J/cm^2 indicated by flow cytometry using Annexin V and propidium iodide (PI) dyes. Results indicate that LILI, when treating lung CSCs, can induce either a bio-stimulatory or bio-inhibitory effect depending on the wavelength and fluence used. This study indicated successful apoptotic induction of lung CSCs. Future experiments should be able to conclude the exact mechanism behind HF-LILI, which can be used in the targeted treatments of CSC elimination, implementing HF-LILI in the same manner as PDT in the absence of a photosensitizer.

Keywords: High Fluence Low Intensity Laser Irradiation; Lung Cancer Stem Cells

1. INTRODUCTION

Cancer is a class of diseases characterised by uncontrolled cell proliferation leading to the formation of masses of tissue known as tumours in solid cancer[1]. With 8.2 million cancer related deaths in 2012, cancer is the leading cause of mortality worldwide[2]. Breast and cervical cancer are among the 4 most common cancer affecting women worldwide. They have reached 522 000 and 266 000 death cases, respectively in 2012. Lung cancer accounts for more cancer-related mortalities worldwide than any other cancer in both men and women[3]. Treatments for lung cancer include chemotherapy, radiation, and surgery. Despite these therapeutic advances, lung cancer still has a poor prognostic outcome, which can be attributed to its metastatic potential[4]. Post therapeutic cancer recurrence due to the failure of conventional therapeutic measure to cure invasive cancer is believed to be caused by cancer stem cells (CSCs). They are thus the main target in the development of innovative approaches for anti-tumour therapies[5]. Low intensity laser irradiation (LILI) has been shown to have a bio inhibitory effect on cancer cells and CSCs if the appropriate light parameters are used. Therefore it could be a potential therapeutic tool for invasive tumour treatment especially when CSCs are the main target[6].

1.1 Cancer Stem Cells

A variety of different types of cells have been found within malignant tumours. CSCs also called tumour or cancer initiation cells (CICs or TICs) represent a minority of undifferentiated side-population (SP) cells among cancerous cells of a malignant tumour possessing stem cell-like properties[7]. They show tumorigenic properties such as the multidrug resistance, the expression of anti-apoptotic proteins, drug efflux pumps, tumour regeneration after treatment, the capacity to initiate tumours mimicking the parent tumour, uncontrolled proliferation and metastasis making them the main target in invasive cancer treatment[8].

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Unlike non-SP cancer cells or differentiated cancer cells that undergo symmetric cell division only giving rise to non-SP cells, SP cells undergo asymmetric cell division giving rise to both SP and non-SP cells[9]. Several signaling pathways that regulate normal functions on embryonic and adult stem cells appear to be altered in CSCs[10]. Stem cells reside in a niche which maintains them in a quiescent state. In CSCs this niche called the CSCs niche may promote enhanced DNA repair. The quiescent phenotype, as well as the high rate of DNA repair may contribute to their therapeutic resistance[11].

1.2 Lung cancer stem cells

Patients with lung cancer are likely to present with early-disseminated disease regardless of tumour size suggesting enrichment of CSCs in these tumours which can be identified and characterized[12]. Pathways such as the Notch, Hedgehog (HH), and Wnt define normal SCs and guide the behaviour of normal pulmonary precursors within several different lineages. Abnormal signaling in these pathways may cause lung cancer arising from inappropriate expansion of pulmonary SCs developing into CSC lineages[13]. In addition to the genes involved in the Notch, Wnt, and HH pathways, there are numerous genetic markers associated with CSCs. Cell surface markers are used to characterize and identify lung CSCs. One such surface marker is CD 133 also known as Promonin-1. This marker is a pentaspan trans membrane protein localized in microvilli and other plasma membrane protrusions[14]. Lung CSCs with the surface marker CD 133 displayed properties that included the increased capability to self-renew, drug resistance and high tumorigenic potential as well as increased expression of stem-ness[15,16,17].

1.3 Low-Intensity Laser Irradiation

Low-intensity laser irradiation (LILI) is a photobiomodulative therapy that makes use of low intensities of light that is emitted coherently at a specific wavelength in the visible spectrum, which consists of red and near-infrared (IR) light between 600 and 1070 nm, also known as the optical window where effective tissue penetration is maximal. This therapeutic model exposes cells or tissues to visible red light and near-IRlight, having either a bio stimulatory or a bio inhibitory effect[18]. The mechanism by which LILI induces a photobiological effect is chromophore stimulation at the mitochondrial level, causing various metabolic effects, depending upon the wavelength and fluence used[19]. Previous studies conducted using LILI with wavelengths between 625 and 675 nm and fluences ranging from 1 to 15 J/cm2 all indicated a bio stimulatory effect showing an increase in proliferation and viability on various cells[20,21,22,23,24]. However, studies indicated biochemical inhibition when using higher wavelengths of 800–830 nm and fluences ‡10 J/cm^2 [21,25,26]. Photobiomodulation relies on specific parameters such as wavelength, fluence, power density, pulse structure, and treatment time when applied to biological tissue. This allows for targeting of specific light-absorbing molecules in specific tissues, operating on the principle of photochemistry, as opposed to photo thermogenesis. The light energy absorbed causes singlet state excitation of oxygen molecules, leading to triplet state excitation causing an energy transfer to ground state molecular oxygen (a triplet) to form the reactive species, singlet oxygen. Alternatively superoxide may be formed as a result of electron reduction. LILI operates at an exact wavelength of light, which influences the depth of tissue penetration. Similar to normal cells, cancer cells also contain with intracellular chromophores. Different cellular chromophores are stimulated at different wavelengths[27,28]. Therefore, the prediction can be made that in targeting cancerous cells, the outcome expected can be controlled by the wavelength as well as by the energy output that will lead to either stimulation or inhibition. The exact mechanism behind the stimulation of the light-absorbing molecules producing these two different effects is still being investigated.

2. MATERIALS AND METHODS

2.1 Cell culture

This study used commercially obtained lung cancer cells (A549, ATCC® CCL-185). Lung cancer cells were cultured in Rosewell Park Memorial Institute 1640 medium (RPMI) (Sigma, R8758) supplemented with 10% foetal bovine serum (FBS) (Biochrom, S0615) and 0.5% penicillin/ streptomycin (Sigma, P4333) and 0.5% amphotericin B (Sigma, A2942). The cells were incubated at 37°C in 5% CO2 and 85% humidity. Control cell lines were cultured in Dulbecco's Modified Eagle's Media (DMEM) (Sigma, D5796) supplemented with 10% FBS, 2mM L-Glutamine (Sigma, G7513), 1% penicillin/ streptomycin (Sigma, P4333) and 1% amphotericin B (Sigma, A2942) for CaCo2 (ATCC®, HTB-37™) which is used as the positive control and DMEM (Sigma, D5796) supplemented with 10% FBS (Biochrom, S0615) and

1% penicillin/ streptomycin (Sigma, P4333) and 1% amphotericin B (Sigma, A2942) for SKUT-1 (ATCC®, HTB114™) as the negative control.

2.2 Isolation of side population cells

Lung CSCs were isolated using the magnetic bead isolation kit (Miltenyi Biotec, QuadroMACS™ separation unit 130-091-051), were they were magnetically labelled with microbead conjugated antibodies directed at the antigenic surface marker of interest. Lung CSCs were enriched using the CD133 MicroBead Kit (Miltenyi Biotec, CD133 MicroBead Kit, human 130-050-801) designed for the positive selection of cells expressing human CD133 antigen.

2.3 Cancer stem cell characterisation

After isolation cells were characterised using flow cytometry to identify the CD133 cell population via antibody identification. 2° Antibody labelling was used to label the isolated population of cells, which were then compared to a positive and negative control cell line, CaCo2 which is used as the positive and SKUT-1 as the negative control.

2.3 Low Intensity Laser Irradiation

Cells were seeded at a concentration of 1 x 10^5 in 3.5 cm diameter treated plastic dishes. Each plate contained 3 ml media supplement consisting of RPMI, 10% Foetal bovine serum and 1% antibiotics. After 24 h incubation at 37°C, the monolayer was rinsed with HBSS and covered with another 3 ml of supplemented medium. Where after the plate was placed under the laser light. The beam of the He-Ne laser was delivered through an optical fibre of 0.5 mm diameter. To obtain an even light distribution on to the cell monolayer. After obtaining the power output using a power meter the exposure time was calculated in order for the cells to receive energy with a fluence of 5 – 20 J/cm^2 and 40 J/cm^2. Laser parameters are indicated by Table 1.

Table 1. Laser Parameters using the 636, 825 and 1060 nm Diode Lasers.

Parameters	Value		
Laser type	Semiconductor (Diode)		
Wavelength (nm)	636	825	1060
Wave emission	Continuous	Continuous	Continuous
Power output (mW)	±85	±85	±85
Power density (mW/cm^2)	9.36	9.36	9.36
Spot size (cm^2)	9.1	9.1	9.1
Fluence (J/cm^2)	5, 10, 20 and 40	5, 10, 20 and 40	5, 10, 20 and 40
Duration of irradiation ± min,sec	8min 54sec, 17min 48sec, 35min 36sec and 71min 13sec	8min 54sec, 17min 48sec, 35min 36sec and 71min 13sec	8min 54sec, 17min 48sec, 35min 36sec and 71min 13sec

2.4 ATP Viability assay

ATP viability measurement was performed using the CellTiter-Glo® luminescent cell viability assay (Whitehead Scientific, Promega, G7573), quantified luminescence was recorded using the Perkin Elmer, VICTOR3™ Multilabel Counter (model 1420) in relative light units (RLUs).

2.5 Alamar Blue proliferation assay

The AlamarBlue® reagent (Invitrogen™, AlamarBlue® DAL1025) was used to perform the experiment, where fluorescence was measured using the Victor-3 (Perkin-Elmer, Separation Scientific) at Ex/Em 560/590.

2.6 Quantitative analysis of cell apoptosis

The Annexin VPI kit (BD Pharmingen™ Annexin V: FITC Apoptosis Detection Kit I 556547) is used as a labelling kit to identify the amount of live and dead cells in a cell population. Live cells exclude the dyes having no stain, whereas apoptotic cells and necrotic cells are stained with Annexin V and PI respectively. This kit was used in conjunction with the C6 Flow Cytometer (BD biosciences, BD Accuri™ C6) which sorts each cell population accordingly.

2.7 Statistics

Results were compiled using Sigma plot version 12. All assays were performed at least four times. All error bars represent standard error of the mean (SEM) (n ‡ 4). For statistical evaluation, Student's paired t-test was used, and significance was defined as a P value. For fluorescence emission intensity analysis, a background subtraction was performed for all of the data.

3. RESULTS

A side population of cells positive for the antigenic marker CD 133 were obtained from a lung cancer cell line A549. These cells were treated with LILI using fluences of 5 J/cm^2 - 40J/cm^2 from lasers with wavelengths of 636, 825 and 1060 nm. Where after post irradiation biochemical assays were conducted after 24 hours. Assays including viability, proliferation and cell death was performed which has an indication of the treatment outcome. These assays presented either with a bio stimulatory or bio inhibitory effect. Statistical significances are indicated on the figures and tables accordingly ($P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)).

3.1 Positive identification of isolated cells

To identify whether the isolated cell population had the CD 133 marker indicating stem-ness the cells were characterised using flow cytometry and antibody labelling. Immunofluorescence labelling using FITC as the fluorescent marker attached to a secondary antibody was used to attach to the antigen CD 133 on the CSC surface. Expression of the marker was seen on the isolated lung CSCs, as well as the positive control cell line CaCo2. No fluorescence was observed from the negative control cell line SKUT-1. Positive expression of the CD 133 antigenic surface marker indicated positive isolation of lung CSCs. As majority of the population were to be identified as CSCs. Results indicated that the cells isolated had been characterised as CD 133 positive shown in Figure 1.

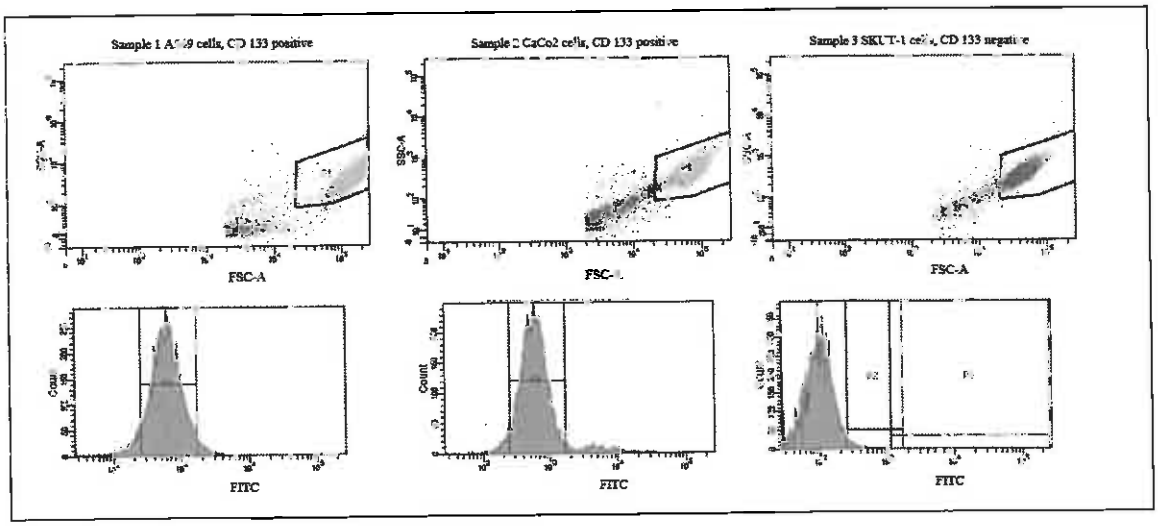

Figure 2. Characterisation of isolated cancer stem cells and control cells.

3.2 Viability study

The number of viable cells that were measured is based on quantitation of the ATP present. Cells that are still metabolically active produce ATP. This production was measured using an ATP absorbance kit measuring levels of ATP per cell. This is a direct indication of mitochondrial activity as energy is produced via the organelle. ATP viability results are shown in Table 2.

Table 2. ATP viability post irradiation.

ATP viability RLUs			636 nm			825 nm			1060 nm		
	5 J/cm2	control	93835,25	±	13928,2263	72615,50	±	15149,3013	72615,50	±	15149,3013
		test	144129,00		25956,2181	80339,25		11340,0547	80339,25		11340,0547
	10 J/cm2	control	94959,75	±	6868,6481	61333,00	±	14462,6258	84253,00	±	3679,5765
		test	119725,25		6455,8605	86549,25		8437,8377	93954,50		9451,495
	20 J/cm2	control	107280,00	±	4593,2365	94470,50	±	10256,7541	93388,75	±	6439,4638
		test	106823,00		18385,1354	94258,50		5947,522	93592,50		13663,4358
	40 J/cm2	control	462512,25	±	36315,9548	39036,75	±	7139,22	42834,00	±	9029,6315
		test	278450,25		55282,3211 * P = 0,032	19258,25		2736,6028 * P = 0,041	27790,25		2895,2668

A definitive increase is seen in all wavelengths when applying energy of 5 – 10 J/cm^2 when comparing test samples to their respective control groups, although the increase does not reach statistical significance. At 20 J/cm^2 all wavelengths indicate that there is neither stimulation nor inhibition reached at the specific energy level and that test and control samples maintain similar mitochondrial levels. Statistical significant differences are seen between test and controls when comparing the energy level 40 J/cm^2 at wavelengths 636 and 825 nm. This indicates that different wavelengths act upon the mitochondrion at specific wavelengths. Having little effect when using a wavelength of 1060 nm.

3.3 Proliferation study

The amounts of proliferating cells were measured using Alamar Blue. This assay measured mitochondrial metabolic activity after treatment as viable cells generate a quantitative measure of proliferation. Measurement was read as an absorbance value seen in Figures 2, 3, and 4.

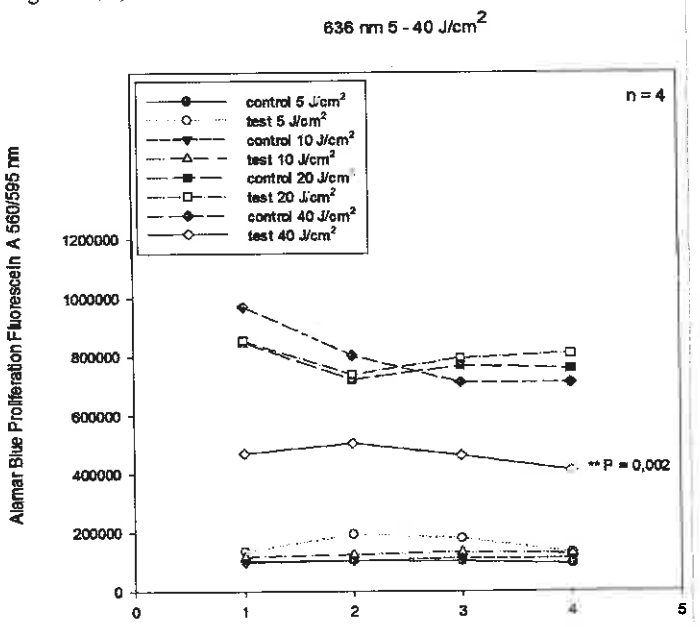

Figure 2. Alamar Blue proliferation at 636 nm.

When comparing the test samples of wavelength 636 nm to their respective control groups statistics show that there is a significance at the energy level 40 J/cm^2 (**P=0.002). This indicates that the higher energy level induced mitochondrial reduction leading to decreased proliferation. An increase in proliferation is noted when viewing energy levels 5 – 10 J/cm^2 and little variation is seen at 20 J/cm^2.

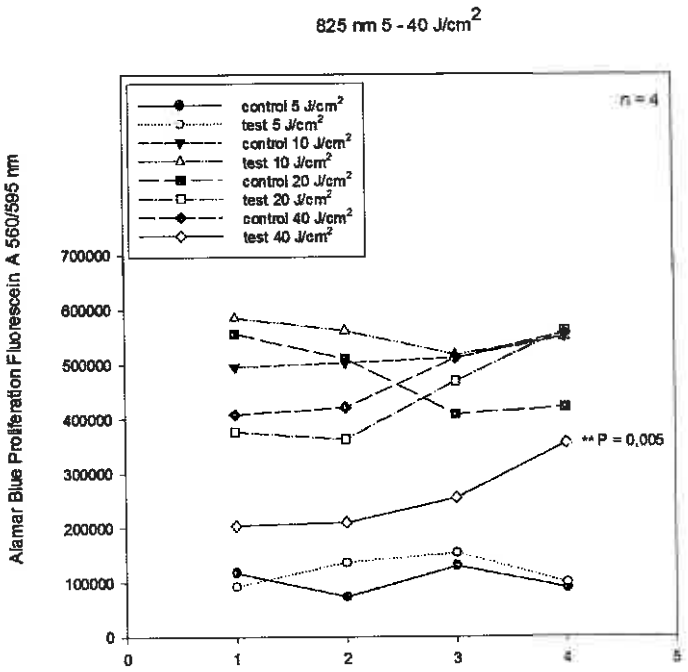

Figure 3. Alamar Blue proliferation at 825 nm.

A similar trend is seen when comparing tests to control groups 5 – 20 J/cm². Having no statistical differences. A substantial decrease in proliferation is seen at 40 J/cm² with a statistical significance of P=0.005 indicating an inhibitory effect on mitochondrial activity of the test group after treatment with LILI.

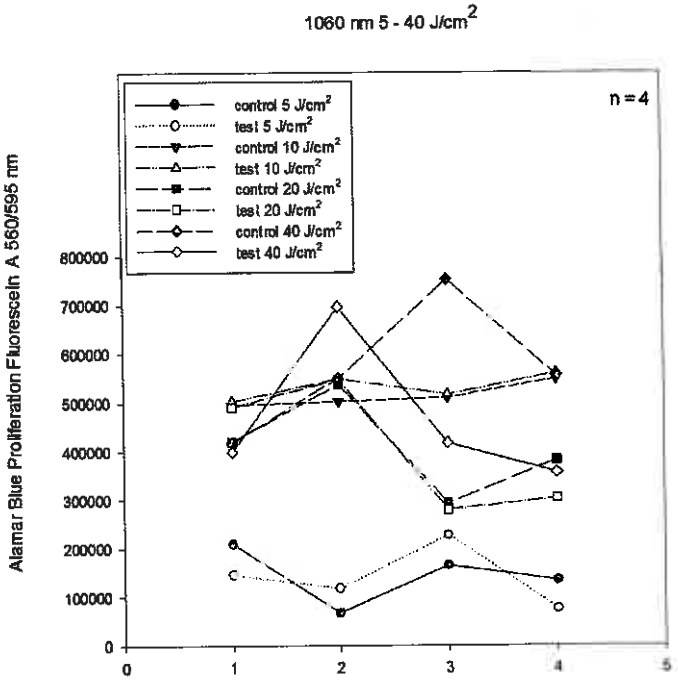

Figure 4. Alamar Blue proliferation at1060 nm.

Minimal increase is seen in proliferation when comparing the test samples to their respective controls having no statistical significance when viewing samples given energy with fluences ranging from 5 – 20 J/cm^2. There is a decrease seen in proliferation when comparing the treatment group of 40 J/cm^2 but is not statistically significant indicating minimal stimulation upon the mitochondrion when using 1060 nm.

3.4 Cell death study

Cell death was measured using Annexin V PI which indicates cell death either as necrotic or apoptotic. The assay uses flow cytometry and fluorescent staining to distinguish between the different cell deaths. As apoptosis is indicated by the annexin and necrosis by PI. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface. Annexin V, a Ca2+-dependent phospholipid-binding protein, has high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposed PS using flow cytometry. PS translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes. Propidium iodide (PI) is used for identification of late apoptotic cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI which then binds to the nucleic acids exposed. Results show that apoptosis was achieved at 40 J/cm^2 when using wavelengths of 636 nm and 825 nm (Figure 5 and 6). Although the population of apoptotic cells was seen to be lower at fluences of 5 – 20 J/cm^2. At 1060 nm little to no apoptosis was seen indicating that this wavelength didn't induce the desired cell death (Figure 7).

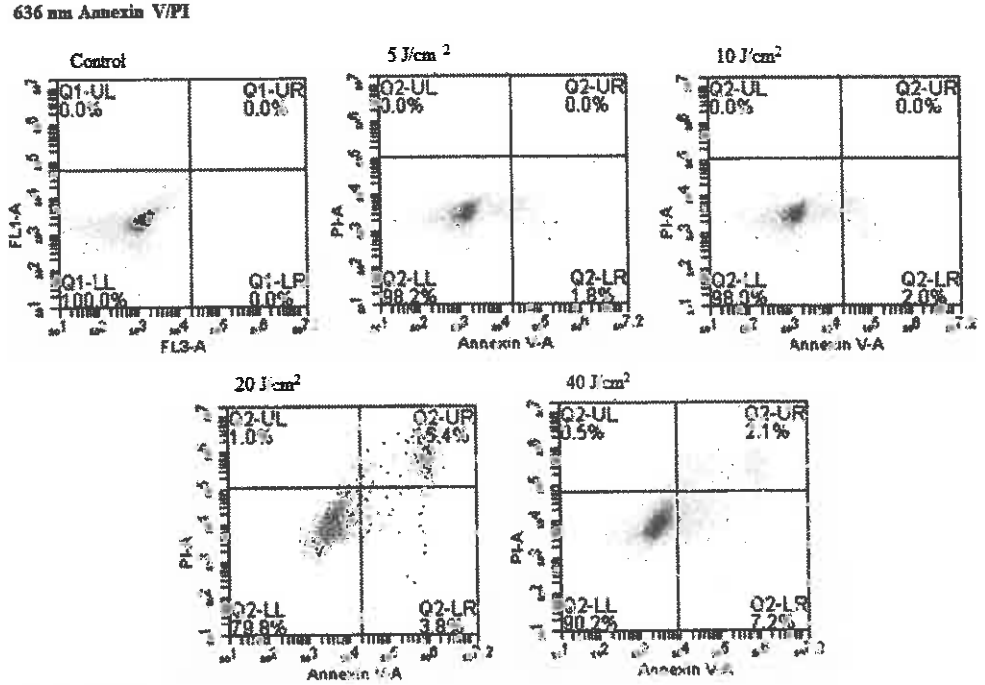

Figure 5. Quantitative cell death analysis at 636 nm.

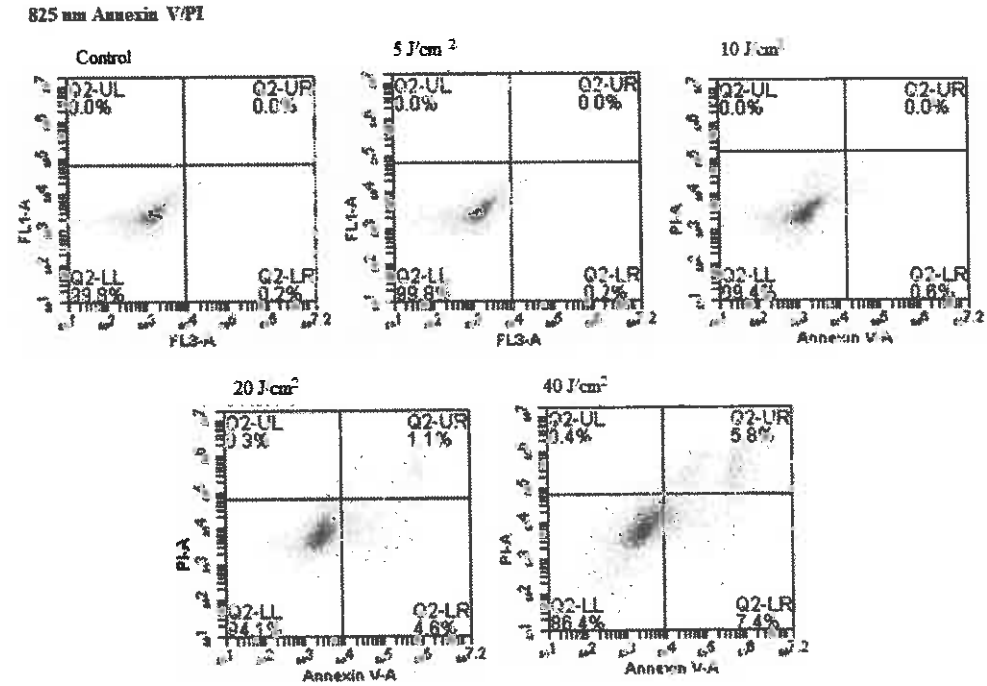

Figure 6. Quantitative cell death analysis at 825 nm.

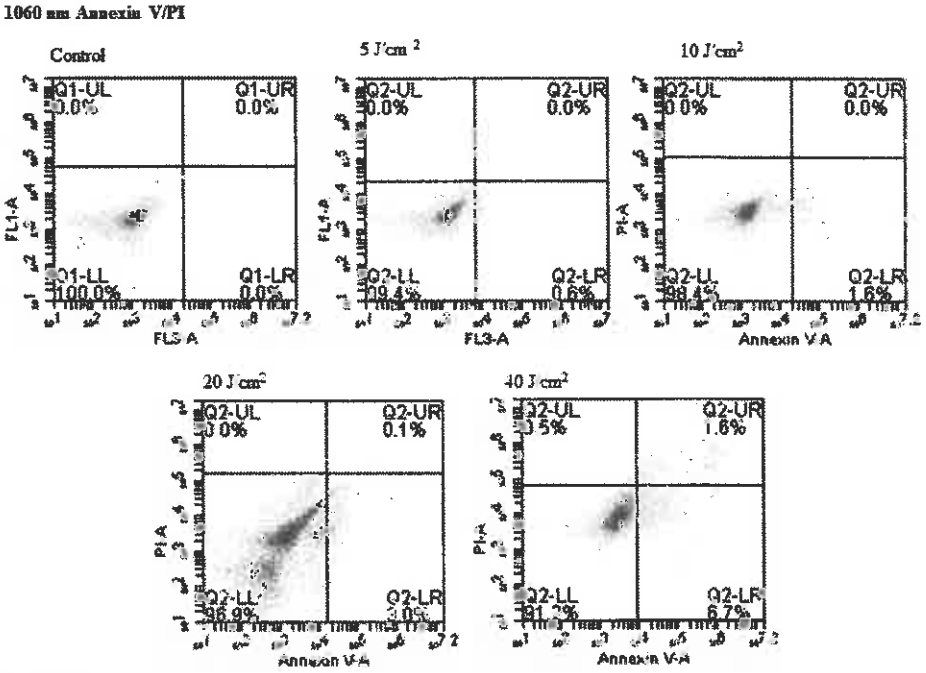

Figure 7. Quantitative cell death analysis at 1060 nm.

4. DISCUSSION AND CONCLUSION

CSCs can be identified and isolated using their antigenic markers[29]. Promonin 1 (CD 133) is a pentaspan transmembrane glycoprotein usually found on cell surfaces[30]. It has been used to identify and isolate different SCs and CSCs[31]. Lung CSCs have previously been isolated using the surface marker CD133[32] and the same antigenic marker was used in this study. Control cell lines used were the CaCo-2 cell line which is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells, which have been found to express CD 133[31]. SKUT-1 is a cell line derived from human uterus leiomyosarcoma cells[33], these cells don't express the surface marker CD 133 as they were used as a somatic cell line and contributed as the negative control cell line.

There were changes seen in cell viability and proliferation. Showing different wavelengths at different fluences having either a bio stimulatory or bio inhibitory effect. These results concur with previous studies conducted indicating that LILI can stimulate cells with a wavelength of 636 nm and low fluence of 5 J/cm^2 [34] as well as wavelenghts of 825 nm and 1060 nm with energy levels of 5 – 10 J/cm^2. CSCs oppose cell death by self-renewal[35]. This is seen even when applying increased energy levels of 20 J/cm^2. Suggesting that irradiation between 5 and 20 J/cm^2 with wavelengths ranging from 636 nm – 1060 nm have little damaging effect but rather a stimulatory effect on the CSCs[34] although bio inhibition can be achieved using high fluence LILI (HF-LILI) at 40 J/cm^2 with wavelengths of 636 nm and 825 nm. These findings are in accordance with previous research stating that parameters such as wavelength, fluence and intensity play an important role in photobiomodulation of cellular metabolism[21,23,24,25]. It should also be noted that when using a wavelength of 1060 nm and fluence of 40 J/cm^2 that it has little outcome on the treated samples. It can be said that CSC photobiomodulation have stimuli in the visible red light spectrum with wavelengths between 636 and 825 nm. Infra-red light has little to no effect on CSC metabolism.

Cell death quantification was achieved using Annexin V PI giving an indication of the cell death pathway that is followed by the lung CSCs after treatment with LILI. Little to no cell death was seen when irradiating the cells at

fluences of 5 – 20 J/cm^2 with wavelengths of 636 – 1060 nm. Although it can be said that HF-LILI which is represented by 40 J/cm^2 does induce cell death via the recommended cell death pathway apoptosis. Necrosis is only seen due to cells going into late apoptosis and eventually dying. These results are also confirmed by the reports suggesting that light is absorbed by chromophores (porphyrins or cytochromes) located in the intercellular organelles such as the plasma membrane, mitochondria or lysomes after HF-LILI, and the activation results in ROS production[36] assisting cell death through apoptosis. Although the exact mechanism behind HF-LILI being able to induce apoptosis is not well understood.

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