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Research Article

Safety and Clinical Impact of a Single Red Light Irradiation on Breast Tumor-Bearing Mice

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ABSTRACT

Low-level light therapy has been used in health care as a therapeutic strategy for different diseases. However, its effects on cancer are controversial. This work evaluated the effects of three energies on breast cancer-bearing mice after a single red light-emitting diode (LED) irradiation. 4T1 cells were inoculated into the mammary fat pad of female BALB/c mice. When tumor volume reached 100 mm^3, animals were irradiated by a LED irradiator (660 ± 11 nm) with energies of 1.2, 3.6, and 6.0 J. Control without irradiation and healthy animals were also evaluated. Mice were monitored regarding tumor volume and total blood count. After euthanasia, their organs were examined. We observed that a single irradiation does not increase tumor volume. All irradiated groups exhibited better clinical conditions than control, which presented a significant decrease in platelet and red blood cell levels compared with healthy mice. The energy of 3.6 J arrested neutrophil-lymphocyte rate besides promoting longer survival and a lower number of metastatic nodules in the lungs. These findings suggest that a single red LED irradiation causes no impact on the course of the disease. Besides, the intermediary dose-effect should be further investigated since it seems to promote better outcomes on breast cancer-bearing mice.

INTRODUCTION

Breast cancer is a noncommunicable disease that constitutes an important public health concern, it is the fifth principal cause of cancer-associated death worldwide (1). Unfortunately, despite high cure rates when there is early diagnosis, the lack of preventive diagnostic campaigns causes the disease to be diagnosed in advanced stages, thus increasing the mortality rate associated with this type of cancer. It is estimated a 33% increase in the number of breast cancer-related deceases for the year 2025 (2).

Treatment is based on the type of breast cancer, its stage, and any other patient's special condition. Frequently, it requires multimode treatment comprising surgery, radiotherapy, systemic treatment with chemotherapy, and/or hormone therapy (2). In its initial stage, treatment is conservative, that is, minimum breast amputation where the tumor is located. In contrast, radical mastectomy is advised when the tumor is large. For both cases, radiotherapy is recommended as an adjuvant to avoid recurrence (3,4). Unfortunately, breast cancer therapies provoke displeasing side effects, which encourage the pursuit of new strategies to improve patient life quality.

Today, light-based technologies are an effective and noninvasive alternative for the treatment of different disorders. Many studies have encouraged the use of this technology in daily medical practice, aiming not to replace but to improve existing conventional techniques. In this context, the use of the low-level light therapy (LLLT) in clinical practice, currently named photobiomodulation therapy (5), has gained great interest due to the worldwide tendency to look for less invasive forms of treatment. Indeed, recent clinical studies and systematic reviews have demonstrated many beneficial effects of LLLT as the promotion of welfare (6), tissue healing (7–9), pain relief and analgesia (10,11), improvement of muscle fatigue (12), recovery of sensory and motor response (13), etc.

However, the use of red and near-infrared light in cancer cells is still controversial. Some studies report that LLLT stimulates the proliferation of cancer cells while others describe the opposite, that is, LLLT promotes beneficial results in preventing tumor progression (14). Although LLLT has been used in the alleviation or prevention of painful side effects arising from breast cancer therapies, such as radiodermatitis (15,16) and lymphedema (17), it could also be used in patients with undiagnosed cancer. Recently, it was reported that LLLT can be used to treat nipple fissure in breastfeeding women (18). Thus, to understand the light effect in cancer cells seems increasingly important.

In this work, we used a murine model of a mammary tumor to investigate the effects of a single application of LLLT on breast cancer. We used the 4T1 mammary carcinoma cell line, which is known to be extremely aggressive and able to promote metastasis from the primary tumor in the mammary gland to multiple distant sites, mainly lungs. Moreover, it presents several features that make it an appropriate experimental animal model for human mammary cancer (19). Besides tumor progression evaluation and metastasis, we aimed to analyze complete blood count and its influence on mouse clinical signs and survival.

MATERIALS AND METHODS

Cell culture. Experiments were conducted with 4T1 breast tumor cells (ATCC ® CRL-2539). The cells were cultured in RPMI 1640 medium (Sigma, USA), supplemented with 10% fetal bovine serum (Sigma,

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USA), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Sigma, USA) at 37 C with 5% CO_2 in humidified air. The cells were cultivated until reaching 70% of confluence, passaged using 0.25% trypsin and 0.03% ethylenediaminetetraacetic acid (EDTA) (Sigma, USA), quantified by the trypan blue exclusion method in a Neubauer chamber and suspended at a concentration of 1×10^5 cells in 40 μl of phosphate-buffer solution (PBS).

Animals. The study was conducted according to the Animal Use Ethics Commission (CEUA) of the Energy and Nuclear Research Institute (IPEN) and approved under number 214/18. For the development of the experimental assay, we used 5–7 weeks old female BALB/c mice ($n = 31$, 7 per experimental group, and 3 healthy mice for the negative control) with a body mass of approximately 20 g. The animals were maintained in a pathogen-free environment at alternating lighting hours (12 h light/12 h dark), with food and water *ad libitum* during all the experimental period.

Tumor induction and quantification. For tumor induction and all experimental manipulation, animals were anesthetized by inhalation with a mixture of 2.5% isoflurane (Isoforine, Cristália, Brazil) and 1.5% for maintenance. Under anesthesia, the animals were trichotomized in the mammary gland region. Thereafter, local asepsis was performed and 1×10^5 cells were inoculated into the 5th left mammary fat pad using 1 mL-syringe and 27-gauge hypodermic needles.

The animals were monitored daily and after 7 days of cell inoculation, the nodule was discernible. At this moment, we initiated the quantitative evaluation of the tumor with the aid of a digital caliper. Tumor volume was determined using equation (20):

$$V(\mathrm{mm}^3) = 0.5\mathrm{L}.\mathrm{W}^2 \quad (1)$$

where V is the volume in mm^3, L is the length and W is the tumor width, both in mm.

Irradiation procedure. After 14 days of cell inoculation, when the tumor volume reached approximately 100 mm^3, we irradiated the tumor. We used as the light source a device containing light-emitting diodes (LEDs) designed for irradiation of small animals (LEDbox, Biolambda Ltd., Brazil). According to the manufacturer, the beam area of the device is 163.5 cm^2. The LEDbox provides light in the red region ($\lambda = 660 \pm 11$ nm) with a uniform optical power of 120 mW, which was checked at 3 points inside the device with a power meter (FieldMate, PM10, Coherent, USA). The LEDbox irradiance (38.2 mW cm^{-2}) was calculated dividing the optical power by the area of the detector head (3.14 cm^2).

The animals were randomly distributed into 4 experimental groups ($n = 7$ animals/group) and covered with a light shield accessory to allow that only the tumor area (0.64 cm^2) received light during irradiation. Thus, the optical power on the tumor was 24.5 mW. Thereafter, mice were submitted to a single LED irradiation with parameters displayed in Table 1.

Clinical monitoring. To verify the clinical signs and tumor response after LED exposure, the animals were monitored twice a week throughout the experimental period. Tumor volume was measured and a trained veterinarian attributed scores to the clinical signals presented by each animal (21). According to the used score system, a higher score means a worsening of the animal clinical condition. The clinical signs that were evaluated and scores are presented in Table 2.

Blood count cells. To identify hematological alterations, we developed a blood count schedule, where the experimental and healthy animals ($n = 3$) were followed in the week preirradiation, and in 1st-week, 2nd-week, 3rd-week, and 4th-week postirradiation. To collect blood samples, the animals were anesthetized as previously reported. Samples (approximately 30 μL) were collected via the caudal vein, and we used 1 μL of 10% sodium- EDTA as the anticoagulant. Samples were analyzed on a hematology analyzer (Mindray, BC 2800 VET) with the mouse reference standard. Total blood encompassed white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts.

Euthanasia and organ examination. Experimental and healthy animals were euthanized in the 5th week after irradiation, using an excess of anesthetics (60 mg kg^{-1} xylazine and 235 mg kg^{-1} ketamine). Lung and spleen were collected and evaluated postmortem regarding metastatic superficial nodules and mass, respectively. Figure 1 summarizes our experimental design.

Statistical analysis. The data obtained were statistically analyzed following Shapiro–Wilk to test normality. We used the one-way analysis of variance (ANOVA) and Tukey test as the post-test to identify differences intergroup at each moment and intragroup over time. For the survival analysis, the Log-rank test was used. Data were considered statistically significant when $p < 0.05$. Data are presented as means ± standard error of the mean (SEM), and all statistical analysis was performed using the Origin Pro 8.5 program.

Table 1. LED parameters and protocol used for the experimental groups

Optical power (mW)					
		120			
Irradiance (mW/cm^2)	38.2				
Beam area* (cm^2)	163.5				
Mode	continuous				
Tumor area (cm^2)	0.64				
Optical power on tumor (mW)	24.5	**Group**	**Energy (J)**	**Radiant exposure (J/cm^2)**	**Exposure time (s)**
		G0	0	0	0
		G1	1.2	1.8	49
		G3	3.6	5.6	148
		G6	6.0	9.4	246

*Informed by manufacturer

Table 2. Evaluated clinical signs and attributed scores

Clinical sign	Rate	Score
Loss of body mass	0	0
	5%	1
	10%	5
	20%	10
Hypokinesia	Normal activity	0
	Reduced activity	5
	Inactive	10
Curvature	Normal posture	0
	Arched posture	1
Piloerection	Yes	1
	No	0

RESULTS

Tumor volume and clinical score

The tumor volume increased exponentially during the experimental period. Although no statistically significant differences have been observed among groups, some particularities were noticed over time. Three weeks following red LED irradiation, G0, and G1 showed a significant tumor increase compared to week 1 and a further significant increase was noticed in the fourth week. In contrast, G3 and G6 showed a statistically significant tumor growth between weeks 2 and 4 postirradiation (Fig. 2).

Clinical signs represent directly the health conditions of animals. We can observe from Fig. 3 that in the first and second weeks postirradiation, the control group exhibited a mean score significantly higher than irradiated groups. However, no statistically significant differences intergroup were noticed at week 3 and week 4 after irradiation. Over time, mice of G0 and G1 showed a significant worsening in their clinical condition at week 3, which further significantly increased at week 4. In contrast, the lower and middle doses seemed to arrest the mouse

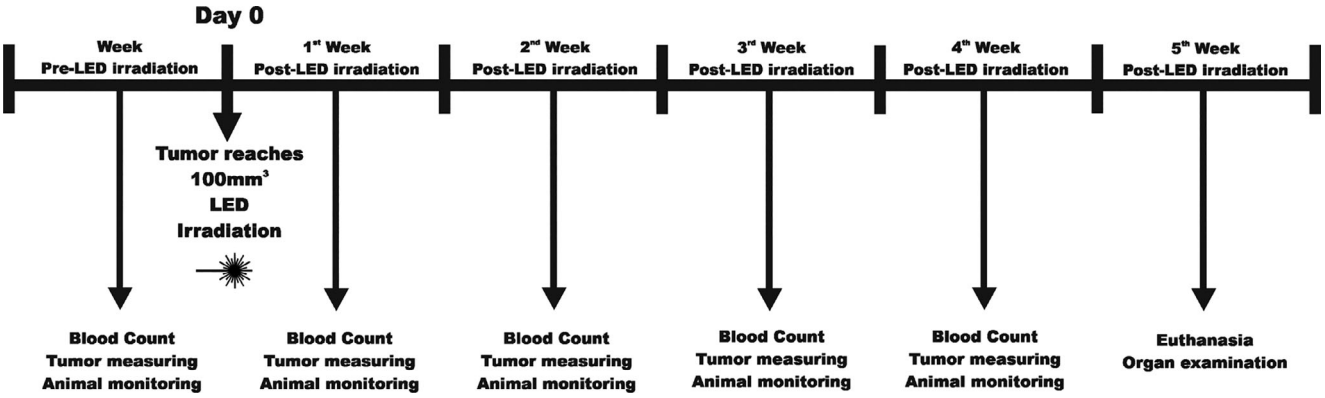

Figure 1. Experimental design of this study

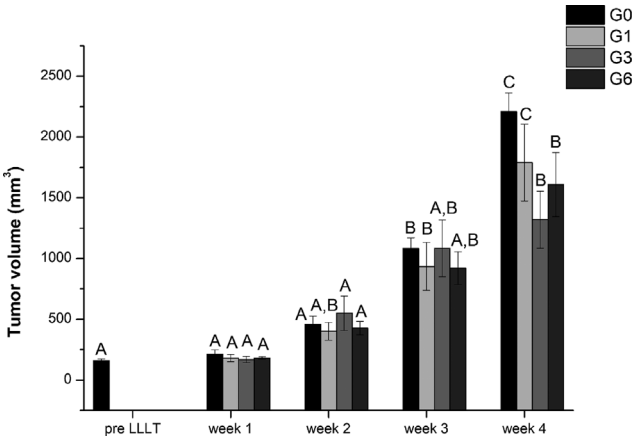

Figure 2. Growth of tumor volume during the experimental period. Different capital letters represent statistically significant differences intragroup over time. Data are presented as mean values ± SEM

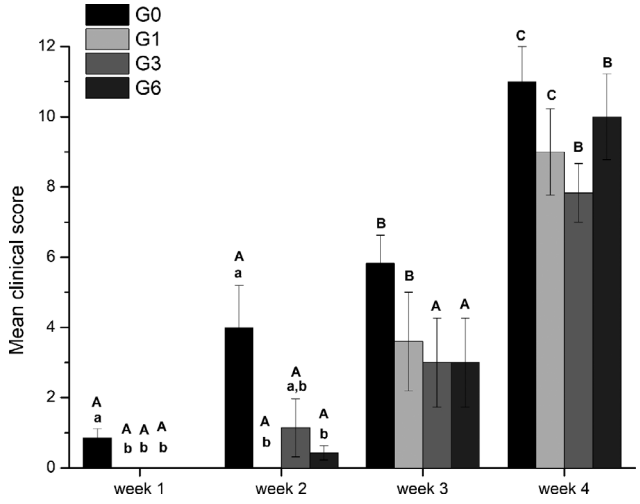

Figure 3. Mean clinical score ± SEM of the groups during the experimental period. Different lowercase letters represent statistically significant differences among groups. Different uppercase letters denote statistically significant differences intragroup over time

clinical worsening, and statistically significant differences were noticed only in the fourth week after red LED irradiation.

Total blood count

We identified significantly lower RBC levels in G0 than healthy mice in the fourth week after the red LED irradiation (Fig. 4). Over time, no statistically significant differences were observed among groups.

There was a significant decrease in PLT counts of control and irradiated groups compared with healthy mice at week 2 (Fig. 5). No statistically significant differences among groups were identified in the other time points. On the other hand, only G0 showed a significant decrease in platelet counts from week 1, which remained low until the end of the experiment.

Figure 6 displays the WBC levels of the groups during the experimental period. Statistically significant differences among groups were noticed at week 3 and week 4 following red LED irradiation. G0 presented a significantly higher WBC level when compared to healthy mice. Over time, G0 and G6 showed a statistically significantly higher number of WBCs in the third week, which remained stable at week 4. In contrast, G1 and G3 showed similar levels of WBCs throughout the experiment.

Figure 7 shows the neutrophil-lymphocyte ratio (NLR) during the experimental period. In the first week following irradiation,

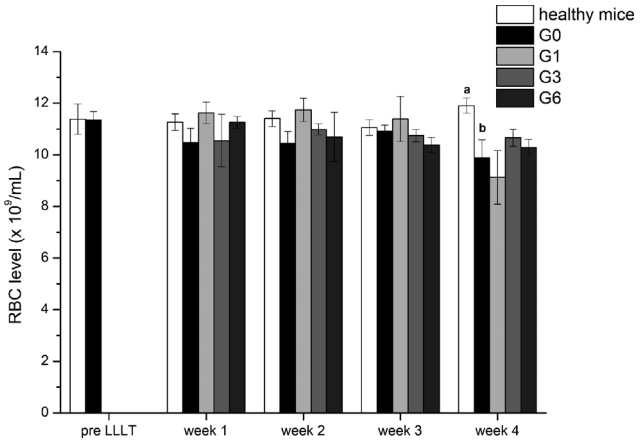

Figure 4. Mean values ± SEM of red blood cell (RBC) levels during the experimental period. Different lowercase letters represent statistically significant differences between control group and healthy mice

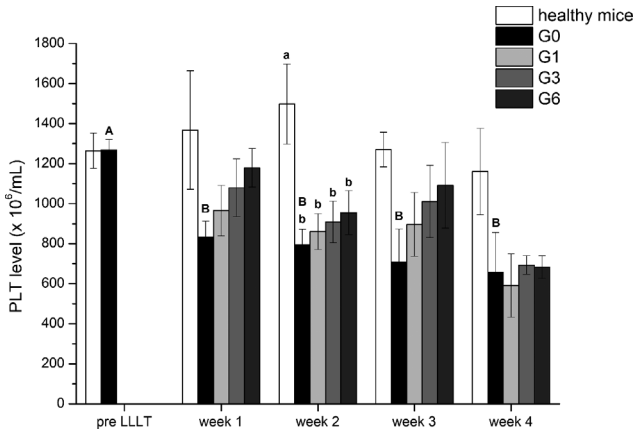

Figure 5. Mean values ± SEM of platelet PLT levels during the experimental period. Different lowercase letters represent statistically significant differences between experimental groups and healthy mice. Different uppercase letters denote statistically significant differences intragroup over time

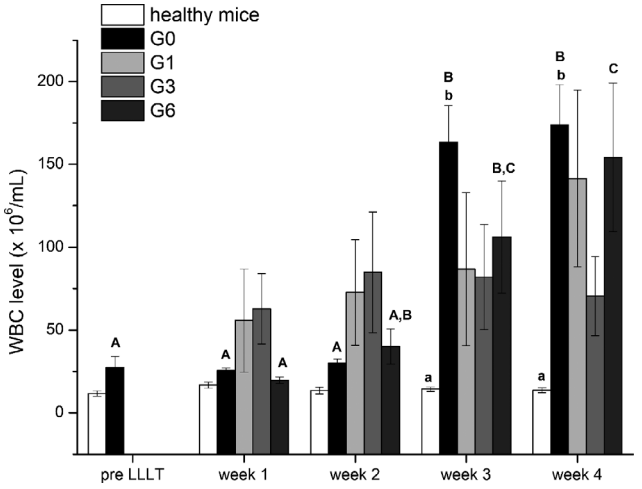

Figure 6. Mean values ± SEM of white blood cell (WBC) levels during the experimental period. Different lowercase letters represent statistically significant differences between control group and healthy mice. Different uppercase letters denote statistically significant differences intragroup over time

G0 presented a statistically significantly higher NLR compared with healthy mice and G1. In the second week, NLR was similar for healthy and G1 but significantly different from G3 and G6. At week 3 and week 4, statistically significant differences were noticed only between healthy and control mice. Interestingly, G3 showed similar NRL over time, while a significant increase was observed for G0 and G6 at week 3, which significantly increased at week 4, and for G1 in the fourth week postirradiation.

Survival and organ examination

Figure 8 shows the survival curve of the animals, which were monitored for 32 days after the red LED irradiation when they were euthanized due to the human endpoint established by ethical concerns. We can observe that mice started to die in the fourth week after the red LED application. On day 21, G1 and G6 had higher mortality rates (28.6%), while G0 and G3 groups

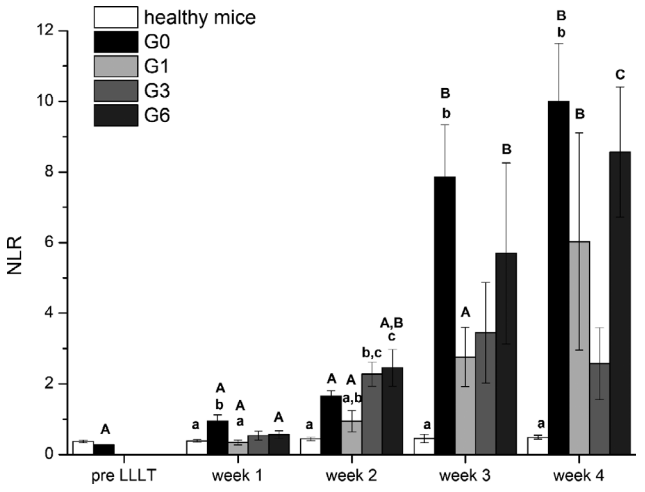

Figure 7. Mean values ± SEM of the neutrophil-lymphocyte ratio (NLR) during the experimental period. Different lowercase letters represent statistically significant differences among experimental groups. Different uppercase letters denote statistically significant differences intragroup over time

reached a lower index (14.3%). In the fifth weekpostirradiation, 28.6% of mice of G0 died while G6 reached 43% of mortality, which remained until the end of the experiment, making it the group with the highest mortality. G3 group showed a longer survival compared with other groups, although no statistically significant differences were noticed.

After euthanasia, mice were anatomized to verify the spleen size and mass, and superficial metastatic nodules in the lungs. We noticed that all experimental groups presented splenomegaly (Figs. 9A and B) with spleen mass significantly greater than healthy mice. It was also observed that G3 presented a significantly lower number of metastatic nodules in the lung when compared to other groups (Fig. 9C).

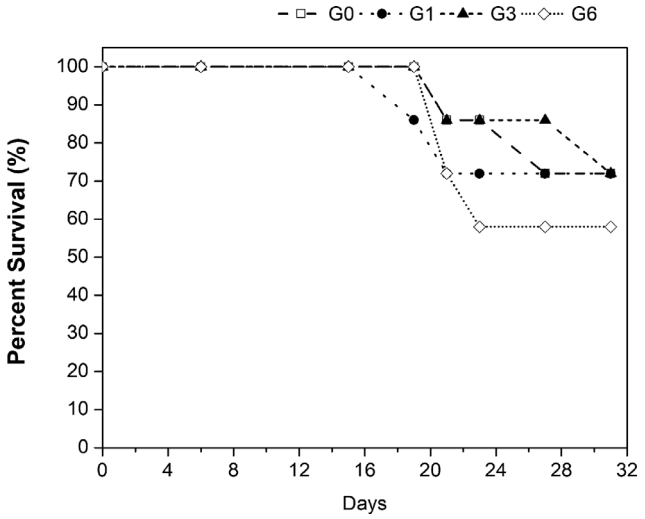

Figure 8. Survival curves of breast cancer-bearing mice for experimental groups (n = 7)

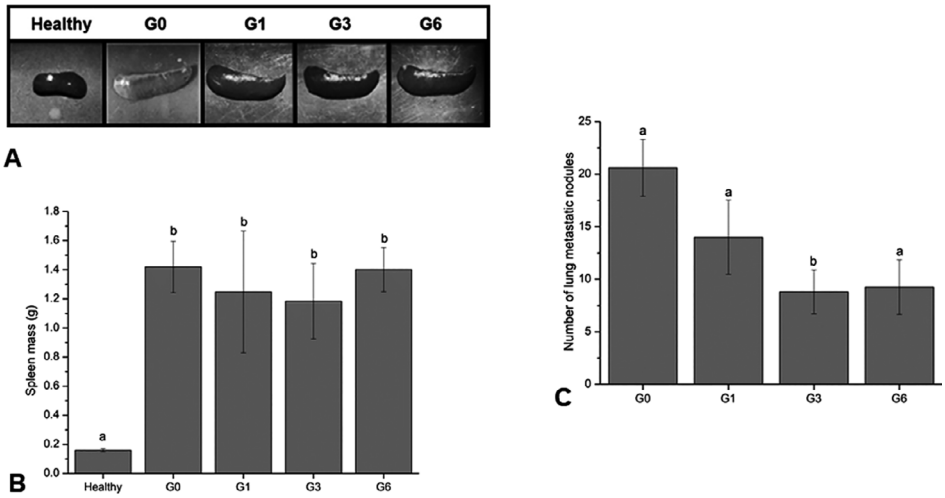

Figure 9. Representative images of the spleen of healthy, control and irradiated groups obtained after mouse euthanasia (A); mean values ± SEM of the spleen mass for healthy mice and experimental groups (B). Different lowercase letters represent statistically significant differences between experimental groups and healthy mice; mean values ± SEM of the number of lung metastatic nodules for experimental groups (C). Different lowercase letters represent statistically significant differences among experimental groups

DISCUSSION

In this work, we successfully have induced breast tumors in female BALB/c mice. Our results showed that a single red LED irradiation is not able to worsen tumor growth, health condition, blood count, and mouse survival rate regardless of the light dose used when compared to the control group. On the other hand, it seemed that the tumor evolution was dependent on the light parameters.

Although no statistically significant differences in tumor volume have been identified between irradiated and control groups at any time point throughout the follow-up period, G0 showed a more pronounced tumor progression over time. These findings are closely related to clinical signs presented by animals since irradiated groups showed lower clinical scores than G0 in the first 2 weeks following light exposure and slower evolution of the worsening of mouse clinical condition.

We decided to evaluate the total blood count since hematological tests are part of protocols for oncological conditions to observe tumor progression and possible complications (22,23). Furthermore, studies show that tumor progression occurs by procedures related to blood cells resulting in changes to their reference levels (24–26).

Our data showed that RBC count was constant throughout the experimental period for all irradiated groups while G0 showed a significant decrease compared with healthy mice in the fourth week after irradiation. Low levels of RBCs indicate anemia, which is highly prevalent in patients with advanced cancer (27,28). Thus, we can assume that single irradiation on the tumor could avoid anemia-related symptoms, like fatigue and weakness.

Platelets are part of blood cells and their main function is to stop bleeding via clumping and forming clots. It circulates in the bloodstream for 8 to 10 days, when they are removed and destroyed by the spleen (29–31). During the tumor progression, PLTs can play a role in the metastatic process aggregating to tumor cells, forming clots, and inducing inflammatory processes. Due to these factors, there is an increase in platelet consumption, resulting in increased platelet production, which can present changes in blood count (32–35). However, our results showed that all experimental groups significantly reduced PLT levels compared with the healthy group at week 2 postirradiation.

On the other hand, leukocytes or WBCs are cells of the body immune system and are part of the blood cells group originating from the bone marrow and spleen (36,37). Particularly for the 4T1 murine tumor model, there is a leukemoid reaction, which increases the WBC count, in addition to splenomegaly (32,33). Regarding WBCs, we detected a significant increase for G0 and G6 at week 3 and week 4, even though only G0 has been significantly different from healthy mice.

Our findings suggest that there were changes in bone marrow function causing an increase in the production of WBCs by the spleen. This increase conducted to spleen hyperplasia and congestion hindering cell circulation, which resulted in PLT sequestration and splenomegaly (28).

Besides, the literature has described a significant relation between NLR and breast cancer stage, that is, an increase of the NLR may be a predictor of an increase in the mortality of patients with breast cancer (38). In our study, we observed that G3 showed similar NLR values throughout the experimental period and higher survival percentage. These data seem to be related to the lower number of metastatic nodules perceived in this group. Indeed, lung metastasis is expected during the development of breast cancer in mice using the 4T1 tumor cell (15). Thus, we hypothesize that the intermediate light dose was able to provide a better outcome for the mice.

Protocols and consensus about the effects of LLLT on cancer are still lacking. Different *in vivo* methodologies and outcomes reported in literature raise questions about its clinical application. Indeed, we noticed that preclinical studies involving LLLT in cancer are scarce (Table 3). In the last 20 years, only 7 articles were published regarding models of gastric adenocarcinoma, melanoma, squamous cell carcinoma, nonmelanoma skin cancer, and breast cancer. From those that used red light, three reported the increase of the tumor volume (39–41), two suggested that the tumor growth depends on radiant exposure (42,43), one reported that LLLT did not influence tumor growth (44), and one showed the decrease of the tumor volume (45). All of them used more than one session of LLLT.

Table 3. Preclinical studies of LLLT in cancer. NR: not reported by authors; SCC: squamous cell carcinoma; UV: ultraviolet

Tumor model	λ (nm)	Optical power (mW)	Irradiance (mW/cm^2)	Exposure time (s)	Radiant exposure (J/cm^2)	Frequence	Outcome
Human gastric adenocarcinoma transplanted in athymic mice (39)	633 (laser)	NR	NR	NR	3.5	Local irradiation on days 1, 3, 5, 8, 10, 12 after transplantation	Increase of the tumor volume
Melanoma induced by inoculation of B16F10 cells in BALB/c mice (42)	660 (laser)	50	2500	60 420	150 1050	Local irradiation daily for 3 days	150 J/cm^2 did not influence tumor growth 1050 J/cm^2 increased tumor volume
SCC chemically induced in golden hamsters (oral cavity) (40)	660 (laser)	30	424	133	56.4	Local irradiation every other day for 4 weeks	Increase of the volume and severity of the tumor
Nonmelanoma UV-induced skin cancer in SKH1 hairless mice (44)	670 (LED)	NR	8	312	2.5	Full body irradiation twice a day for 37 days	LLLT did not influence tumor growth
Breast cancer induced by inoculation of 4T1 cells in BALB/c mice (41)	405 532 632 (lasers)	1 - 3	NR	600	NR	Local irradiation 3 times a week (10 sessions)	405 nm decreased tumor volume 532 did not influence tumor growth 632 increased tumor volume*
Melanoma induced by inoculation of B16F10 cells in C57BL/6 mice (45)	660 800 970 (lasers)	100 1000 2500	50 200 200	60 30 30	3 6 6	Local irradiation daily for 4 days	Decrease of the tumor volume
SCC chemically induced in C57BL/6 mice (oral cavity) (45)	970 (laser)	2500	200	30	6	Local irradiation daily for 4 days	Decrease of the tumor volume
Melanoma induced by inoculation of B16F10 cells in BALB/c mice (43)	660 (laser)	50	2500	60 180 420	150 450 1050	Local irradiation daily for 3 days	150 J/cm^2 did not influence tumor growth 450 and 1050 J/cm^2 increased tumor volume

*Data informed by authors although no statistically significant differences were reported between red and control group.

Regarding breast cancer, although Khori and collaborators reported that the red laser applied 3 times a week in 10 sessions was able to increase the breast tumor volume in mice, no statistically significant differences were showed between the red and control groups (41). Additionally, it is noteworthy that all studies applied LLLT in more than one session. However, it is well known that the number of sessions is critical for LLLT, which possesses a biphasic dose-response.

On the other hand, Bamps and collaborators used head and neck squamous cell carcinoma and applied single irradiation delivering either 1 J cm^{-2} or 2 J cm^{-2} (46). They observed that 1 J cm^{-2} promoted an increase in cell proliferation, while no effect was observed with 2 J cm^{-2}. The authors associated the proliferation with high levels of pAKT, pERK, and Ki67 protein expression. Indeed, elevated pAKT and pERK are linked with poor prognosis in breast cancer (47) so Kim et al. have suggested the search for potential therapies acting as inhibitors of pERK (48).

Herein, we reported that a single red LED irradiation does not cause any negative impact on breast cancer, regardless of the light dose. We also noticed that the middle dose promoted better clinical conditions, sustained blood cell levels, and NLR over time, and showed higher survival and lower number of lung metastatic nodules than other groups. Overall, our data indicate LLLT in a single application as a safe procedure and motivate further steps toward pursuing an effective protocol and understanding its biological effects to use it as a noninvasive therapeutic tool to help fight breast cancer.

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