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Evaluation of the Proliferative Effects Induced by Low-Level Laser Therapy in Bone Marrow Stem Cell Culture

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Abstract

Objective: The objective of this study was to evaluate the effect of laser irradiation on dog bone marrow stem cells. ***Background data:*** Low doses of low-level red laser positively affect the viability of mesenchymal stem cells, and also increase proliferation. ***Methods:*** Low-level laser (wavelength, 660 nm; power output, 50 mW), was applied to dog bone marrow stem cell cultures (DBMSC). The energy densities delivered varied from 1 to 12J/cm^2. The effect of the laser irradiation was evaluated on cell proliferation measured with the MTT colorimetric test, cell cycle phase, and on lipidic peroxidation (free radical production). ***Results:*** The results indicate that laser irradiation to DBMSC did not change the morphology of the cells, but significantly increased their viability and the number of cells at the G2/M phase with 6, 10, and 12 J/cm^2. On the other hand, malonaldehyde production was significantly enhanced with 8 J/cm^2. ***Conclusions:*** The parameters used to irradiate DBMSC increased significantly proliferation without producing high levels of reactive oxygen species (ROS).

Introduction

Low-level laser (LLL) is a photonic radiation without destructive potential, which generally causes cell stimulation. Its photochemical mechanism can be compared with that described in photosynthesis, when the light absorbed induces a chemical reaction,[1] The cellular and molecular mechanism of LLL suggest that photons are absorbed in the mitochondria by cytochrome-c oxidase,[2] stimulate adenosine triphosphate (ATP) production and low levels of reactive oxygen species (ROS) (free radicals), and activate transcription factors such as nuclear factor kappa beta (NF-kB), inducing transcription of many products responsible for the beneficial effects of LLL. Free radicals are known to stimulate cell proliferation at low levels, but to inhibit and even kill cells at high levels.[1] Nitrous oxide (NO) competes with oxygen at the site of cytochrome c oxidase. LLL releases the NO leaving, this site free for oxygen binding and consequent ATP production. LLL produces NO at other sites (hemoglobin and nitrosylated myoglobin). The production and release of cyclic NO that reacts with monophosphate guanine (cGMP) cause vasodilation. It is possible that the release of low levels of NO is beneficial for cells, whereas higher levels can be harmful.[1]

Low doses (0.5–5 J/cm^2) at low power (20–50 mW) of low–level red laser, 635–660 nm, positively affect cell viability of mesenchymal stem cells from adipose tissue,[3] heart,[4] dental pulp,[5] and bone marrow,[6–8] and also increase proliferation of adipose tissue,[3] dental pulp,[5] and bone marrow.[6]

LLL applied to cell cultures including stem cells, induced higher ATP and RNA levels and increasing DNA synthesis, without cytotoxic effects. Effects depend upon the wavelength (600–700 nm) and energy density (0.5–4 J/cm^2).[9] Low doses of laser promote secretion of growth factors vascular endothelial growth factor (VEGF) and nerve

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growth factor (NGF), and facilitate myogenic,[6] osteogenic,[10] neurogenic, and osteoblastic induction[7] compared with unirradiated osteoblasts.[11] LLL applied to fibroblasts can promote the healing process because its photomodulatory effect induces liberation of related biomolecules,[12] resulting in enhanced cell differentiation.[7]

LLL applied to bone marrow mesenchymal stem cells before implantation into infarcted myocardium can reshape the hostile environment, increasing the number of new capillaries and their survival rate, decreasing apoptosis, and enhancing their therapeutic potential.[13] Infarcted mice treated with bone marrow stem cells irradiated with LLL had a 76% reduction of the infarct size and also had 75% of ventricular dilatation compared with the control group.[14]

The objective of this study was to evaluate the proliferative effects and lipidic peroxidation production of dog bone marrow stem cells (DBMSC) irradiated with different energy densities of LLL and to investigate the dose-response.

Materials and Methods

This work was approved by the ethics committee of Cruzeiro do Sul University, São Paulo, SP, Brazil.

Laser device

The laser device was a Thera Lase (DMC, São Carlos, Brazil). Electric tension was 90–240 V, fuse was a 3 A, maximun power output was 9 W, and the active medium was InGaAlP.

Irradiation parameters

Irradiation parameters were continuous beam, visible light, red laser; wavelength was 660 nm; and power output was 50 mW. The energy densities used were 1, 2, 4, 6, 8, 10, 12 J/cm^2 compared with an untreated control. Total exposure time varied from 0 sec (control group) to 90 sec (12 J/cm^2). Spot area was 0.38 cm^2, and the laser stick cross-section area was 0.028 cm^2.

Cell seed assay

Cells were plated in columns, six wells in each column, in 96 well plates at a density of 10^4 cells/well into flat microtiter plates, and incubated for 1 day at 37°C in a humidified incubator containing 5% CO_2.

Irradiation assay

Plates were taken from the incubator to the sterile chamber, over autoclaved black paper, at room temperature. The cover plate was removed, and LLL was applied 24, 48, and 72 h[15] after cell seed, using a holder to position the laser stick at the entrance of each well. Unirradiated columns were covered by autoclaved alluminium. A built-in power meter was used to analyze and correct the power output as each energy density was changed. After each irradiation, cells were returned to the incubator.

Cell culture

DBMSC were characterized and donated by Wenceslau CV, as part of a PhD thesis, at Instituto Butantan, Sao Paulo, Brazil.[16] DBMSC positively reacted to CD44, anti-vimentin, anti-nestin, anti-cytokeratin 18, and anti-Oct3/4 antibodies, and were negative to hematopoietic specific markers anti-CD45, and anti-CD13. Expression of Oct-4, and SOX2 was verified by reverse transcription polymerase chain reaction (RT-PCR).[17]

MTT assay

Twenty-four hours after the last treatment, DBMSC cells were exposed to 5 mg/mL MTT for 3 h, and the precipitated formazan was dissolved in 0.1N HCl in isopropanol and measured at 540 nm with a microplate reader Thermo Plate (Rayto Life and Analytical Sciences C. Ltd., Germany) spectrophotometer.[18]

Cell cycle phase assay

Control cells were treated with phosphate-buffered saline (PBS) alone. Supernatant and adherent cells were collected

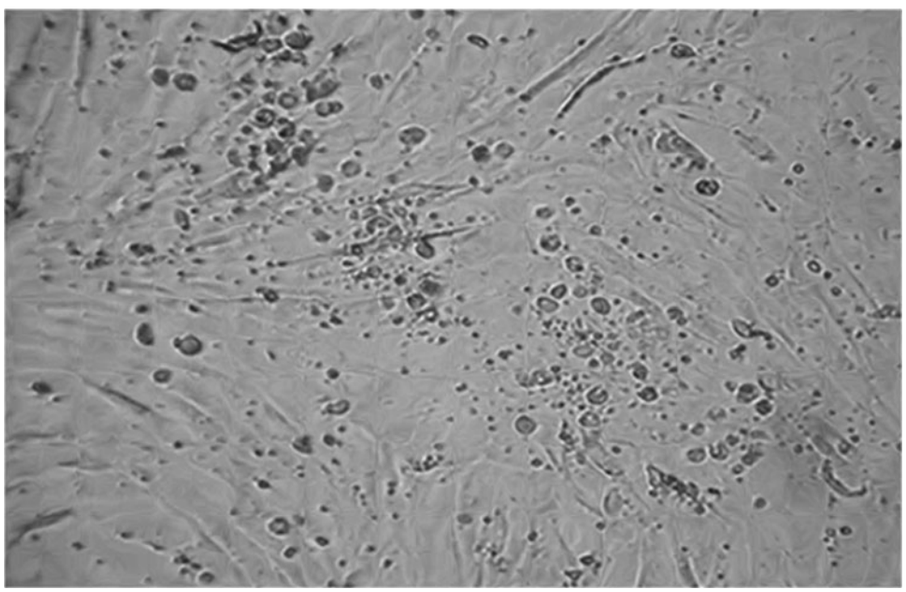

FIG. 1. Dog bone marrow stem cell cultures (DBMSC) showing confluence; control untreated group.

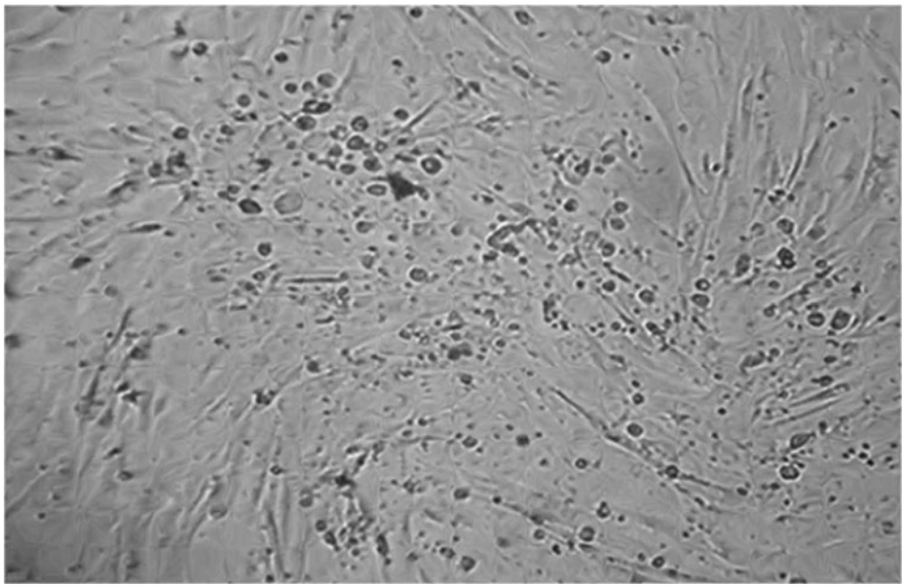

FIG. 2. After laser treatment with 12 J/cm^2, still exhibited a fibroblast-like morphology, small, elongate and fusiform, with small cytoplasmatic projections, similar to preirradiation appearence.

and washed in PBS. Cells and apoptotic bodies were harvested by centrifugation at 3000 rpm for 10 min. The cell pellet was fixed in ice-ethanol 70% and maintained overnight at −20°C. Prior analysis, PBS containing 1.8 μg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO) and 0.1 mg/L ribonuclease-A (Sigma Chemical Co., St. Louis, MO) was added to the cell pellet and incubated in the dark for 20 min at room temperature. At least 10,000 events were acquired using Cell Quest Software. DNA content was measured in the FL2 channel on FACScalibur flow cytometer (BD, USA). The percentage of apoptotic cells and cells in the cell cycle phases G0/G1, S, G2/M, and sub-haploid cells/DNA fragmented was determined using the ModFit 2.9 software.

Lipid peroxidation assay (malondialdehyde [MDA] formation)

Twenty-four hours after the last laser treatment, the supernatant was transferred into a 96 hole flat bottom culture plate kept under −20°C MDA. Supernatant levels were

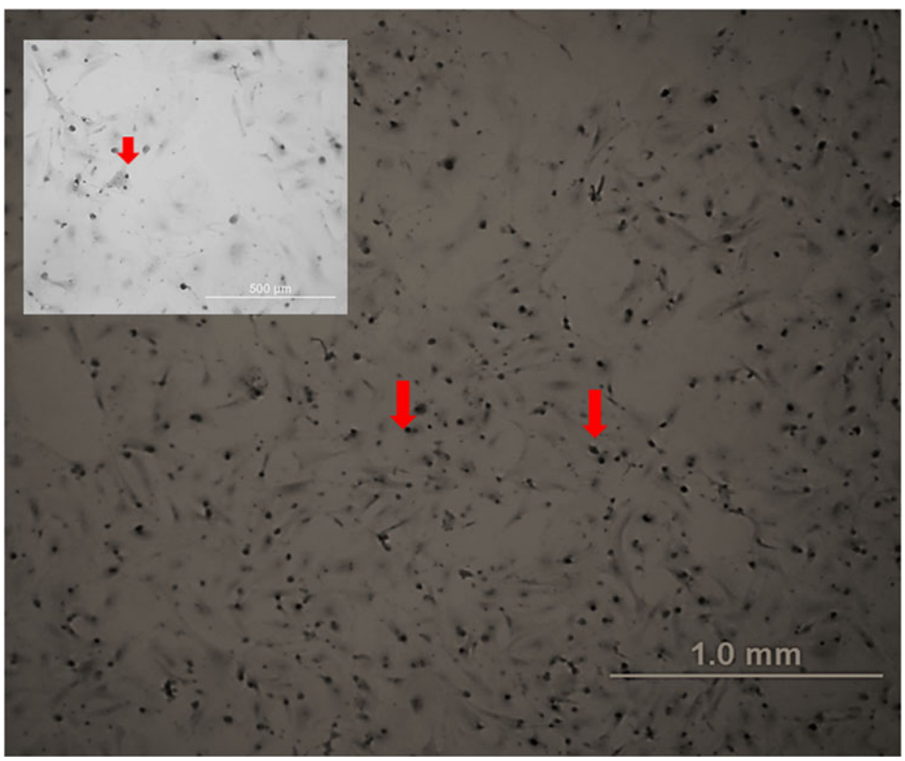

FIG. 3. Laser treatment with 12 J/cm^2 after MTT. Note the formation of formazan crystals.

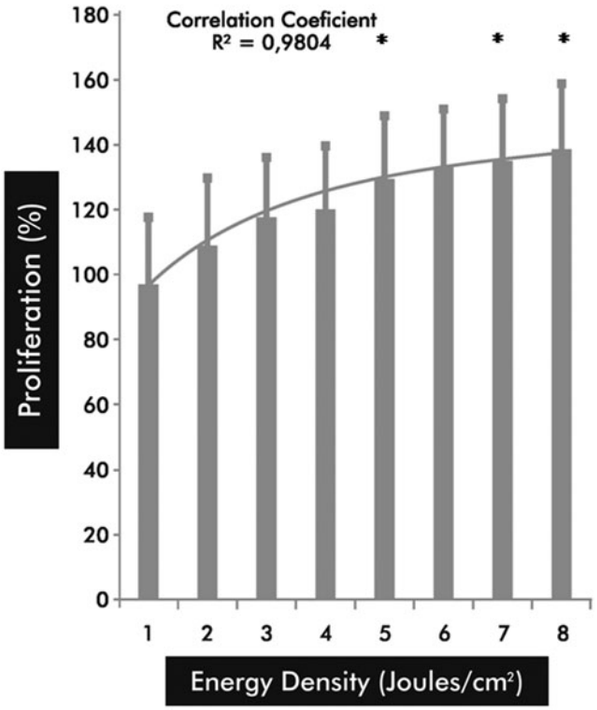

FIG. 4. Proliferation in percentage (%) and laser energy density in Joules. $R^2 = 0.9804$. Group 1 is the untreated control, group 2 is laser treated with 1 J/cm^2, group 3 is laser treated with 2 J/cm^2, group 4 is laser treated with 4 J/cm^2, group 5 is laser treated with 6 J/cm^2, group 6 is laser treated with 8 J/cm^2, group 7 is laser treated with 10 J/cm^2, and group 8 is laser treated with 12 J/cm^2. Note a direct relationship between laser energy density and proliferation. ANOVA: analyzed Group 8 was statistically significant ($p < 0.05$) compared with group 1, and groups 5, 7, and 8 were statistically significant ($p < 0.05$) compared with group 3.

determined by thiobarbituric acid reactive substances (TBARs) assay. Aliquots of 200 μL of supernatant were added and briefly mixed with 200 μL of trichloroacetic acid at 30%, 0.9 mL of TRIS-HCl (pH 7.4), and 200 μL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 60 min. Afterward, samples were centrifuged at 8000 rpm for 5 min and the A535 nm of the supernatant was recorded. The signal was read against an MDA standard curve, and the results were expressed as nmol/mL.[19]

Statistics

Proliferation data were submitted to ANOVA and Tukey–Kramer tests using Graphpad Prism version 5.0. MDA results were submitted to variance ANOVA and Bartlett tests using Graphpad Prism version 5.0.

Results

Morphologic aspects of treated cells

After laser treatment, adherent DBMSC continued to exhibit a fibroblast-like morphology, being small, elongate, and fusiform with small cytoplasmatic projections (Figs. 1 and 2).

DBMSC cells after MTT assay presented formazan crystals (Fig. 3)

MTT cell viability

Variance analysis ANOVA revealed that Group 8 (12 J/cm^2) was statistically significant ($p < 0.05$ compared with group 1 (control not irradiated), and that groups 5, 7, and 8 (6, 10, and 12 J/cm^2) were statistically significant ($p < 0.05$ compared with group 3 (2 J/cm^2). Moreover, a positive direct relationship ($r^2 = 0.98$) between laser energy density (J/cm^2) and cell proliferation (%) could be observed (Fig. 4).

Cell cycle

The quantity of cells at G2/M increased as energy density increased, indicating cell proliferation. In the unirradiated group (control), most of the cells were at G0/G1, and almost one third were at G2/M. After laser irradiation, almost all cells were at G0/G1. Using 2J/cm^2, most of the cells were divided between the S and G2/M phases; after 6J/cm^2, the number of cells at S phase started to decrease, and at G2/M they started to increase; using 12J/cm^2, one third of the cells were at the S phase, and in almost two thirds, they were at G2/M (Fig. 5).

Lipoperoxidation production

Higher values of MDA were obtained using laser energy densities 2 and 10 J/cm^2, but were not statistically significant after ANOVA and Bartlett test. As results were not significant, it was possible to notice that higher laser energy densities did not increase the number of free radicals produced; therefore, the group using 12 J and the untreated control group presented similar levels of peroxidated free radicals (Fig. 6).

Discussion

Experiments using DBMSC *in vitro* are the first step to test *in vivo* implants, using the dog as an animal model, for tissue engineering.

There was a direct relationship between laser energy density and cell proliferation. MTT absorbance increased with laser energy density; moreover, cell cycle showed that the number of cells at G0/G1 decreased, and that those at G2/M increased with laser energy density.

Our experiments using a wider range of energy densities (control, and 1, 2, 4, 6, 8, 10, and 12 J/cm^2) showed proliferative effects only with higher energy density levels (6, 10, and 12 J/cm^2). Morphologically analyzed, the cells did not undergo differentiation into any cell type or into neoplasic cells.

Irradiation of stem cells has been reported in the past 10 years. We used bone marrow stem cells, but experiments were also found in cardiac,[4] dental pulp,[5] and mesenchymal stem cells,[3–7,10,13,14] with the last being obtained from bone marrow[6,7,10,13,14] or adipose tissue.[3] Moreover, it is noted that they came from different donor sources. We used dog stem cells, but stem cells from other donors have also been used, such as rats, mice, and humans.

Other authors had an approach similar to ours regarding the wavelength (635 nm,[3,6,10] 660 nm,[5] and 680 nm[20]).

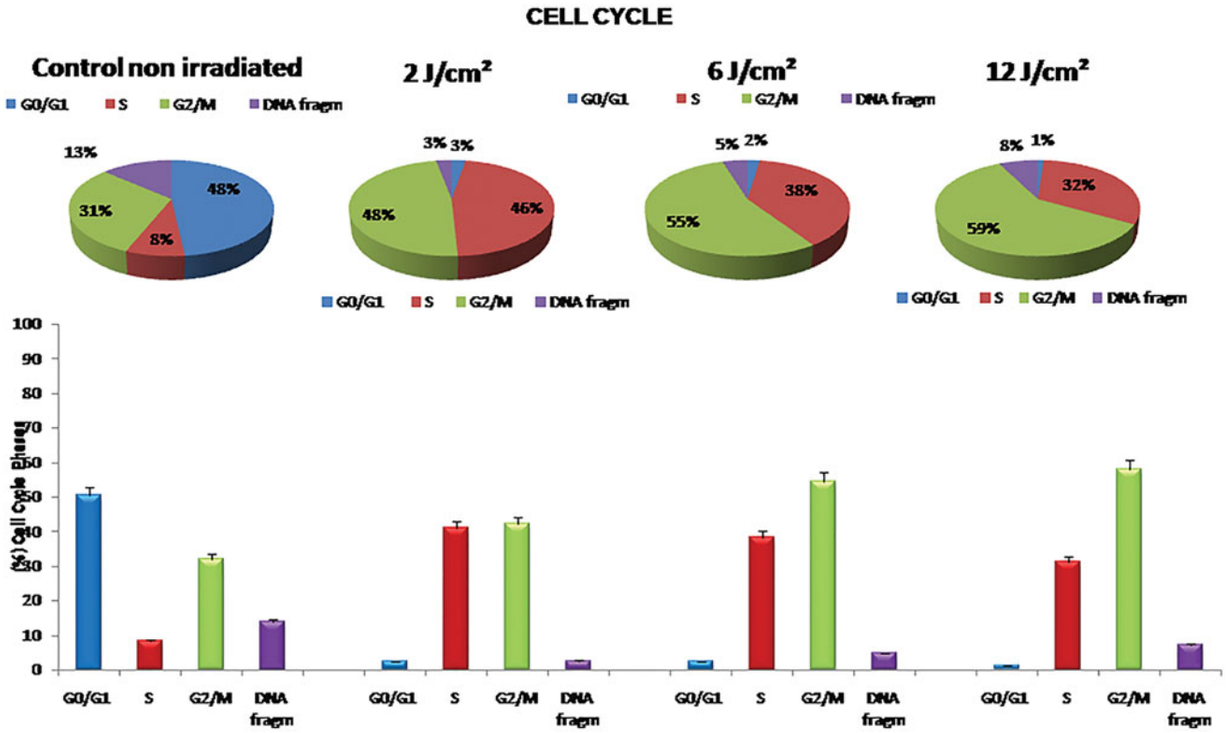

FIG. 5. Cell cycle. The quantity of cells at G2/M increases as energy density increases. Left, unirradiated control, in blue; most of the cells at G0/G1, in green. Almost one third are at G2/M. Left center, 2 J/cm^2, in blue. Almost all cells were at G0/G1, with most of the cells divided between red, at the S phase, and green, at the G2/M phase. Right center, 6 J/cm^2, in blue, with almost all cells at G0/G1, in red. The number of cells at the S phase started to decrease and in green, the cells at G2/M increased. Right, 12 J/cm^2, in blue, with almost all cells at G0/G1, in red. The number of cells at the S phase is one third of the population, and, in green, cells at G2/M are almost two thirds of the population. ◀AU11

We used red laser (660 nm). One study that used the infrared wavelength range (808 nm)[20] did not find proliferative effects on irradiated cultures compared with the controls. More recently one experiment used infrared laser (780 nm) to test if different energy densities had proliferative effects at lower energy densities (1–3 J/cm^2.[21]

The use of wavelengths in the red range seems to be appropriate for studies of cells in culture. As the penetration of the beam is a negligible factor in these conditions, model predictions and computer simulations recommend wavelengths ranging from 600 to 680 nm, and energy densities ranging from 0.3 to 4.0 J/cm^2 for enhancing proliferation of mesenchymal stem cells in culture.[22]

Regarding the energy density applied, authors mostly used the interval between 3 and 5J/cm^2 because they are traditionally found in the literature as showing proliferative effects,[3–7,9,10,12–14,,20,22,23] but higher energy densities (11.7 J/cm^2), also induced proliferative effects.[24] Our experiment found proliferative effects after 6 J/cm^2.

Our treatment pattern, laser irradiation after 24, 48, and 72 h, was also used successfully by other authors.[15,21]

We tested a wide variation of energy densities (control, and 1, 2, 4, 6, 8, 10, and 12 J/cm^2) searching for a dose response, and some experiments had a similar approach using similar energy densities (0.5, 1.0, 2.0, and 5.0 J/cm^2,[6] and 0.5, 1.5, 3, 5, and 7 J/cm^2).[21]

In general, stem cells are sensitive to culture medium and can present different behaviors when its composition is changed.[17] These data are important to make a comparative analysis. It is important to emphasize that large differences between declared and actual power output of the device were found after calibration with power meter.[20] For better results, in this work, a built-in power meter was used to analyze and recalibrate the power output during laser delivery as each parameter was changed.

The study of the production of oxygen-free radicals was performed by demonstration of the presence of MDA, which is a product of peroxidation of biological membranes. ROS are a group of molecules, such as superoxide ions, hydroxyl radicals, and hydrogen peroxide, which interact with proteins and nucleic acids. Recently, their role in modulation of cell metabolism has been highlighted. LLL therapy has proven to be an effective agent in modulating the production of these radicals, and, more broadly, in the modulation of the oxide-reductive potential toward a more oxidative cellular environment. This modulation may involve the induction of the activity of several transcriptional factors including NF-kB.[1]

An oxide-reducing cellular environment is of great importance in the modulation of metabolism, especially because of the stimulation of various substances such as the nicotinamide adenine dinucleotide phosphate (NADP/NADPH) system and the glutathione thioredoxin system. A graphic can represent the oxide-reducing gradient and its relationship with different cell cycle phases. At the oxidative zone are the proliferative effects and events following

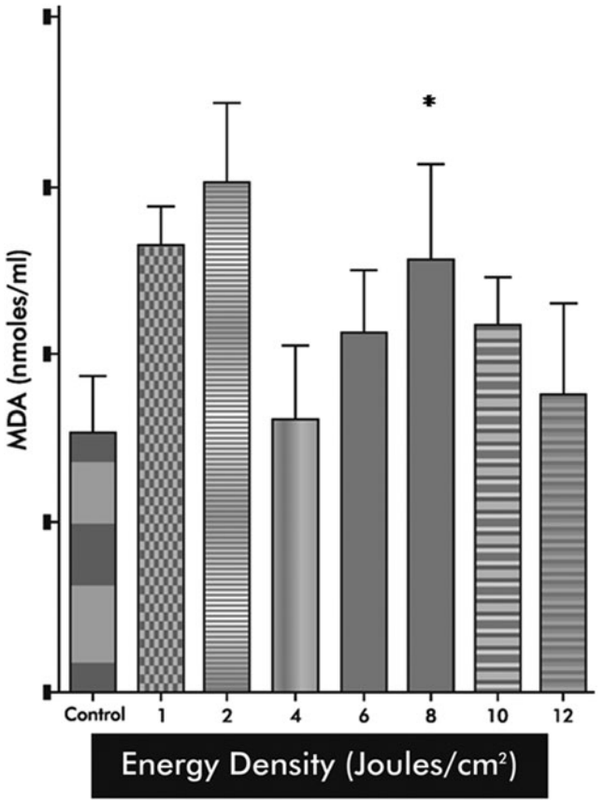

FIG. 6. Peroxidated free radicals (nmoles/mL) and laser energy density (J/cm^2). There were no statistically significant variations among groups.

cellular differentiation. On the other side, the reducing zone, are the apoptosis and necrosis phenomena,[25] Our results show that peaks of cell proliferation are related to discrete quantities of MDA. Therefore, based on our results, it is possible to think that excessive amounts of oxygen free radicals can be harmful to the process of cell proliferation, whereas low quantities would act as cellular microselectors.

Conclusions

Different LLL energy densities (12, 10, and 6 J) increased the proliferative capacity of DBMSC, and this increase had a significantly positive correlation between the energy density applied and the proliferative response. The different energy densities did not induce significantly increased lipoperoxidated free radicals, and the increase in the proliferation capacity was related to a nonstatistically significant decreased production of these radicals.

Author Disclosure Statement

No competing financial interests was reported

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