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Low-Level Laser Irradiation Modulated Viability of Normal and Tumor Human Lymphocytes In Vitro

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Abstract

Introduction: Laser radiation is a promising strategy against various malignancies. Recent studies have shown that the application of low-power laser therapy (LPLT) at different doses and exposure times could modulate the growth dynamic of tumor cells. Based on the type of laser, LPLT could potentially trigger cell proliferation, differentiation, and apoptosis in different cell lines.

Methods: In this study, MTT assay was used to monitor the effect of low and high laser intensities on the viability of normal and cancer lymphocytes. The protein levels of Ki-67 (a proliferation marker) and Caspase-3 (an apoptosis factor) were measured in human peripheral mononuclear cells (PBMCs) and the B-lymphoblastic cell line (Nalm-6) using flow cytometry after being-exposed to 630-nm LPLT at low (2, 4, 6, and 10 J/cm^2) and high (15, 30, 60, and 120 J/cm^2) energy densities in a continuous mode for 48 and 72 hours.

Results: By using higher energy densities, 60 and 120 J/cm^2, a significant decrease was shown in the viability of Nalm-6 cells, which reached 6.6 and 10.1% after 48 hours compared to the control cells ($P<0.05$). Notably, Cell exposure to doses 30, 60, and 120 J/cm^2 yielded 7.5, 12.9, and 21.6 cell viability reduction after 72 hours. The collected data showed that the high-intensity parameters of LPLT (15 to 120 J/cm^2) promoted significant apoptotic changes in the exposed cells coincided with the activation of Caspase-3 compared to the none-treated control cells ($P<0.05$). The data further showed the stimulation of the Ki-67 factor both in primary PBMCs and the lymphoblastic cell line treated with LPLT at energy densities of 4 and 6 J/cm^2 ($P<0.05$), indicating enhanced cell proliferation. Similar to Nalm-6 cells, primary PBMCs showed apoptosis after 48 hours of being exposed to doses 60, and 120 J/cm^2, indicated by increased Caspase-3 levels ($P<0.05$). As expected, the Nalm-6 cells were resistant to cytotoxic effects of laser irradiation in the first 48 hours ($P>0.05$) compared to normal PBMCs. The exposure of Nalm-6 cells to low-intensity laser intensities increased a proliferation rate compared to the PBMCs treated with the same doses.

Conclusion: We showed the potency of LPLT in the induction of apoptosis and proliferation in human primary PBMCs and Nalm-6 cells in a dose and time-dependent manner after 72 hours.

Keywords: Low-power Laser therapy; Leukemia; Peripheral blood mononuclear cells; Proliferation; Apoptosis.

Introduction

Acute lymphoblastic or lymphocytic leukemia (ALL) is typically characterized by the uncontrolled expansion of immature progenitors of lymphocyte lineage. According to statistics, 80% of affected children and less than 40% of adults are cured after different modalities.[1,2] Recent studies have shown that low-power laser therapy (LPLT) could be touted as a promising strategy against various cancer types.[3-7] Some recent data have shown that LPLT is applicable at different doses or exposure times to inhibit and/or promote cell growth. In addition, the type of radiation, the pulse or continuous wave laser, could also affect the dynamics of target cells. In this regard, low-level laser radiation has the potential for triggering different cellular bioactivities, mainly proliferation, differentiation, and apoptosis.[7-9] It has been shown that low-level laser therapy stimulates cell metabolism in different cell types such as HeLa cells, Schwann cells, and fibroblasts.[10-12] Data from *in vitro* and *in vivo* experiments showed that LPLT had healing effects on tissues like skin, cartilage, tendons, muscles, and nerves by promoting photoreceptors.[13-16] These elements have a capacity for absorbing lights

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and initiating a metabolic cascade reaction inside the mitochondria that is governed by an enzyme, namely cytochrome C oxidase.[17] In contrast to the beneficial effects of LPLT, the promotion of pro-inflammatory mediators and consequent cell death were also seen after applying distinct doses and laser intensities.[18] Both stimulatory and inhibitory effects of light radiation are generated by the same photoreceptors inside the cells.[18,19] For instance, the exposure of human lung adenocarcinoma cells to laser radiation at 120 J/cm^2 induced apoptosis by promoting mitochondrial-Caspase-3 signaling pathways.[6,7] Wu et al proved that LPLT radiation increased the production of reactive oxygen species (ROS) and activated apoptotic factors, notably Bax in multiple cancer cell lines, including ASTC-a-1, HepG2, HeLa, and A549 cells.[6,20] In this study, we evaluated the proliferation and apoptosis of human primary peripheral mononuclear cells (PBMCs) and B-lymphocytic cell lines (Nalm-6) after being exposed to high and low energy densities of LPLT in an *in vitro* condition during 48- and 72-hours post-irradiation.

Materials and Methods

Sampling and Cell Expansion

We purchased the ALL cell line (Nalm-6; NCBI code: CCRF-CEM) from the Iran National Cell Bank (Pasteur Institute, Tehran) and maintained it in an RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% Pen-Strep solution (Biosera). Human PBMCs were also isolated from the blood samples of healthy donors using Ficoll-Paque Plus (Ref no: 002041600, Inno-train) and then diluted with an equal volume of phosphate-buffered saline (PBS). The samples were gently overlaid to the same volume of Ficoll-Hypaque solution and centrifuged at 400 g for 40 minutes at 4°C. After the completion of centrifugation, PBMCs at the interphase between Ficoll-Hypaque and PBS were carefully collected by sterile pipettes and washed twice with PBS. To expand the cells, freshly isolated mononuclear cells were suspended in a culture medium of RPMI-1640 supplemented with 10% FBS and 1% Pen-Strep solution and transferred into Ultra-Low adherent culture plates (Greiner; Germany). The culture plates were maintained at a conventional atmosphere with 5% CO_2 and 90% humidity. The cell media were replaced every 3-4 days.

Laser Radiation Procedure

We used continuous-wave red laser light using a GaAlAs diode laser (Model: Mustang 2000+, Russia) at a wavelength of 630 nm. The output power was adjusted to 34 ± 2 mW with the acupuncture nuzzle of 6.6 mm in diameter. The Gaussian laser-beam feature was applied to all cells. For laser treatment, 5×10^5 cells from the primary cells and Nalm-6 line were seeded separately in Ultra-Low adherent 96 well plates (Greiner; Germany). The laser beam area was 0.33 cm^2 and the power densities reached 99.43 mW/cm^2 at energy densities of 2, 4, 6, 10, 15, 30, 60, and 120 J/cm^2. Radiation time was adjusted to a range of 20 to 1200 seconds for different time periods. The cells were exposed to low-level laser radiation for two consecutive days and subjected to MTT and flow cytometry analyses.

MTT Assay

We performed a conventional survival assay, namely MTT analysis, in order to assess the dose-dependent proliferative and cytotoxic effects of LPLT. MTT analysis was performed using the 3-[4, 5-dimethylthiazol-2-yl] -2, 5 diphenyltetrazolium bromide (MTT, Sigma). The plates were incubated at 37°C with 5% CO_2 for 4 hours. During this procedure, vital cells absorbed MTT and produced formazan crystals. After 4 hours, we added 100 µL solubilizing solutions, 10% sodium dodecyl sulfate in 0.01 M HCl. Then, the plates were placed on the shaker and agitated for 20 minutes to dissolve the formazan crystals. The final absorbance was measured at different wavelengths ranging from 570 nm to 630 nm. Finally, cell viability was calculated based on the percentage of the time-matched control group.

Antibodies and Flow Cytometry Analysis

Following the irradiation protocol, the treated cells were analyzed by flow cytometry to evaluate the protein levels of Caspase 3 and Ki-67. The cells were collected and washed several times using PBS and then were permeabilized by using the Cytofix/Cytoperm solution (Cat No: 51-6896KC, BD Biosciences) at 4°C for 20 minutes. The washing procedure was again repeated by using the Perm/Wash solution (Cat No: 51-6897KC, BD Biosciences) followed by incubating the cells in a 20 µL PE-conjugated mouse anti-human Caspase 3 antibody (Cat No: 550914 BD Pharmingen active caspase-3 apoptosis kit) and a 5 µL PE-conjugated mouse anti-human Ki-67 antibody (Cat No: 12-5699-42; eBioscience, Inc.; USA) for 20 minutes at room temperature. After being washed twice with PBS, the cells were analyzed by the BD FACSCalibur system (BD Bioscience) and the collected data were processed by using FlowJo software ver. X.0.7.

Statistical Analysis

The data are expressed as mean ± SD. The statistical analysis was done by using one-way ANOVA and the Tukey's HSD test. $P<0.05$ was considered statistically significant.

Results

The Effects of LPLT on the Viability of PBMCs and Nalm-6 Cells

We used the MTT assay to determine the rate of cell viability in PBMCs and Nalm-6 after exposure to different laser intensities. Cell irradiation at energy densities of 4 and 6 J/cm^2, but not 2 J/cm^2, was shown to stimulate

PBMCs proliferation after 48 hours, reaching 11.6 and 9.9% respectively compared to the control group ($P<0.05$; Figure 1A). These values were 8.7, 20.9, and 30.4% after 72 hours at energy densities of 2, 4 and 6 J/cm^2 ($P<0.05$; Figure 1A). The use of the same protocol at energy densities of 2, 4, 6 and 10 J/cm^2 stimulated Nalm-6 cell proliferation and yielded similar results (7.4, 14.9, 13.6, and 7.9% respectively) compared to the non-treated control cells ($P<0.05$; Figure 1B). Based on the data from the MTT panel, cell exposure to different laser intensities ranging from 2 to 10 J/cm^2 increased the viability of Nalm-6 cells to 11.6, 49.4, 24.9 and 10.6% respectively after 72 hours ($P<0.05$; Figure 1B).

The viability of PBMCs significantly decreased in groups 60 and 120 J/cm^2 (8.4 and 13.2%) after 48 hours compared to the control group ($P<0.05$; Figure 1A). Higher cytotoxic effects were observed in these cells at energy densities of 15, 30, 60 and 120 J/cm^2 72 hours post-irradiation (8.8%, 12.8%, 18.4% and 26.2% respectively) compared to the control cells. Similar to these results, a significant decrease (6.6% and 10.1% respectively) in the survival of Nalm-6 cell lines was observable at energy densities of 60 and 120 J/cm^2 after 48 hours compared to the control Nalm-6 cells ($P<0.05$; Figure 1B). As expected, Nalm-6 treatment with the doses of 30, 60, 120 J/cm^2 contributed to 7.5, 12.9, and 21.5% cell reduction after 72 hours ($P<0.05$; Figure 1B). Based on our results, it seems that the primary cells are more sensitive to stimulatory and inhibitory effects of laser irradiation at distinct doses in comparison with the immortalized cell line, Nalm-6.

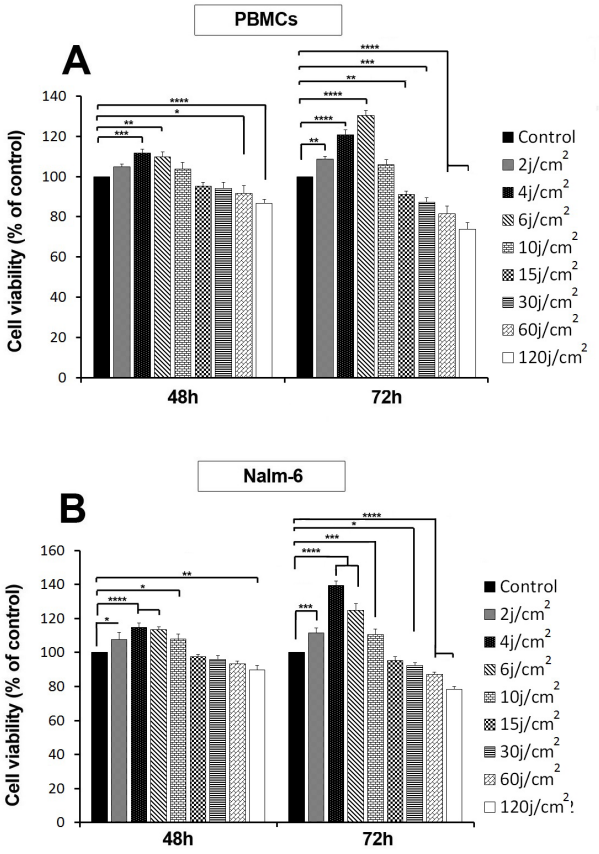

Figure 1. Measuring PBMCs and Nalm-6 Survival Rate by MTT Assay After the High- Intensity and Low-Intensity Laser Radiation (A-B). Data are expressed as mean ± SD (n = 6). Values correspond to mean ± SD. The cell viability of both cell types in the LLLI-treated group showed a significant difference compared with the control group (non-irradiated). Differences between the control and treated groups are significant at $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ (ANOVA and the Tukey HSD test).

The Induction of Apoptosis at High- Fluence LPLT

Following cell exposure to higher laser intensities at the range of 30, 60 and 120 J/cm^2, we evaluated apoptosis by monitoring the intracellular levels of Caspase-3 (a marker of apoptosis) via flow cytometry analysis. 48 hours after irradiation, Caspase-3 positive PBMCs were 1.5%, 2.5%, and 2.9% and the cells from group 60 and 120 J/cm^2 showed significant changes compared to the control PBMCs ($P<0.05$; Figure 2A-B). These values reached significant levels (14.9%, 55.6%, and 60.7%) after 72 hours compared with the control group ($P<0.05$; Figure 2A-B). In contrast to PBMCs, the treatment of Nalm-6 with the same doses caused a non-statistically difference in the percent of the Caspase-3 positive cells (1.1%, 1.6%, and 1.9%) after 48 hours. It seems that these values increased to significant levels (3.9%, 6.3%, and 15.8%) after 72 hours in the cells from 30, 60 and 120 J/cm^2 groups ($P<0.05$; Figure 2C-D). It was shown that the induction of apoptosis and Caspase-3 activity correlated with the doses and time periods. In addition, these data showed that tumor cells were more resistant to similar energy intensities than normal PBMCs. In addition, the cytotoxic and promotive effects of laser irradiation were determined by time.

Cell Proliferation Stimulated by LPLT at Low Energy Density Doses

The analysis of the data from flow cytometry analysis showed that there was a significant increase in the percent of Ki-67 positive PBMCs (23.7, and 15.6%) 48 hours after being exposed to doses 4 and 6 J/cm^2 ($P<0.05$; Figure 3A-B). 72 hours after irradiation, we found an increase in the proliferation of Ki-67 PBMCs (26% and 35.4%) compared to the non-treated control group. In Nalm-6 cells, the application of doses 4 and 6 J/cm^2 caused enhanced cell proliferation (7.6% and 10.2%) in comparison with the control group ($P<0.05$; Figure 3C-D). By the time, the percent of Ki-67 positive Nalm-6 cells reached 6.5 and 10.2% compared to the control group ($P<0.05$; Figure 3C-D). These data stand for a fact that the use of lower LPLT doses could increase the rate of cell proliferation by time in either normal or cancer cells.

Discussion

In favor of the clinical approach, red or infrared laser diodes at a range of 10 to 100 mW have routinely been used for therapeutic approaches.[21] However, such laser radiation

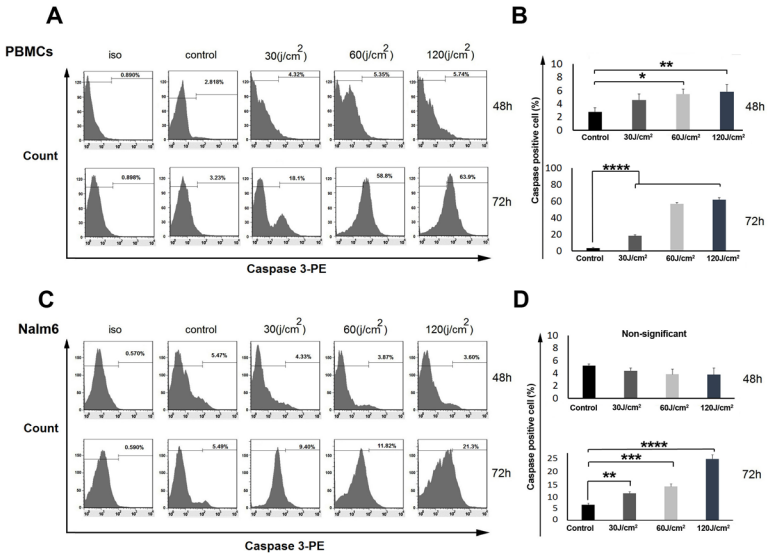

Figure 2. Measuring Apoptosis (Caspase-3 Levels) in Laser-Irradiated Cells by Flow Cytometry Analysis (A-D). Flow cytometry analysis revealed the induction of Caspase-3 in PBMCs (A-B) Nalm-6 cells (C-D) after exposure to higher laser doses, 30, 60 and 120 J/cm^2, analyzed after 48 and 72 hours. Data are expressed as mean ± SD (n=6). Differences between the control and treated groups are significant at $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ (ANOVA and the Tukey HSD test).

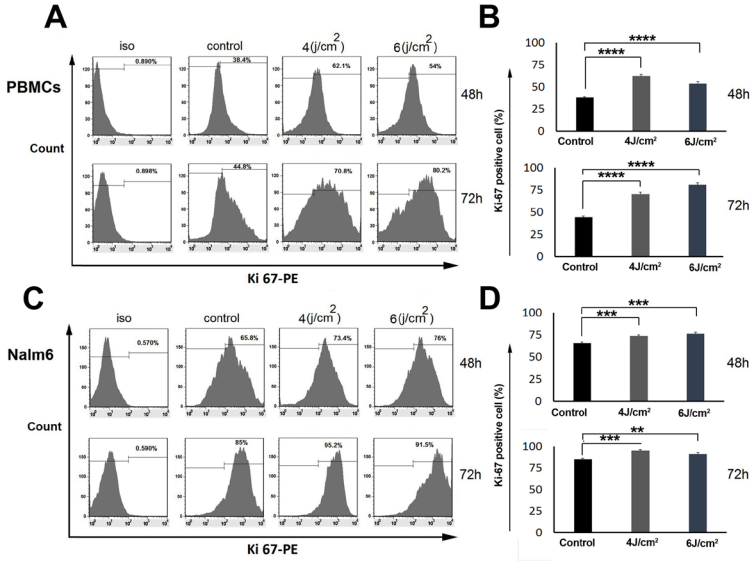

Figure 3. Flow Cytometric Analysis of Cell Proliferation By Monitoring Ki-67 in PBMCs (A-B) and Nalm-6 Cells (C-D) Exposed to Lower Laser Energy Intensities After 48 and 72 Hours. Data are expressed as mean ± SD (n=6). Differences between the control and treated groups are significant at $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ (ANOVA and the Tukey HSD test).

as an alternative clinical modality for the inhibition of cancers is still under investigation. Notably, results have shown the inhibitory effects of laser irradiation on cancer cell proliferation.[22] In this regard, Wang et al have shown that human lung adenocarcinoma cells (ASTC-a-1) enter apoptosis 6 hours after exposure to high-intensity LPLT (60 and 120 J/cm^2).[5] They also noted the efficiency of Caspase-3 induction by using the fluorescence resonance energy transfer method.[5] These modalities contributed to the superior effect of phototherapies such as high-intensity LPLT on human cancer cells. It was shown that LPLT at higher energy intensities was able to generate large amounts of singlet oxygen that in turn initiated the oxidation of a large variety of biological molecules in DNA damage and apoptosis initiation.[23] Also, Huang and co-workers confirmed the involvement of the Akt/GSK3β pathway and the expression of Bax in cells soon after cell exposure to LPLT.[24,25] In laser-irradiated cells,

viability diminished by the abrogation of Nox4 and surviving activities.[26] Another possible mechanism that makes the cells sensitive to irradiation correlates with the reticulum endoplasmic stress and the induction of autophagy modulators such as activating transcription factor-4.[27,28] It was recently shown that the treatment of human ovarian cancer and endothelial lineage with laser irradiation at high energy intensities was able to bring about oncostatic effects by the simultaneous expression of heat shock protein 70 after 72 hours.[29] In addition to different cytotoxic properties, various mechanisms for the mitogenic effects of LPLT have also been suggested at specific intensities.[30,31] Therefore, it is reasonable to claim that the cell survival rate is dependently related to the irradiation intensity and the time period.

Our results showed that Ki-67 expression was stimulated in both primary PBMCs and lymphoblastic cell lines, especially at energy densities of 4 and 6 J/cm^2. In line with our results, Prabhu et al have also shown that the Ki-67 and proliferating cell nuclear antigen (PCNA) expression increased by laser exposure in rat models for healing.[32,33] They have also claimed that a single exposure of cells to 2 J/cm^2 was able to increase cell proliferation of basal cells, whereas the application of a higher dose at 10 J/cm^2 inhibited the expression of Ki-67 and PCNA after 10 days. In this regard, Taniguchi et al showed that the upstream signaling cascade of PCNA and Ki-67 in synovial fibroblasts played a role in the activation of p15 during photo bio-stimulation.[34] In parallel, the application of He-Ne laser radiation (632.8 nm) increased the translational expression of cyclin D1 in mouse satellite cells.[35,36] Other experiments revealed that photobiomodulation stimulated mitochondrial enzymes in the respiratory chain, affecting electron transfer and increased the intracellular calcium content. These changes, in turn, enhance cell proliferation.[17,37,38] It is believed that the mitochondrial cytochrome C oxidase could absorb red to near-infrared light, which is one of the respiratory chain components, resulting in the generation of ROS and adenosine triphosphate (ATP).[38]

Conclusion

In conclusion, we showed that both apoptosis and proliferation could be induced in the primary PBMCs and the B-lymphocyte cell line (Nalm-6) by LPLT at different energy intensities and time periods. We also confirmed that the bio-stimulatory effect of LPLT on PBMCs and Nalm-6 cells was dose- and time-dependent and in doses of 2, 4, 6 and 10 J/cm^2 could increase the cell proliferation rate while higher energy rates ranging from 15 to 120 J/cm^2 were able to stimulate cell apoptosis. We concluded that special doses of LPL radiation must be selected for therapeutic goals and achieving a desirable modulatory effect on the proliferation and apoptosis activities of cancer cells.

Ethical Considerations

All procedures performed in this study involving human participants were in accordance with the ethical standards of the research committee of Tabriz University of Medical Sciences (ethical code: TBZMED.REC.1394.585) and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

Conflict of Interests

The authors declare no conflict of interest.

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