Inhibitory Effect of Photobiomodulation on the Proliferation Rate of the U87 Glioblastoma Cell Line

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ABSTRACT— Photobiomodulation therapy (PBMT) or Low level light Therapy (LLLT), is the stimulatory effect of light on the cell behavior. It has been considered as a potential therapeutic intervention. Glioblastoma is a malignant primary brain tumor without any effective treatment. This in vitro study investigated the effect of PBMT on proliferation rate and vital activity of human glioblastoma U87 cell line. Three different wavelengths were considered: 632 nm (red light, 2.1 mW/cm^2), 534 nm (green light, 1.2 mW/cm^2), and 457 nm (blue light, 6.5 mW/cm^2). The cell behavior was studied during a period of four hours up to 60 hours after irradiation. The irradiated cells were inspected by different assays for cell count, cell viability, cell death, and free radical production rate and were compared with the control non-irradiated ones. The results show a reduction in cell viability for all the three wavelengths. However, the effect is more pronounced for blue light. Cell death assessments, staining and flow cytometry, and NBT assay shows that blue light is not lethal, but that it reduces the free radical production rate. Temporal analysis shows that the maximum effect on cell proliferation will be observed around 48 hours after irradiation. It could be concluded that light, particularly shorter wavelengths, has an inhibitory effect on the in vitro proliferation rate of U87 cell line by affecting the energetics of the cell. The effect is stimulatory and persistent for periods comparable to cell doubling time.

KEYWORDS: Brain cancer, Glioblastoma, Low level light Therapy (LLLT), Light-tissue interaction, Photobiomodulation therapy (PBMT), Photobiostimulation, U87 cell line.

I. INTRODUCTION

Glioblastoma (GBM) is the most common malignant primary brain tumor [1], [2]. GBM has a dismal prognosis and it is very hard to treat it. The reason lies in the fact that it is very heterogeneous with multiple clones within one tumor containing varied genetic imbalances. The conventional therapies for GBM are surgery, radiotherapy, and temozolomide (TMZ) chemotherapy [3]. Most chemotherapeutic agents have no real beneficial effect on GBM patient's survival time [4] and even with improved surgical techniques and post-operative radiotherapy, the average survival time does not exceed 15 months [5]. Therefore, the need for new approaches to GBM treatment remains as intense as ever.

Low level light Therapy (LLLT), recently named photobiomodulation therapy (PBMT) or photobiostimulation, has been considered as a possible subsidiary treatment of some cancer types. It is a non-thermal interaction of cells or tissues with visible and near-infrared light, that is, 400-1200 nm. The term "low level" refers to the light energy densities less than 50 J/cm^2 or power densities less than 100 mW/cm^2 which are relatively low compared to the energy of the other forms of laser therapy which are used for ablation, cutting, and thermal coagulation. These low level energies can modu-

late the activity of the target cells [6]–[8]. Normally, lasers and light emitting diodes (LEDs) are used as the non-thermal and narrow wavelength spectrum light sources for PBMT [9]. The effect of PBMT varies in different cell types and depends on many of irradiation parameters including coherence, wavelength, energy, fluence, irradiation time, etc. It has been revealed that there is an optimal value for each parameter in any particular application. Higher or lower values may have no therapeutic effect [6], [10]. Despite a large number of studies, the mechanisms associated with the PBMT is not yet fully understood. However, the basic biological mechanism is thought to be through the absorption of light by endogenous chromophores, in particular, cytochrome c oxidase [10].

Photobiomodulation is noninvasive, fast, and safe. It also seems that does not interfere with medications. At the tissue level, it has a major role in the stimulation of wound healing, reducing inflammation, edema, and pain and relieving some neurological problems [11], [12]. At the cellular level, it is used to induce differentiation of stem cells, to increase or to inhibit cell proliferation, to change the activity of cells, and to induce cell death [13]–[16].

Based on these facts, an in vitro study was planned to investigate the effect of low level light irradiation on the proliferation rate of U87 glioblastoma cells. The effect was studied for several light wavelengths and different endpoints.

II. MATERIALS AND METHODS

A. *Cell Culture*

Human GBM cell line U87 (Pasture Institute, Tehran, Iran) was cultured in 25 cm^2 flasks with Dulbecco's Modified Eagle's Medium (DMEM, Product Code: 11966025, Invitrogen Inc. Gibco BRL, Gaithersburg, MD, USA), containing 10% (v/v) fetal bovine serum (FBS, Product Code: 12306C-500ML, Sigma-Aldrich Co., St. Louis, MO, USA) and 1% (v/v) penicillin-streptomycin solution (Product Code: P4333-100ML, Sigma-Aldrich Co.), and incubated in an atmosphere of 5% CO_2 at 37±0.2 ºC. The medium was changed every 3 days. After semi-confluency of flasks (80% of flask surface covered by a monolayer of cells), subculture was done and the cells were trypsinized (Product Code of Trypsin: T4549-100ML, Sigma-Aldrich Co.). Cells were plated at a cell density of 12×10^3 cells in each well of the 96-well plates and 80×10^3 cells in 35 mm dishes. Irradiated groups and control non-irradiated group consisted of 4 wells in square shape of multiwell plates or one 35 mm dish depending on the assessment. Irradiation was done 24 hours after seeding. The cells in different stages, after seeding, before treatment, and after treatment were visually investigated for the confluency, adherence, and morphology.

B. *Optical Setup*

The optical system consisted of an optomechanical setup and an LED-based light source. The optomechanical setup provided the necessary framework for applying the irradiation onto multiwell microplates or dishes that was compatible with the cell growth requirements. Its compact design allowed us to locate it within the CO_2 incubator. The light source was composed of multiple power LEDs (Huaxing, China) and appropriate lenses and diffusers to obtain the desired uniform distribution of light energy at the dish position. The light passed through an IR filter before impinging on the cells to eliminate any possible thermal effect. The intensity of light controlled by adjusting the LED currents by a programmable source and was monitored during the experiment to reach the desired stability. Light intensity was measured by a photodiode-based meter and its spectrum was registered by a spectrometer (Thorlabs, Germany).

C. *Assessment*

MTT Assay

The effect of PBMT on viability of glioma cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells to a purple formazan product that is insoluble in aqueous solutions. The amount of

MTT-formazan produced can be determined spectrophotometrically once solubilized in a suitable solvent. Cell proliferation is then determined indirectly by MTT dye reduction [17]. For the assessment, cell culture medium of each well was replaced by 200 µL of 1 mg/mL MTT (Product Code: M5655-1G - Sigma-Aldrich Co.) in DMEM and then was incubated at 37 ºC and 5% CO_2 for 2 hours. The reduced MTT formazan crystals were dissolved by 200 µL dimethylsulfoxide (DMSO) (Product Code: D8418-500ML, Sigma-Aldrich Co.). The optical density (OD) was then read at 490 nm by an Enzyme-Linked Immunosorbent Assay (ELISA) reader (Pharmacia Biotech, Stockholm, Sweden). The assay was done at the 4, 6, 8, 10, 12, 16, 20, 24, 36, 42, 48, and 60 hours of incubation after irradiation. The ratio of irradiated group OD to the control group OD was considered as a representation of viability. The viability percentage was plotted against the incubation time after irradiation.

Staining

The Trypan Blue assay (TB) was used for the cell counting and the investigation of cell death. The assay is based on the principle that normal cell or those that are in the early phase of apoptosis have intact plasma membrane. So they exclude trypan blue and remain unstained; whereas, dead cells or those that are in the late apoptotic stage are permeable to the dye and become blue [18]. At the 24 and 48 hours of incubation after irradiation, irradiated and control cells in 35 mm dishes were harvested by trypsinization, followed by mixing with an equal volume of trypan blue (Product Code: T6146, Sigma-Aldrich Co.). The resulting solution was incubated for 30 min and loaded into a hemocytometer counting chamber and the total number of cells, the number of viable (unstained) cells, and the number of nonviable (stained) cells per milliliter was counted. Four separate counts were performed for each dish. The experiments were performed in triplicate. The viability percentage was defined as the number of viable cells divided by the total cell number x 100. The number of cells per milliliter in the irradiated dish was compared with the ones for control dish and graphs were plotted for cell viability and cell number.

Flow Cytometry

Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Cat. No. APOAF) was used in order to identify apoptotic and/or necrotic cells. It is based on the membrane alteration of cells and their permeability during early and late stages of apoptosis. Briefly, control and irradiated cells were incubated with a solution containing annexin V-FITC and 7AAD (500 µL of binding buffer 1X plus 5 µL of annexin V-FITC plus 10 µL of 7AAD) after being extensively washed with PBS. After an incubation period of 10 min at room temperature, cells are washed again with binding buffer, then fluorescence-activated cell sorting (FACS) was performed by using flow cytometer (BD Biosciences, San Jose, CA, USA).

NBT Assay

The free radical production rate of cells could be detected by nitroblue tetrazolium (NBT) assay. The assay is based on the NBT reduction to the insoluble blue-black formazan due to the transfer of the electron from NADPH to NBT [19]. The spectrophotometric measurement of absorbance (OD) at specified wavelengths could be used to detect the rate of NBT reduction, i.e. formazan production, and to obtain an indirect measure of the cell energetics. To do the assay, at different incubation times after irradiation, cell culture medium of each well was removed and 200 µL of NBT (Product Code: M5655-1G - Sigma-Aldrich Co.) solution (335 µg/mL in DMEM) was added to it. After 2 hours incubation at 37 ºC and 5% CO_2, the resulting formazan was dissolved in 200 µL of 46% v/v DMSO + 54% KOH (2M). The absorbance of control and irradiated groups at 630 nm were determined by the ELISA reader.

D. *Treatment*

To determine the effect of light on the proliferation rate, vital activity, and death rate of the cells, two main experiment groups, control group (Ctrl) and irradiated groups (Light) were assessed by relevant tests. Both groups had similar conditions and had undergone the same

processes except for light treatment that was specific to the irradiated group. Light Groups consisted of blue light group (BL), green light group (GL), and red light group (RL). Each wavelength had its separate control group and was studied in an independent experiment. In addition, to study the temporal response of cells, various subgroups with different end times, 4, 6, 8, 10, 12, 16, 20, 24, 36, 42, 48, 60 hours, were designed for any specific light group. For example, BL-4 group for studying cells four hours after irradiation by blue light. Upon 24 hours incubation after seeding, all of the light groups were illuminated for a definite time of 15 minutes and at every designated interval time, the relevant group was taken out for doing different assays. Every experiment was done in triplicate.

The irradiation parameters for different wavelengths are shown in table 1. The energy densities were chosen based on pilot experiments and previous studies. These optimum values give the most profound effect in each case. The power densities were adjusted to give the desired energies for a fixed irradiation time.

Table 1. Irradiation parameters

Wavelenght λ (nm)	Width Δλ (nm)	Irradiation Time (min)	Power density (mW/cm^2)	Energy density (J/cm^2)
457 (blue)	±10	15	6.5	5.850
534 (green)	±15	15	1.2	1.800
632 (red)	±5	15	2.1	1.890

E. *Statistical Analysis*

Statistical analysis was performed using ANOVA method. Data are presented as the mean ± standard error of the mean (SEM). $p<0.05$ was considered as the significance level. Recording, descriptive and analytical statistical analysis and visualization of data were performed using Gnumeric software.

III. RESULTS

A. *Viability Test- MTT Assay*

The viability of treated U87 cells relative to control groups for different temporal endpoints was evaluated using the MTT assay. The temporal response in 24 hours time interval was determined by doing assay on 4, 6, 8, 10, 12, 16, 20, and 24 hours groups. The results for different wavelengths are shown in Fig. 1a. The viability percent appears to be time-dependent and shows an early reduction followed by a tendency to restoration. The time of maximum reduction and its value is different for various lights. Decreasing the wavelength, the effect is shifted away, such that it occurs around 8 hours for the red light, 12 hours for the green light and it continues to decrease in the 24 hours interval for the blue light. Further study of blue light for times of 36, 42, 48, and 60 hours, shown in Fig. 1b, reveals that the effect is more prolonged, about 48 hours after incubation and more profound, up to 18 percent compared to 7 and 6 percent for red and green lights, respectively. So, the incubation time of 48 hours after radiation was chosen as the endpoint for other assays of blue light.

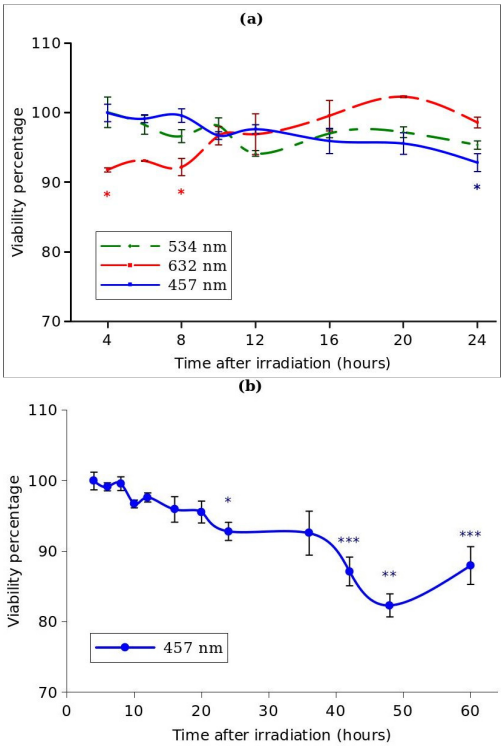
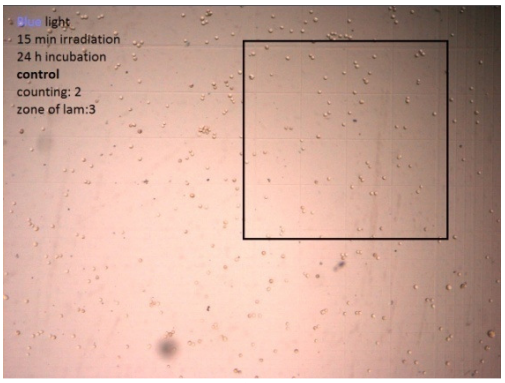


Fig. 1. MTT viability test results. a) Relative vital activity of U87 cells treated with light with different wavelengths during a period of 24 hours compared to control non-irradiated groups. b) The temporal changes of viability of treated cells with blue (457 nm) light during a longer period. (Note that *means

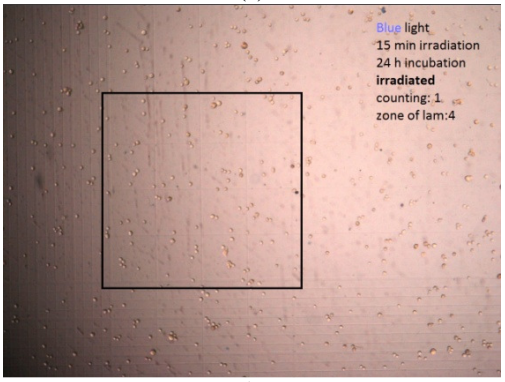
significant at p<0.05, **means significant at p<0.01, and *** means significant at p<0.001).

B. *Cell Counting- Trypan Blue Assay*

To reveal the effect of light on the cell count and cell death, trypan blue staining was done 48 hours after treatment with blue light. Figure 2 shows the image of cells in control and irradiated groups under the trypan blue test. The results of the test are shown in Fig. 3 that compares the cell numbers and cell death in the irradiated and non-irradiated groups. It is evident that the ratio of the dead cells to the total cells is actually the same in both groups, so light has not induced any lethal effect. But the number of cells has been significantly reduced, about 13%, in the irradiated group that means light had an inhibitory effect on the proliferation rate of cells. The results are in conformance with the viability test and show that the reduction in total vital activity is related to the cell number reduction.



(a)



(b)

Fig. 2. The sample images of cells under the trypan blue assessment for control and irradiated groups.

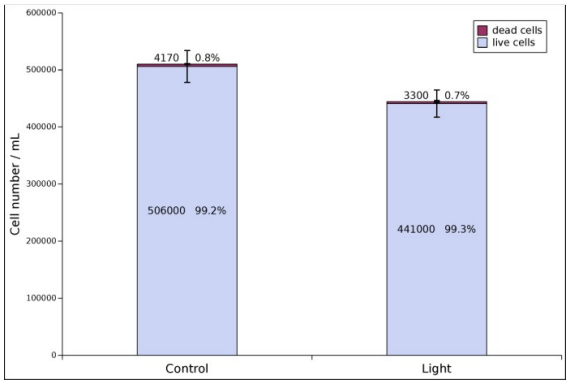


Fig. 3. Cell counting results of trypan blue assay. The number of live and dead cells and their percentages has been shown for two experimental groups. Light group denotes cells exposed to blue light irradiation for 15 minutes. Control group is the similar non-irradiated cells. The assay has been done 48 hours after the treatment.

C. *Cell Death Analysis- Flow Cytometry*

Flow cytometry analysis was done to determine the death rate and to analyze the ratio of apoptotic and necrotic deaths. The data of flow cytometry assay are shown in Fig. 4. The Annexin V-FITC values are plotted against 7AAD values for both of the non-irradiated control group and irradiated group. The Q3 region represents the live cells and shows a tiny difference between the two groups, around 92% in control group and 90% of cells in light group. The Q1 region shows the dead cells due to necrosis, about 6% in the control group and 5% in the light group. The apoptotic death is demonstrated in Q2 and Q4 regions. The apoptosis rate for two groups is a little bit different and irradiated group shows a very small increase, about 1.8% more than control in early apoptosis (Q4 region) and 1.2% in late apoptosis (Q2 region).

The results show that there is not any considerable difference in cell death between control and light treated groups and the light in doses used does not induce necrotic or apoptotic death. In summary, the effect of light on cell death is negligible, an outcome that is consistent with the results of trypan blue staining.

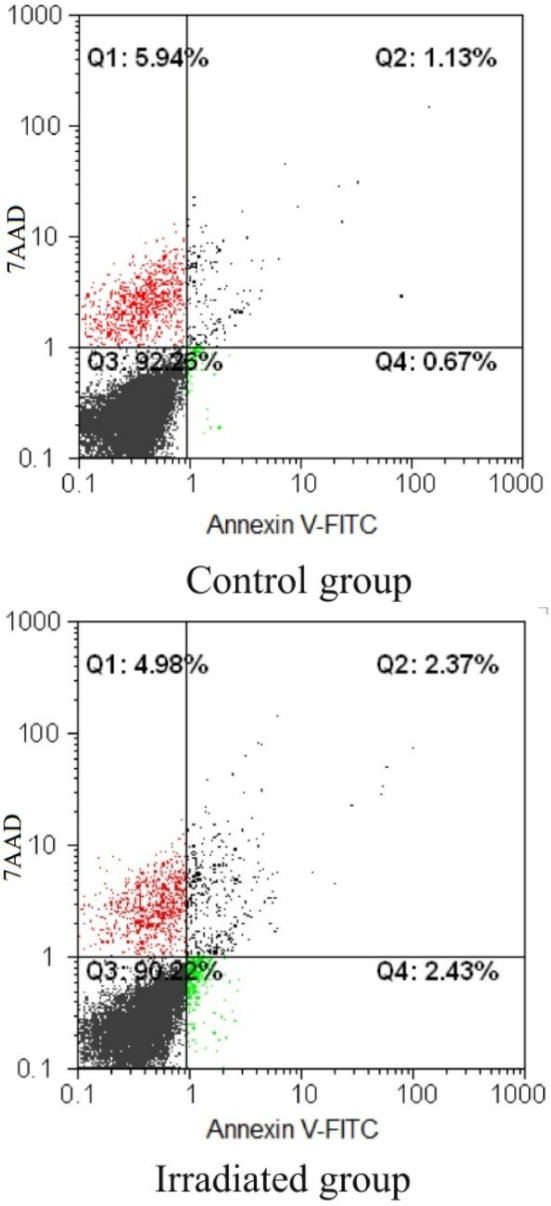


Fig. 4. Flow cytometry analysis of cell death in different experiment groups. The Q3 portion shows the live cells distribution. The necrotic dead cells, the cells in early stages of apoptosis and the cells in the late stages of apoptosis are shown in Q1, Q4, and Q2 regions, respectively. The analysis has been done on cells 48 hours after treatment.

D. *Free Radical Production Rate- NBT Assay*

To study the effect of light irradiation on the energetic of the cells, the NBT assay was done on corresponding light and control groups.

Temporal changes were measured during the first day after treatment and are shown in Fig. 5(a). The absorbance of light groups relative to the absorbance of control groups is depicted. It shows an initial modest decrease followed by an increase that peaks around the 16 hours after irradiation and a subsequent decrease to around the 85% of the control group.

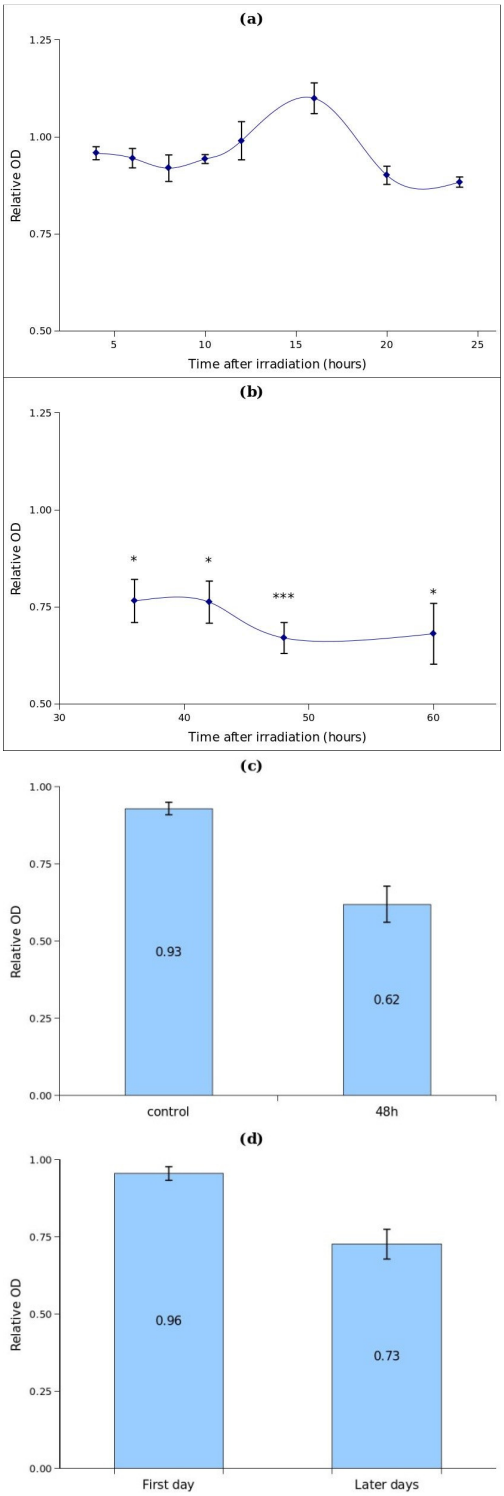


Fig. 5. Results of NBT assay on cells treated with blue light. a) Temporal changes during the first day

after irradiation. The optical density (OD) at 630 nm of irradiated group relative to the control non-irradiated one is shown against the time. b) The relative OD for longer times, extending from 36 to 60 hours. c) The comparison of NBT assay values of control non-irradiated group and light-treated group at the endpoint of 48 hours. d) The average value of NBT assay during the first day is compared to its value during second and third days for the blue light treated cells. (Note that *means significant at $p<0.05$ and ***means significant at $p<0.001$)

Longer term investigation, presented in Fig. 5(b), shows more decrease down to a nearly constant value of 70% during the next two days. The values for a chosen endpoint of 48 hours has been shown on Fig. 5(c) for the light and control groups that exhibit a significant decrease in the NBT reduction in treated groups. The overall effect of light on free radical production rate during the first day after treatment has been compared with the subsequent days in the part (d) of Fig. 5. It shows that the effect has been stabilized during the second day.

IV. DISCUSSION

The current study was aimed at the investigation of the photobiomodulation effects of light on the vital activity of human glioblastoma cell line U87. It was carried out to probe the effect of low level light irradiation at different wavelength diapasons on the proliferation rate of the cell line. The cell viability, the cell death, and the energetics of the cells were studied.

Few studies have investigated the effects of PBMT (LLLT) on cancer cells, especially brain cancer cells. Sroka et al. observed that 511 nm light irradiation decreases the mitosis of glioblastoma cells (U373MG) [20]. Murayama et al. investigated the effect of LLLT on GBM cells (A172) using 808 nm diode laser (15 mW/cm^2 and 20, 40, and 60 min irradiation) and observed a decrease in the cell proliferation rate [21]. In another study, Ang et al. studied the effect of LLLT with the wavelength of 405 nm and the power of 27 mW on this cell line. 48 hours post treatment, they observed induced necrosis and apoptosis following 1, 2, 3, 5, 10, 20, 40, and 60 minutes irradiation [16]. The same group studied the effect of phototherapy with 532 nm ND:YVO4 laser using the power of 60 mW and the irradiation times of 20, 40, and 60 minutes and showed that it significantly stimulate the cell proliferation rate of A172 cells [22].

The mechanisms involved in the biostimulation of photons are not yet fully understood. The most probable mechanism is the induced changes in the electron transport chain of the cytochrome c oxidase enzyme in the mitochondrial membrane [23], [24]. This chain provides the required energy for the ATP production. The light interaction increases mitochondrial membrane potential and alters the ATP, nitric oxide, superoxide and free radical generation rate. All of these are related to the cell energetics and its overall effect could be observed in the vital activity and proliferation of cells. In general the photobiostimulation does not have any lethal effect and does not lead to an increased level of apoptosis. The effects are light power and energy dose dependent that largely varies for different type of cells and tissues. It is known that there is an optimum value for the energy [23]. The very low doses do not trigger any observable effect. Higher energies have a stimulatory effect and the very high doses of light energy act as inhibitor. The effects appear at different time scales after irradiation. Due to different pigments, molecules, and cellular organelles that are involved in the process, a wavelength dependency is also expected and observed [24].

Considering the obtained results of the current study, there is a significant reduction in vital activity of U87 cell line due to the treatment with light. The assays show that light has no lethal effect and does not significantly increase the cell death. So, the reduction in vital activity could be attributed to the decrease of cell energetics as shown by the observed step-down in free radical production rate of cells irradiated by light. The effect was observed for light with different wavelengths, all having a common trend. The effect is stimulatory and develops after the treatment, so it can be labeled as photobiomodulative. It is long-lasting compared to the duration of 15 minutes irradi-

ation and lasts to time intervals comparable to cell doubling time. The effect is enhanced by increasing photon energy, that is, decreasing the light wavelength, not to be confused with irradiated energy or light dose, so that the blue light (457 nm) has the most intense consequence. Additionally, decreasing the wavelength causes the effect to appear later but it lasts more. Regardless of the wavelength used, the effect finally starts to diminish.

In summary, our experiments showed a time dependent decrement of cells viability based on MTT assay, and a low decrease in the cell counts without increasing the dead cell proportion based on the trypan blue staining. The non lethal effect of irradiation was supported by flow cytometry cell death analysis that shows no alteration of cell death pattern. The NBT assay showed a reduction of the free radical production rate that supports the idea of probable alteration of cythchrome c oxidase activity due to light as the involved mechanism.

Comparing with previous studies, our results is in agreement with the above mentioned works of Sroka et al. [20] and Murayama et al [21] that show a decrease in proliferation rate, but they differ from observed increase in proliferation of Fukuzaki et al. [22]. Also, it was not observed any induced death as reported by Ang et al. [16]. These discrepancies may be attributed to the difference in cell lines and different light doses and wavelengths used. Since the electron transport chain of the mitochondria, via the photoreceptors of cytochrome C oxidase, plays the main role in the photon absorption of cell and consequent PBM processes, it is expected that cells that are richer in mitochondria, including neural cells, absorb light more efficiently and the inhibitory effect of light begins at lower doses. The current study should be expanded focusing on different cell lines.

V. CONCLUSION

Our results from this in vitro study show that PBMT treatment of human glioblastoma cell line U87 decreases their vital activity through reducing the proliferation rate of cells by affecting their energetics. It has no lethal effect on cells but it stimulates processes that restrain the proliferation mechanisms. It is not wavelength-specific but decreasing the wavelength, the effect becomes more profound and more persistent.

The following restrictions must be taken into account. First, the results are based on an in vitro study and could not be readily generalized to in vivo or ex vivo conditions or to the tissues. Second, the effect is observed for a specific cell line and may be different for the others. Third, it is well known that the PBMT effect is dose-dependent, so the results could not easily be generalized for much lower or much higher doses. In addition, the possibility of changing the regime of irradiation for obtaining enhanced effects could be considered for further studies, for example, the intermittent, periodic or sporadic, irradiation may lead to more profound and more lasting effects.

ETHICAL ASPECTS

This article does not contain any studies with human participants or animals performed by any of the authors.

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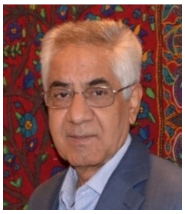

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