Original Article

The Effect of Low-Level Laser Therapy on Human Leukemic Cells

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Abstract:

Introduction: Laser phototherapy is used for the treatment of chemotherapy-induced oral mucositis in patients with leukemia, although there are limited data supporting the safety of this method. This study aimed to evaluate the effect of different doses of low-level laser on proliferation of acute myeloid leukemia (AML) cell line (KG-1a) in vitro.
Methods: A plastic flask containing 5,000,000 KG-1a cultured cells was provided by Iran Pasteur Institute. KG-1a cell line has been produced from the bone marrow aspirate of a 59-year-old white male with acute myelogenous leukemia. Upon completion of the proliferation steps of KG-1a cell line, 7×10^4 cells were placed in 96-well tissue culture plates. All the surrounding wells were filled with Wright-Giemsa stain in order to prevent laser from scattering to the neighboring wells. In total, 28 plates were prepared using this method. After a forty-eight hours incubation period, irradiation was performed in continuous mode with an infrared laser of 810nm wavelength. After 24 hours, cells cultures were exposed to one, two, or three applications of laser irradiation. Irradiation exposures were performed at energy densities of 5, 10, and 20 J/cm^2. Each experiment included 18 replicates for each application of laser and 6 replicates of negative/untreated controls. For experiments with two and three repeated exposures, the irradiation applications were separated by 48 hours. All the culture plates were incubated for seven days. Cell proliferation was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay after seven days. Spectroscopy (620nm) was used to determine the optical density (OD) of both irradiated and control samples.
Results: Significant increase in cell proliferation was seen only after two exposures at energy density of 20J/cm^2 (P=0.021).
Conclusion: Although LLLT is commonly used to treat radiotherapy- or chemotherapy- induced mucositis, as long as further studies demonstrate that different wavelengths and doses of laser phototherapy are safe and effective in treatment of mucositis, clinicians should remain cautious regarding the use of this treatment modality to treat patients with malignancies.
Keywords: low-level laser therapy; leukemia; cell culture.

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Introduction

Low-level laser therapy (LLLT) has been used in medicine since the late 1960s [1]. In LLLT, monochromic light with long wavelength in the range of red and near infrared is used to cure different soft tissue and neurologic conditions in a non-thermal and non-destructive fashion [2]. The first reports of LLLT use in 1960s and 1970s were

interpreted with great suspicion and since then many in vitro studies, animal models, and clinical studies have been conducted in order to evaluate the potential beneficial effects of laser irradiation [1].

Today, LLLT is used with great success in many dental and medical procedures [3]. Treatment with low-level laser has become popular in a variety of clinical applications such as pain management and promotion of wound healing.

Low-level laser therapy is greatly effective in reducing inflammation after injury, accelerating soft tissue healing and inducing angiogenesis [4]. It is believed that the effects of LLLT on wound healing are the result of increased cell proliferation [5]. It is suggested that the observed healing is due to photochemical interactions [3]. Since the evaluation of effective variables related to pain and tissue repair in animal studies or clinical trials is difficult, it is imperative to perform in vitro studies to better understand the effects of laser therapy.

In the last 25 years, in vitro studies have become popular worldwide. This is especially true for studies evaluating drug interactions and anti-tumor procedures. Recently, in vitro studies have been used to evaluate the effects of laser therapy on different cells. Using this method, one can evaluate the effects of different agents on cell lines of different origins. Another unique advantage of this methodology is to avoid possible adverse events associated with human and animal models. There is no agreement regarding the stimulatory effects of laser on cells, as there are reports of both stimulatory and inhibitory effects of low-level laser on cell cultures [5].

External factors such as energy, wavelength, potency, and also photoreceptors, which are part of the respiratory cycle, might be associated with inhibitory or stimulatory effects of laser irradiation. In other words, the effect of laser is dose–dependent [5].

The potential for cell proliferation after irradiation with low-level laser has raised concern about the possibility of increased tumor growth in neoplastic diseases. The stimulatory effects of irradiation with 809nm laser on the proliferation of epithelial carcinoma cell in vitro have been previously exhibited[4]. However, to date there is no evidence that LLLT could induce carcinogenesis.

The use of malignant cells in cell cultures has been reported elsewhere [5]. In previous studies, various tumor cell lines were irradiated with low-level lasers, under different conditions, with conflicting results [6]. Reports showed increased cell proliferation in cultures prepared from melanoma, cervical, and breast cancer cells after irradiation with argon laser [5]. In one study using 805nm laser for phototherapy on gingival squamous cell carcinoma cultured cells (ZMK), urinary tract carcinoma cells (J82), and normal epithelial cells of urinary tract (HCV29), an increase in mitosis of HCV29 and J82 was observed with $4J/cm^2$ and a decrease in mitosis was observed with $20J/cm^2$. Gingival SCC cells showed a decrease in number of mitosis with both doses [7]. Differences in genotype and phenotype in cell lines, as well as different parameters used in LLLT could explain these results.

It remains unclear how laser affects normal and malignant cells; thus, many clinicians are uncertain about the application of LLLT as a treatment modality [4]. Therefore, we believe that before using laser as a cancer treatment modality, the effects of LLLT on tumor growth should be studied in both in vitro environment and animal models.

Due to the increased evidence for the efficacy of laser therapy and the better understanding of its effects, in recent years investigators have examined new clinical applications of laser. One of these novel clinical approaches is the application of laser to heal injured epithelium. Recently, low-level laser has been extensively used in the prevention and treatment of mucositis caused by radiotherapy and chemotherapy in patients with leukemia [8-10]. However, in our review, we were not able to find any studies, which evaluated the effects of laser on malignant blood cells. Therefore, it is imperative to determine the safety and efficacy of laser therapy by evaluating its dose-response characteristic in vitro, before widespread application of LLLT as therapeutic modality to treat cancer.

While the effect of laser on cell proliferation remains controversial, LLLT is commonly used to treat mucositis secondary to chemotherapy. Therefore, our aim in this study was to evaluate the effect of LLLT on the proliferation of leukemic cells. Our goal was to find a suitable and reliable alternative for management of chemotherapy- or radiotherapy-induced oral mucositis in patients with leukemia.

Methods

Cell culture

A plastic flask containing 5,000,000 KG-1a cultured cells was provided by Iran Pasteur Institute. KG-1a cell line has been produced from the bone marrow aspirate of a

59-year-old white male with acute myelogenous leukemia. The cells showed great morphologic polymorphism. Myeloblasts and promyelocytes formed the majority of the cells, while few mature granulocytes, macrophages, and eosinophils were also present.

In order to provide adequate cells for the study, the cells were grown in plastic culture flasks in a medium containing RPMI 1640 Gibco with 10% fetal bovine serum (FBS), 100µg/ml streptomycin, 100 IU/ml penicillin G and two mmol/lit L-Glutamine at 37ºC in a humidified atmosphere containing 5% CO_2.

Before any experimentation, all the cultures were examined under light microscopy. The viability of the cultures was confirmed by Trypan blue exclusion test of cell viability, as this substance easily enters the nonviable cells, leaving the normal ones colorless. We used the following formula to calculate cell viability:

Viability = number of vital cells × 100 / number of dead cells

Forty-eight hours before phototherapy, a cell suspension containing 7×10^4 cells/100µl was prepared. Flat-bottom 96-well tissue culture plates were used for the irradiation experiments. One hundred microliters of the suspension was placed in six wells in order to provide maximal distance between cells to prevent light from scattering to other wells.

All the wells surrounding the cell-containing well were filled with the Wright-Giemsa stain in order to prevent laser from scattering to the neighboring wells. In total, 28 plates were prepared by this method. The plates were incubated at 37ºC and one atmosphere pressure with 5% CO_2 for seven days.

Laser phototherapy procedure

We used continuous wave infrared laser for irradiation (wavelength 810nm, 0.1w, Wuhan Gigaa Optronics Technology Co., Ltd. Diode, China).

The focal point of the probe was 0.5 cm and equal to the diameter of culture plate wells. The probe was attached to the floor of the wells.

After 24 hours, cultures of cells were exposed to one, two, or three applications of laser irradiation. Irradiation exposures were performed at energy densities of 5, 10, and 20 J/cm^2. Each experiment included 18 replicates for each application of laser and 6 replicates of negative/ untreated controls. For experiments with two and three repeated exposures, the irradiation applications were separated by 48 hours. All the culture plates incubated for seven days.

Evaluation of proliferation

On day seven post-irradiation, the cell proliferation was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay [5]. The MTT colorimetric test is an established and very popular method for quantification of viable cells in proliferation and cytotoxicity studies specially in vitro studies. The basis of this test is on mitochondrial enzymes function to form a soluble blue formazan product from yellow tetrazolium salt, MTT. The amount of formazan product is directly proportional to the number of living cells, present in the time of MTT exposure [11].

Plates were incubated at 37ºC with 5% CO_2 for four hours. During this time period vital cells absorbed MTT and produced violet crystals.

After four hours, the plates were centrifuged at 2000 rpm for five minutes. Subsequently, the overlying liquid (supernatant) was removed and 100µl acidic isopropanol was added.

Next, the plates were placed in the shaker for 20 minutes in order to dissolve the violet crystals. After complete dissolution of the crystals in isopropanol, the contents of each well were transferred to a special 96-well plate. Spectroscopy (620nm) was used to determine the optical density (OD) of both irradiated and control samples.

Statistical analysis

We used SPSS version 11.5 to determine the statistical significance in cell proliferation outcomes after laser irradiation.

Descriptive statistics were used to describe the means and standard deviation (SD) of the percentage. Two-way ANOVA was used to compare different groups. One-way ANONA with Tukey's Honestly Significant Difference (HSD) test was performed to determine the level of significance, when comparing energy density and number of exposures of irradiated cells versus the untreated controls.

$P<0.05$ was considered statistically significant (95% confidence interval).

Results

There were no changes in the growth rate of KG-1a cells treated with 5J/cm^2 and 10J/cm^2, irrespective of irradiation frequency ($P=0.161$ and 0.284, respectively) (Table 1 and 2). The cells that were irradiated with 20J/

Table1. Mean and standard deviation (Std.) of OD at energy density of 5J/cm^2

Frequency of laser irradiation	Mean	Std.
One	0.5458	0.06775
Two	0.4971	0.11245
Three	0.4985	0.1037

Table 2. Mean and Std. of OD at energy density of 10J/cm^2

Frequency of laser irradiation	Mean	Std.
One	0.5156	0.11400
Two	0.5716	0.16769
Three	0.5714	0.19821s

Table 3. Mean and Std. of OD at energy density of 20J/cm^2

Frequency of laser irradiation	Mean	Std.
One	0.5447	0.25502
Two	0.7423*	0.19181
Three	0.5516	0.31361

*Statistically significant

cm^2 showed significant increase in cell proliferation after two exposures (P= 0.021) (Table 3).

When comparing different frequencies of irradiation, there were no statistically significant differences in proliferation after one and three exposure at energy densities of five and 10J/cm^2 (P=0.85 for both) (Table 1 and 2). When cells were irradiated on two separate occasions, significant increases in cell proliferation occurred at energy density of 20J/cm^2 (P<0.001) (Table 3).

All data are presented as mean with standard error of the mean.

Discussion

In this study, we evaluated the effect of different exposure and doses of irradiation with laser on KG-1a cell lines. This study is one of the few studies, which have investigated the effect of repeated exposure with special doses of laser phototherapy that are commonly used in clinical practice, in an attempt to identify the effects on proliferation and growth of AML cells. In clinical practice, clinicians treat patients in repeated sessions with the same laser device. Frequency of laser exposure is unique for each patient according to his/her disease condition. The best treatment result is obtained when the clinician has adequate knowledge of the disease and its treatment, and pay great attention to the sign and symptoms and patient's history. It is generally advised to begin treatment with moderate doses of irradiation at close succession, instead of high doses with long intervals between treatment sessions [12].

Although many studies have supported the use of LLLT in the treatment of radiotherapy- or chemotherapy-induced mucositis in patients with leukemia, there are no well-designed, randomized controlled trials which have evaluated the effect of laser therapy on malignant blood cells [8, 9, 10]. This study is novel in that it evaluates the effects of repeated exposure to different doses of irradiation on AML cell line.

From the previous studies, it is evident that exposure to different doses and wavelengths of laser can induce stimulation, inhibition, or no change at all in both normal and malignant cells. However, these findings have not prevented clinicians from using laser phototherapy to treat mucositis caused by chemotherapy or radiotherapy. Few studies have been performed to assess the safety of laser and it does not appear that survival or disease recurrence is influenced by laser phototherapy [13]. We agree with Werneck et al. who, proposed irradiating a cancer lesion may result in tumor progression [14]. Since laser phototherapy causes increased tumor proliferation in vitro, it is necessary to reevaluate the safety and the efficacy of its use in treatment of a patient with malignant disease.

In this study, we used infrared laser with an application scheme similar to that used in clinical practice. Two or three appointments per week are usually arranged for phototherapy to prevent reaching the cumulative effects and inhibitory dose levels of laser. Daily irradiation with laser with stimulatory doses can cause overstimulation or inhibition. The biologic effects of laser usually remain from nine to 72 hours. This time can be used to determine the interval between treatment appointments. If the treatment course is longer than four weeks, the irradiation can be done twice per week. If the treatment course is shorter than three weeks, it may be increased up to three times per week, as the treatment outcomes are improved by increasing the frequency of treatment courses [15].

We irradiated the plates one, two, or three times every other day with energy density of five, 10, or 20 J/cm^2. Interestingly, we found that the effect of laser on KG-1a cell growth differs in different conditions of irradiation. Cell cultures that were irradiated one or three times did not show any significant changes in cell proliferation, regardless of the dose of irradiation.

These findings are similar to the results of Powell et al., who irradiated human breast epithelial cells (9Bre 80hTERT), and malignant melanoma cells (MDA-MB-435S) with 780, 830 and 904nm lasers and did not find any changes in cell proliferation, irrespective of the wavelength and the energy density of the laser [13].

We also found that when KG-1a cells were irradiated twice, they showed significant cell proliferation compared

to both control cultures and those irradiated one or three times.

When comparing the three different energy densities that were used in this study, only $20J/cm^2$ was able to significantly increase cell proliferation. Increased KG-1a cell proliferation after two irradiations with infrared laser was consistent with the results of Powell et al., in which proliferation of breast carcinoma cells (MCF-7) increased after two treatments with 830nm laser. MCF-7 cells also showed significant cell proliferation after two treatments with 904nm laser, at energy densities of 0.5 and $1J/cm^2$ [13]. Normal and neoplastic cells may have different sensitivities to laser Irradiation. This may depend on different parameters used in the studies, as well as cell cycle and physiologic status of the cells [4]. Normal peripheral blood mononuclear cells showed increased proliferation after three treatments with He-Ne laser with the wavelength of 632.8nm, at energy densities of 2.55 and $5.1J/cm^2$, and decreased proliferation at energy density of $7.64J/cm^2$ [3]. However, in our study, proliferation of malignant blood cells (AML) after three treatments with 810nm laser at energy densities of five, 10 and 20 J/cm^2 did not show any significant increases.

These results emphasize that the effect of various combinations and characteristics of laser irradiation on cell proliferation, both in vitro and in vivo, needs to be further evaluated. Also, the reason for the observed responses, as well as the combination of safe dose and wavelengths need to be further evaluated in clinical application.

A number of authors have postulated that cellular responses to laser phototherapy in designated wavelengths and doses can be influenced by cell/tissue specificity and modified intracellular genotypic and physiologic characteristics. Different wavelengths possess different effects on cells; while some may have direct photochemical effect on the mitochondria, others may result in a photophysical effect on the cell membrane [16].

It has been proposed that the effects of laser phototherapy are dose-dependent. Renno reported that the possibility of getting a response in cells receiving higher doses is greater compared to those receiving lower doses [4]. In our study, we found a significant difference in proliferation of KG-1a cell line at different energy densities (five, 10 and $20J/cm^2$) in the group that showed increased cell growth. It seems that increasing the irradiation frequency to two courses can affect the response of KG-1a cells at specific doses ($20J/cm^2$).

We only found a few studies that have investigated the effect of repeated application of laser phototherapy on malignant cells [13, 16, 17]. Pinheiro et al. irradiated H.EP.2 cell line (laryngeal SCC type 2) with 635nm and 670nm lasers and showed that the cell number increased significantly after multiple irradiations with 670nm laser at energy densities of $0.04\text{-}4.8j/cm^2$ over seven days. Such results were not seen with 635nm laser or in the control group [17].

The biostimulatory effect of low level laser irradiation is thought to be secondary to the absorbance of laser by the components of the respiratory chain, such as flavin dehydrogenases, cytochromes, and cytochrome oxidases. This was confirmed by Friedman and Lubart [18]. It has also been found that the stimulatory and the inhibitory effects of visible and near infrared lights on growth of certain organisms are caused by the same mechanism [7]. Photochemical and photophysical effects may be the result of light absorbance by chromophores. These effects, isolated or together, can stimulate the mitochondrial membrane, so both the membrane potential and photonic gradient would increase, leading to a change in the optical properties of the mitochondria. This change leads to increased activity of NADH, which results in increased generation of RNA, ATP and protein synthesis by the mitochondria [16]. Also it is possible that membrane permeability and transport are affected. Na^+/H^+ ratio and the activity of Na^+- K^+/ ATP-ase is increased which in turn influences Ca^{2+} flux. The Ca^{2+} flux affects the cyclic nucleotide levels which modulate DNA and RNA synthesis, leading to changes in the cell cycle [18]. Furthermore it was reported that light absorption in visible light and infrared spectrum can increase the intracellular PH which stimulates mitosis. The absorption of near infrared light (λ=820nm) by cells resulted in an increase of the ATP production within the mitochondria [7].

The advantage of our study was the use of only six wells of the 96-well culture plate, as well as the use of a light absorbing substance in all of the wells surrounding the cell-containing ones to prevent energy transfer. Moreover, the laser probe was attached to the floor of the plate well. This prevented light from scattering to the surrounding wells; while in previous studies light scattering and energy transfer had not been addressed. In a study by Pinheiro et al. 24 wells of a 96-well culture plate were used to irradiate H.EP.2 cells; but the other wells did not contain any other material [17]. Also, Frigo et al. allowed 5 centimeters of space in all directions between wells and also placed an aluminum sheet halfway in order to prevent light scattering between wells [6].

Although the experiment was done in a controlled environment, where temperature, light, and humidity were controlled, it is possible that other factors such as growth limitation by cell contact inhibition in the culture

medium may have affected our results. Repeating the experiment while controlling for this can provide more information regarding its effect on the study process.

Our results are consistent with the general findings of other researchers, and show that KG-1a cell line (AML cells) behaves in a similar manner as other malignant cells. Thus, we conclude that the response to the stimulatory effects of low-level laser depends on the individual cells and the range of doses; which can be subtle and quite specific. Overall, our results show that the growth of malignant blood cells can significantly increase using special doses and frequencies of irradiation.

Although cell culture is an important method for studying basic biological processes and understanding the possible cell reactions to treatment, the previous studies have shown differences between in vitro and in vivo studies [6]. So, we should be careful before generalizing in vitro results, as cell-matrix interactions and cell behavior in the complex environments of tissues can produce unexpected reactions. In the environment of tissues, there are many factors that contribute to tumor growth, and laser irradiation can modulate most of them. For example, low-level laser can enhance angiogenesis, growth factor synthesis, and inflammatory metabolites and immunological cells [6].

In conclusion, as long as further studies demonstrate that different wavelengths and doses of laser phototherapy are safe and effective in treatment of mucositis, clinicians should remain cautious regarding the use of this treatment modality to treat patients with malignancies.

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