

Laser light induced modulations in metabolic activities in human brain cancer

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The role of low optical or near infra-red laser intensity in suppressing metabolic activity of malignant human brain cancer (glioblastoma) cells was investigated through the application of either a continuous wave 633nm Helium - Neon or a 1,552nm wavelength pulsed picosecond laser. Human glioblastomas were exposed in their growth culture medium with serum for several energy doses. For both types of laser exposures the glioblastomas exhibited a maximal decline in the metabolic activity relative to their respective sham control counterparts at 10 J/cm^2 . The cellular metabolic activities for various treatment doses were measured through the colorimetric MTS metabolic assay after the laser exposure. Interestingly, addition of (the enzyme) catalase in the growth medium prior to the laser exposure was found to diminish the laser induced metabolic suppression for all fluence treatment conditions, thus suggesting a functional role of H_2O_2 in the metabolic suppression. Taken together, our findings reveal that optical or near-IR low level light exposures could potentially be a viable tool in reducing the metabolic activity of cancers; evidence at hand implicates a role of light induced H_2O_2 in bringing about in part, suppression in the metabolic activity. Due to the cellular “biphasic” response to the laser exposure, further research needs to be undertaken to determine exposure parameters which would optimize metabolic and cellular growth suppression in-vivo.

1. Introduction

Wavelength, fluence and intensity have been noted as important light exposure parameters through previous investigations playing an important role in biomodulations which bring about various biological effects [1]. A feature in low level light exposure, as noted through past research, is a “biphasic” biological response to intensity and most notably in the light energy dose, i.e., the fluence [2]. Consequently, for a specified optical or infra-red wavelength and for a specified low intensity there exists an optimal fluence

value for light exposure to produce a maximum modulation for a specified biological response / effect. A few noteworthy bio-effects due to low level light exposure which have been reported in the literature include: (i) modulation in gene expressions [3], (ii) increase in the intracellular calcium levels [4], (iii) increase in the mitochondrial metabolic activity [5] and in enhanced production levels of ATP [6], (iv) with a concomitant increase in cellular proliferation [7].

Findings from Tiina Karu's group on correlations of light irradiated (cellular proliferation) action spectra and the absorption spectra of intact (human cervical cancer) HeLa cells has suggested the hypothesis that absorption of light by certain chromophores within cells do bring about light induced modulations in cellular proliferation. Karu's investigations had found that the intact cell's visible absorption spectrum resembled the absorption spectrum of cytochrome C which is an integral part of the respiratory chain found in the mitochondrion [8]. This observation had led to a hypothesis that selective chromophores within the mitochondria are responsible in light absorption. Additional investigations found that the light absorbed by the mitochondria sped up the electron shuttle within its inner membrane compartments and consequently led to enhancement in the ATP production. As a natural consequence to the enhancement of the respiratory chain activity is the enhancement of the interplay between electrons and molecular oxygen within the mitochondria [9]. Thus, enhancements in the concentration levels of reactive oxygen species (ROS) such as the superoxide anion O_2^- , and H_2O_2 have been observed to be elevated due to light absorption within the mitochondria. Lubart and coworkers have found elevated concentrations of H_2O_2 due to white light exposures from cardiomyocytes [4]. In past literature, enhancement of ROS levels have been shown to activate selective transcription factors which would "turn on" or "turn off" genes and their protein products within the cells [10, 11]. Different cell types have varying degree of responses at their optimal low light level exposure settings.

In 1967, Mester and co-workers [12] performed exploratory research to test if non-ionizing radiation from lasers could induce cancers in mice. They concluded that laser irradiation does not cause cancer, but instead, it was noted to speed up the rate of hair growth from the shaved regions on the mice which were laser tested for the possible induction of cancers. Reports on low level light bio-effects on cancer cells are sparse and

have primarily entailed monitoring proliferation / mitotic rates from a limited number of cancer cell lines (such as the He-La cervical cancer cell lines, the KB cell line, the melanoma cell lines) with several different and discrete exposure parameters in wavelength, intensity, and the amount of energy deposition on the cancer cells (findings summarized in [13]). In this communication, we report on the biphasic role of red and near infra-red low laser intensity in suppressing the metabolic activity of the malignant human glioblastoma. The cellular metabolic activities for various treatment doses were measured through the colorimetric MTS metabolic assay. Addition of the enzyme catalase in the growth medium prior to the laser exposure was found to partially block the laser induced metabolic suppression for the fluence value where maximum suppression were measured. This finding has led us to formulate a working hypothesis on the functional role of laser light induced H₂O₂ in bringing about the biphasic metabolic response.

2. Materials and Methods

Cell line maintenance

Human malignant (brain cancer) glioblastoma was purchased from the American Type Culture Collection (Rockville,MD) and grown and maintained in T-75 flasks under incubation conditions of 5% CO₂ at 37°C. The cells were maintained in ATCC formulated DMEM / F12 growth medium with 10% of fetal bovine serum and 50 Units/ml of penicillin and streptomycin antibiotics.

Cell preparation

When the adherent glioblastoma cells reach 50 – 60% confluence within the T-75 flasks, the cells were trypsinized and brought into suspension. The cells were spun down and the (trypsin) supernate was discarded. The cells were resuspended in fresh growth media at an initial working concentration 75 K/ml. The cell suspension was then transferred into single well's of the 96 well plates with a transfer volume of 0.2 ml or 15K cells seeded per selected well. The cells were seeded into every other well in order to ensure no possible overlap in the laser light exposure. The cells within the 96 well plates were returned back into the incubator for approximately 24 Hrs before the laser treatments.

Cell exposure

Laser exposure set-up is shown in the schematic diagram in Fig. 1 below. A near infra-red 1,552nm wavelength pulsed pico-second laser, Raydiance, Inc., was utilized in exposing cells within the selected wells of the 96 well plates with fixed laser parameters of : 2.93 picoseconds pulse width, 25kHz repetition rate, and with 1.6 micro-joule pulse energy. The measured average power delivered at the underside of the well = 30.0 milliwatts (25% of laser energy was lost due to external optics) with a spot size of 5.75mm in diameter (measured through knife – edge technique), yielding an average laser intensity of 115 milliwatts/cm².

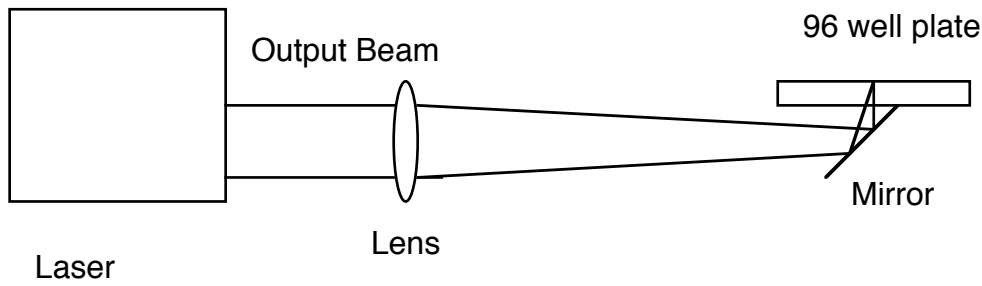


Fig. 1 Schematic diagram of the experimental set-up.

Each alternating column of the 96 well plate (with the exception of column#1 which served as the control / sham exposed condition) received a pre-determine fluence dose of exposure for those wells within the column which contained cells. Fluence levels were varied by keeping the aforementioned laser parameters fixed and changing the irradiation time of exposure. The selected times of laser exposure ranged from 1 second to 7.25 minutes with the corresponding fluence range from 0.115 J/cm² to 50 J/cm². All exposures were done at room temperature (~ 22°C), and the average duration that the 96 well plates were left out at room temperature for laser treatment was approximately 1 hours. Upon completion of the experiment, the 96 well plates were returned to the incubator.

Cell exposure to CW 633nm He-Ne laser

In-vitro human glioblastoma exposures to continuous wave red He-Ne laser light were performed under an identical exposure set – up as that for the pulsed 1,552 nm laser mentioned above. The measured average power delivered at the underside of the well was adjusted through a neutral density filter to a value of 14.5 milliwatts with a spot size of 4.00mm in diameter yielding an average laser intensity (identical to that of the pulsed 1.55 micron laser) of 115 milliwatts/cm² . For the purpose of comparisons between the two different modes of exposure the selected exposure times of He-Ne laser was identical to that of the pulsed near infra-red laser exposure ranging from 1 second to 7.25 minutes with the identical corresponding fluence range from 0.115 J/cm² to 50 J/cm².

Addition of catalase to the growth medium just prior to laser exposure

Independent experiments of laser treatments were also undertaken in the presence of externally added catalase (from human erythrocytes, Sigma-Aldrich, USA) with the same stock of cells utilized in laser treatment. Just prior to the laser exposure, 900Units of catalase were added to the 0.2ml of growth medium in each well.

External addition of H₂O₂ into the growth medium

Independent H₂O₂ experiments were simultaneously undertaken within the same cell stock as that utilized in laser experiments. Glioblastomas were exposed to selective concentrations of hydrogen peroxide. Identical cell preparation protocol was followed as that for the laser treatments. Cells were incubated for approximately 24 Hrs before the H₂O₂ treatment. Glioblastomas were exposed to exogenously added H₂O₂ concentrations ranging from 0.5micro-Molar to 1milli-Molar for an incubation period of 24Hrs.

Measuring cellular metabolic activity

The metabolic response of human glioblastomas to various laser fluences or externally added known concentrations of H₂O₂ were assessed with a non-radioactive colorimetric cell metabolic tetrazolium compound (MTS) assay (Promega, Madison, WI), 24 Hrs or 72 Hrs after the treatments. On the day of measurement, the 96-well plates

were removed from the incubator and 20 micro-liters of the MTS solution was added to each cell containing well. Thereafter, the plates were immediately returned to the incubator for three hour incubation period.

Functionally, the MTS readily permeates through the cell membrane and is metabolized and is converted into formazan by living cells. Conversion into formazan induces a maximum change in absorption at 490nm wavelength.

Three hours after the addition of MTS, absorption measurements were made at 490nm with a 96 well plate reader, and the average absorbance value at 490nm of the treated cell's metabolic activity was computed with standard deviations and compared to the sham exposed average absorbance value with its standard deviation. The percentage of treated cell's metabolic activity was computed relative to the sham exposed metabolic activity.

3. Results and Discussion

The near infra-red pulsed laser exposures on the human glioblastoma cells induced a decline in the metabolic activity relative to their control (sham exposed) counterparts between the fluence values of 1.0 J/cm^2 to 10 J/cm^2 . Maximum suppression in metabolic activity was noted at 10 J/cm^2 . See Fig. 2 below. Interestingly, as the near infra-red laser light dose was further increased beyond 10 J/cm^2 the metabolic activity was found to return towards the control level. See Fig 2 below.

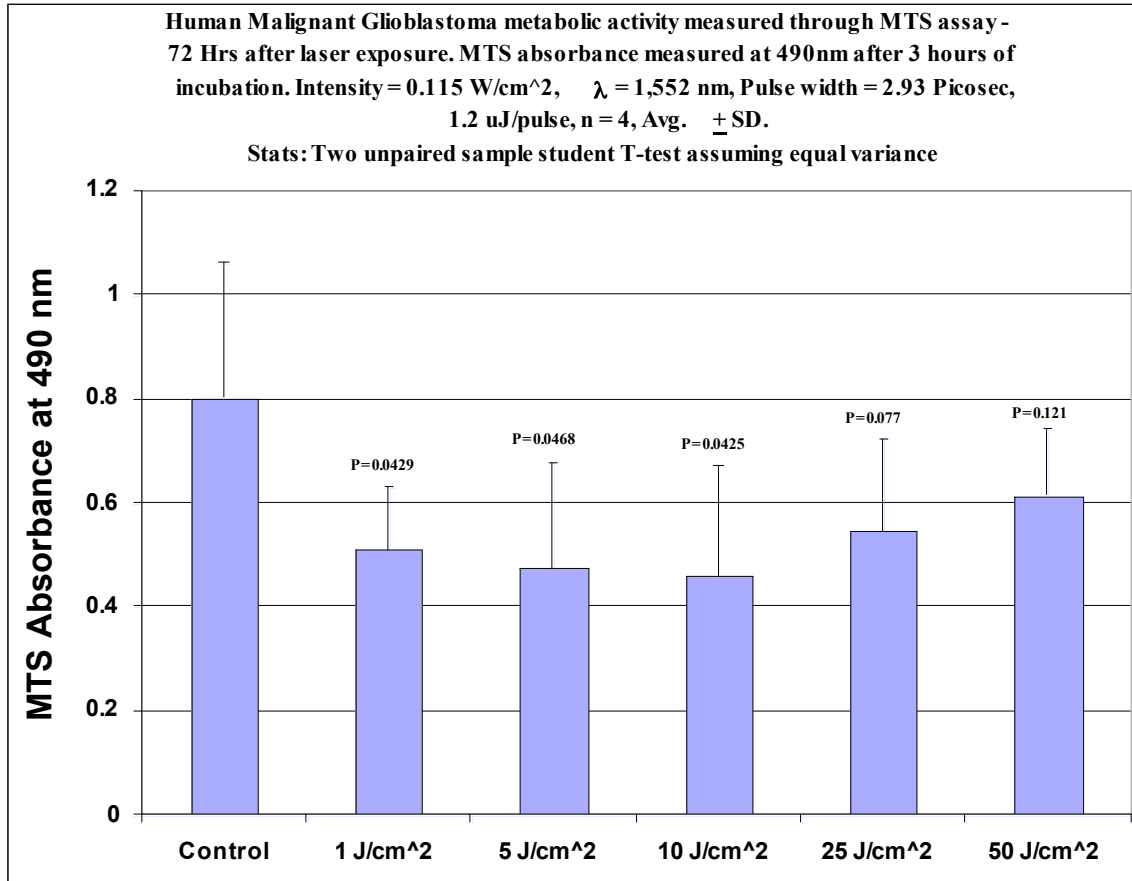


Fig.2 Glioblastoma metabolic activity measured 72 hours after laser exposure through MTS assay. Unpaired sample student t-test statistics were computed between the control and the laser treated conditions.

Addition of the H₂O₂ scavenger enzyme catalase in the growth medium prior to the laser exposures was found to diminish the extent of the laser induce metabolic suppression, thus implicating a role of H₂O₂ in diminishing cellular metabolic activity. See Fig. 3 below.

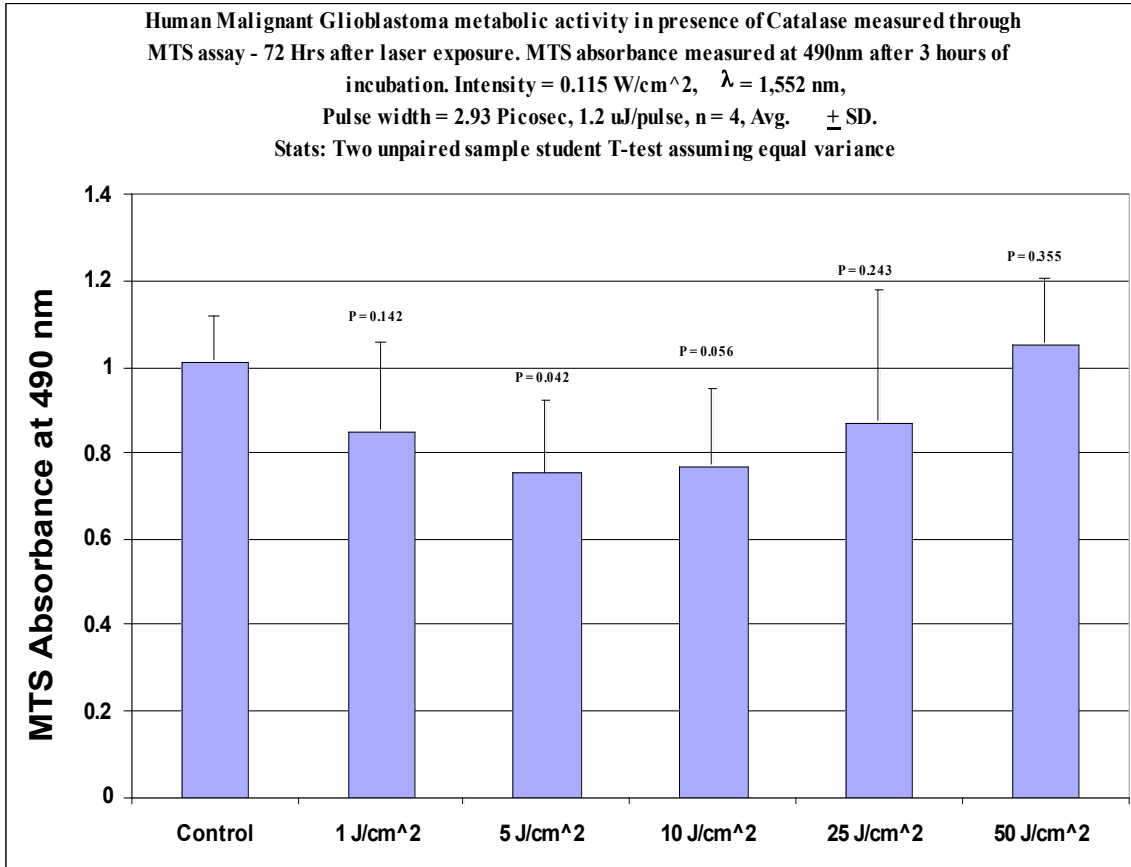


Fig. 3 Glioblastoma metabolic activity measured 72 hours after laser exposure on in presence of [Catalase] = 900Units/well. Unpaired sample student t-test statistics were computed between the control with catalase and the laser treated conditions in presence of catalase.

In Fig. 4, comparative analyses are made between the two data sets of the metabolic activities with the sham and laser treated conditions (between the absence and presence of catalase). Unpaired sample student T-test statistics are computed between the absence and presence of catalase for sham and laser treatment.

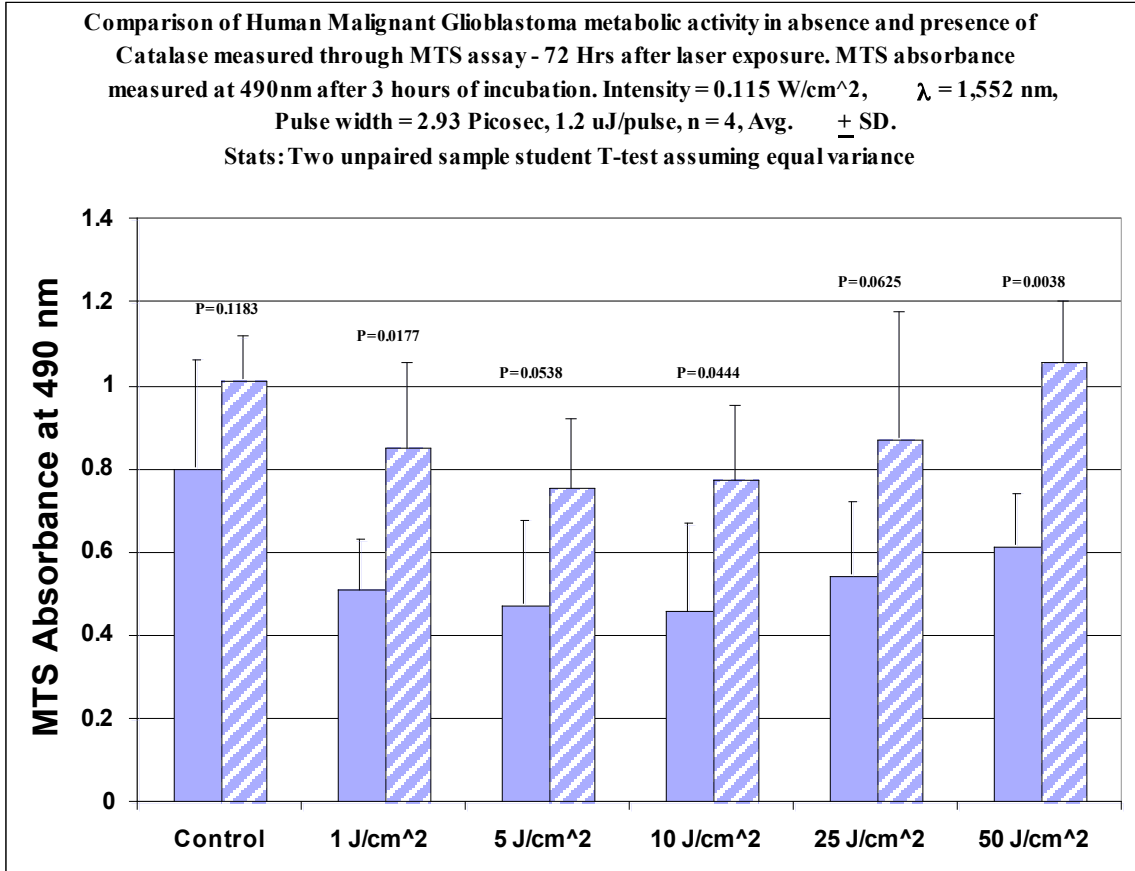


Fig. 4 MTS measured glioblastoma metabolic activity in the absence and presence of catalase for sham and laser treatments. Solid bars represent without catalase in growth medium and stripped bars represent with catalase in the growth medium. P-values were computed between the absence and presence of catalase conditions.

Four of the five laser treatment conditions exhibit statistical significance ($p \sim 0.05$) in the ability of catalase to significantly diminish the laser induced reduction of metabolic activity. Normalization of all data from Fig. 4 relative to the control without the catalase condition is shown in Fig. 5 below.

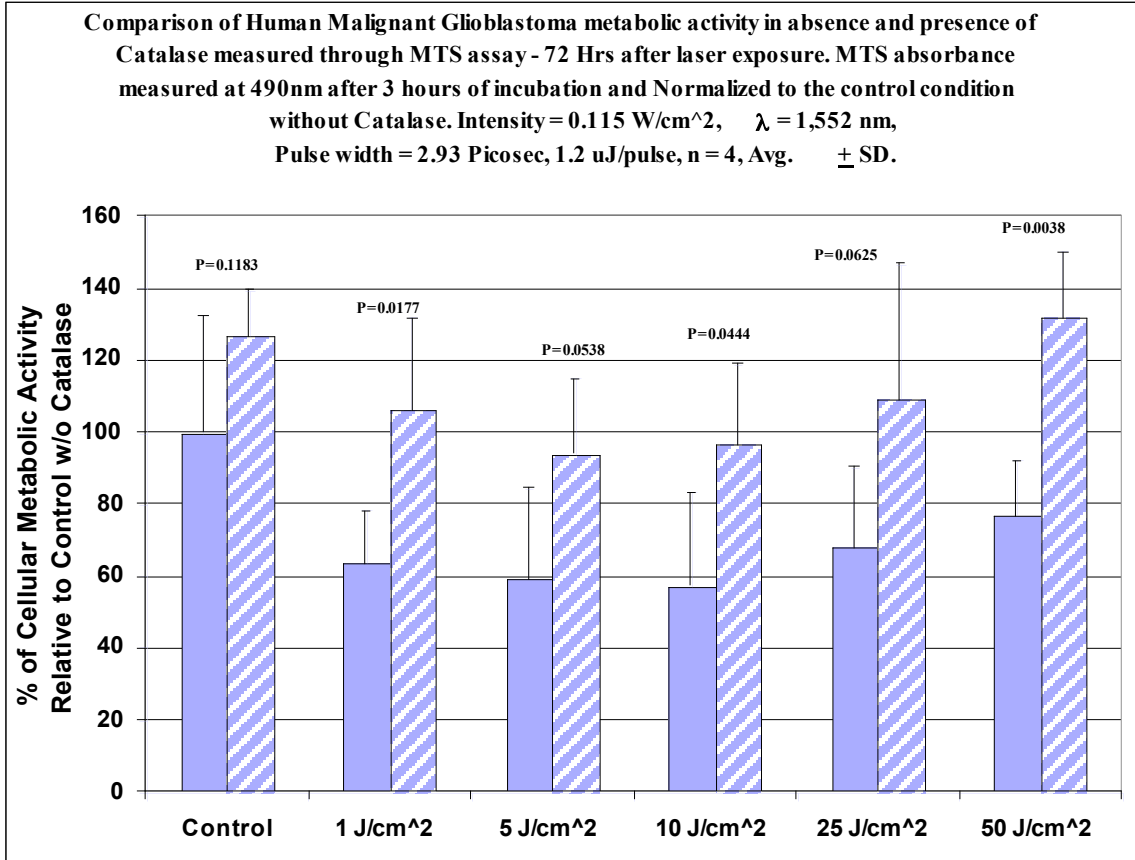


Fig. 5 Comparison of normalized glioblastoma metabolic activity measured 72 hours after laser exposure. Data sets normalized to the control condition without catalase.

Similarly, continuous wave 633nm He-Ne laser treatments were also observed to induce suppression in metabolic activity for fluence values of 10 and 25 J/cm². See Fig. 6 below.

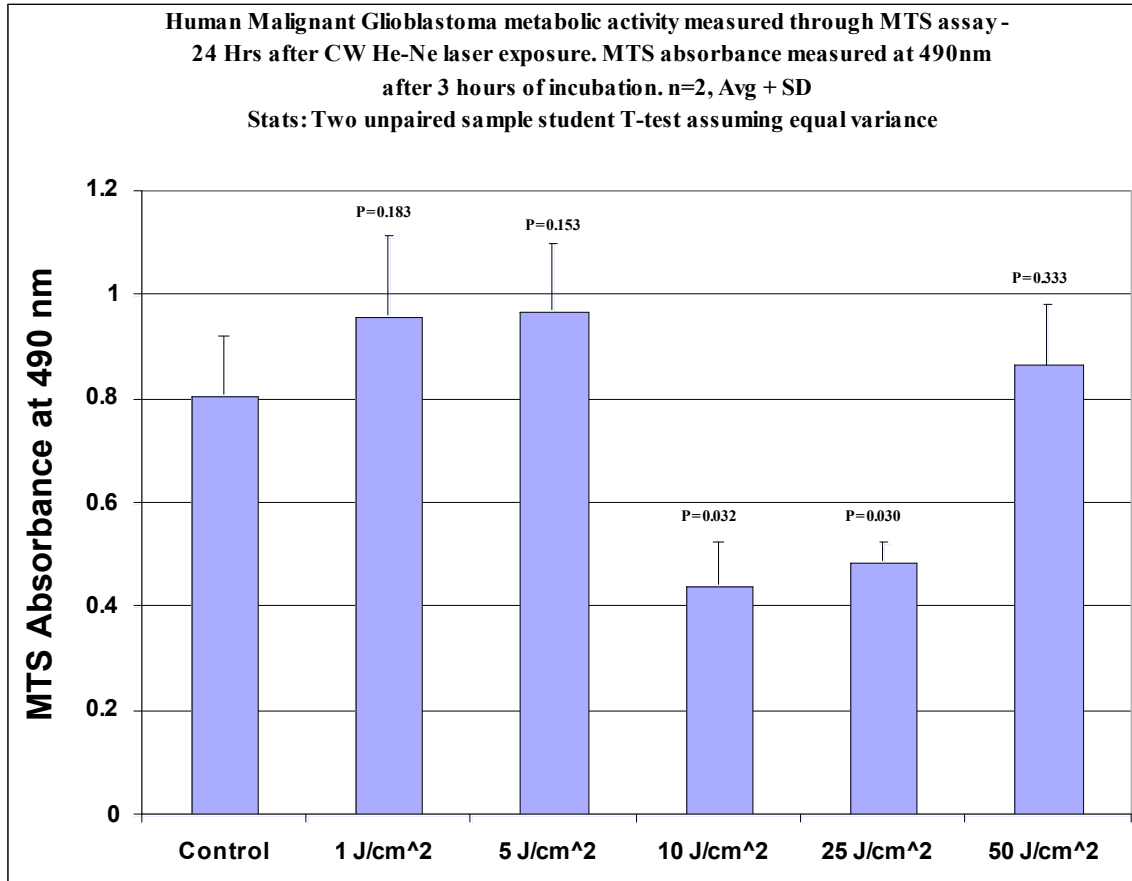


Fig.6 Glioblastoma metabolic activity measured 24 hours after He-Ne laser exposure through MTS assay. Unpaired sample student t-test statistics were computed between the control and the laser treated conditions.

In view of our laser treatment findings and the fact that the catalase scavenging activity of H₂O₂ must reside outside of the cell membrane (since catalase is a fairly large protein and is not known to permeate through the cell membrane), we hypothesize the existence of a sensitive relationship between (laser induced / enhanced production of) extra-cellular H₂O₂ and its partial control over of the cellular metabolic activity.

The metabolic response of Human glioblastoma and Human leukemia HL-60 cells to exogenously added H₂O₂ (without any irradiation) is shown in Fig. 7 and Fig. 8, respectively. The metabolic response of these cancer cells due to the addition of H₂O₂ was found to be consistent with the responses found for fibroblastic cell lines, i.e., the metabolic responses are found to be stimulatory at low concentrations of H₂O₂ and inhibitory at higher concentrations [10].

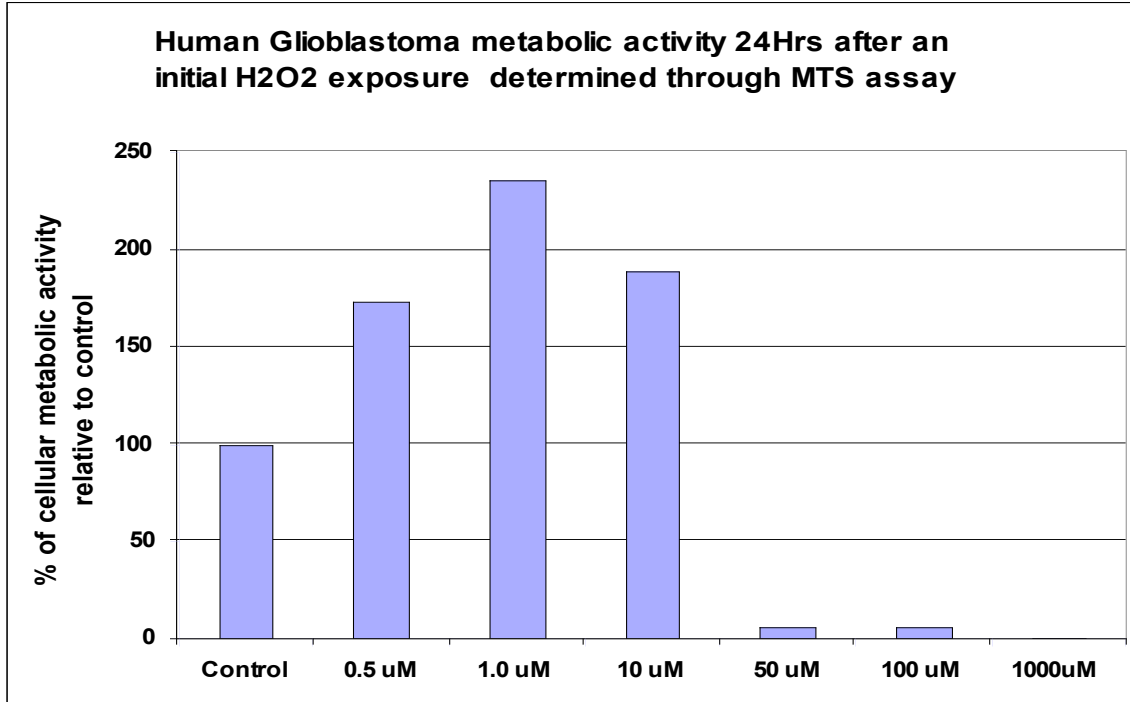


Fig. 6. H₂O₂ dose response curve of human glioblastoma metabolic activity as determined through MTS assay.

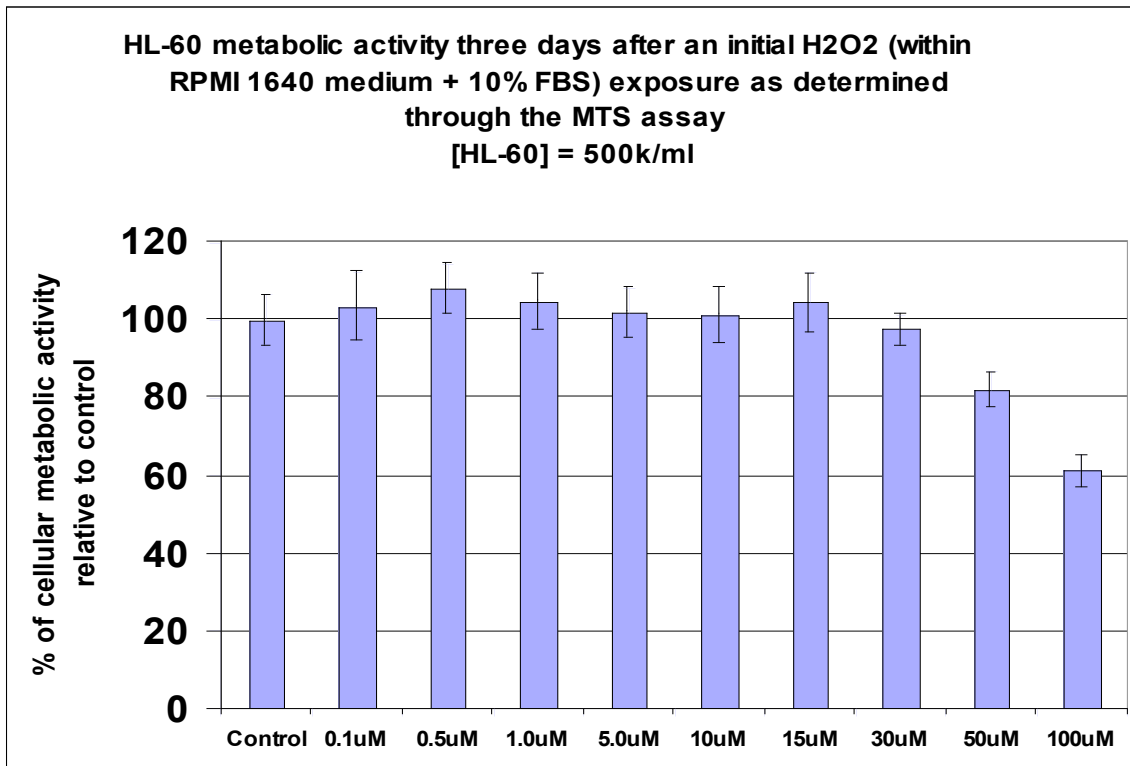


Fig. 7. H₂O₂ dose response curve of human leukemia HL-60 cell line metabolic activity as determined through MTS assay.

4. Discussion

The optical and near infra-red exposure finding (Fig. 2 and Fig. 6) has revealed a biphasic response in the glioblastoma metabolic activity. Although the bulk growth medium temperature immediately after the laser irradiation did not appreciably change ($\Delta T \sim 0.5^\circ\text{C}$), one plausible and speculative mechanism for the metabolic activity trend to return towards the control condition could be attributed to the intra-cellular temperature levels which may potentially reach hyperthermic levels in which case the cellular constitutive heat shock proteins would be immediately called into action to protect the integrity of cellular and mitochondrial proteins and the DNA from thermal damage. Alternatively, elevated and sustained levels of long lived reactive oxygen species, such as hydrogen peroxide, could be expected to mediate similar pro-active responses from the constitutive heat shock proteins.

In view of the fact that the catalase scavenger activity must reside outside of the cell membrane (since catalase is a fairly large protein and is not known to permeate through the cell membrane), we hypothesize that the mode of action for H_2O_2 to influence the cellular metabolic activity is likely to be through the H_2O_2 induced oxidation of cell membrane proteins or lipids on the outer surface of the cell membrane, perhaps resulting through the activation of PKC dependent pathways.

The most likely source in the production of H_2O_2 is within the mitochondria, due to the shuttling of electrons through the respiratory chain complexes, where certain fraction of the total number of shuttling electrons are picked up by molecular oxygen to yield super oxide anions O_2^- which would combine with protons would yield H_2O_2 . It is plausible that light absorption of appropriate chromophores within the respiratory chain complexes could speed up the electron shuttle processes which in turn would lead to faster rates of turn over from ADP into ATP along with the concomitant increase in the generation of super oxide anions O_2^- leading to greater production of H_2O_2 . The experimental evidence at hand strongly supports a dominant role of light generated H_2O_2 in bringing about metabolic modulations. We postulate that the strength and the overall bio-effect, i.e, stimulatory vs. inhibitory due to light induced H_2O_2 generation to depend upon two critical parameters, namely, (i) the efficiency of the cells to generate H_2O_2 through (narrow band wavelength) light absorption and (ii) the total number of cells

present to share the light induced / enhanced H₂O₂ production. From past literature [10], approximately, 6×10^9 to 3×10^{10} H₂O₂ molecules per biological cell have been noted to stimulate fibroblast cellular proliferation, whereas, an order of magnitude greater in the number of H₂O₂ molecules will initiate cells into a temporary growth arrest phase. Permanent growth arrest results when the H₂O₂ molecules are between 5.4×10^{11} to 8.43×10^{11} molecules per cell. Apoptosis is noted when the number of H₂O₂ molecules greater than 9×10^{11} molecules per biological cell.

5. Summary / Conclusions

Maximum metabolic suppression was observed at $10\text{J}/\text{cm}^2$ for the human glioblastoma. Addition of catalase in the growth medium prior to the laser exposure was found to diminish the laser induced metabolic suppression for all fluence treatment conditions, thus suggesting a functional role of H₂O₂ in the metabolic suppression. Taken together, our findings reveal that optical or near-IR low level light exposures could potentially be a viable tool in reducing the metabolic activity of cancers. Further research needs to be undertaken to determine exposure parameters which would optimize metabolic and cellular growth suppression in-vivo.

Acknowledgements

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