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PHOTOBIOMODULATION OF LEUKOCYTES CULTURED WITH TNF ALPHA AND TITANIUM DISCS

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ORIGINAL ARTICLE

ABSTRACT

Objective:

This work evaluated cell proliferation, cell cycle, and lipoperoxidation of populations of neutrophils and lymphocytes, in contact with titanium discs, in an inflamed environment, irradiated with LLL (low-level laser).

Background:

Dental implants, and titanium screws, used in dental and maxillofacial surgery, can osseointegrate, and support loads, The osseointegrated implant surgery causes trauma and initiates an inflammatory process.

Laser photobiomodulation can affect living tissues, accelerating the inflammatory phase and the repair process.

Method:

lymphocytes and neutrophils were cultured with TNF-***α*** (tumor necrosis factor-alpha) to simulate an inflamed environment. They were cultured with titanium discs to simulate osseointegrated implants. To evaluate the laser photobiomodulation effects, neutrophils received 1 irradiation of LLL, and lymphocytes received 3 irradiations of LLL, (every 24 hs) of 50mW, 660 nm AsGaAl (aluminum gallium arsenate) red laser, for 50 sec., 2.5 J and 88.33 J/cm² per session.

The cell cycle was analyzed in flow cytometry, and the lipid peroxidation was quantified. Results were statistically evaluated using unpaired one-way ANOVA and Tukey-Kramer analysis (*) $p<0.05$ at graphpad Prism 8™.

Results:

All the treatments increase the levels of ROS inside the neutrophils and lymphocytes.

All the groups present changes in the cell cycle increasing DNA synthesis and mitosis, except for the neutrophils that when laser irradiated are not distinct from the control group, and for the lymphocytes that when cultured with TNF-***α*** itself increase apoptosis.

Conclusion:

Laser photobiomodulation after implant placement increases lymphocyte and neutrophil DNA synthesis and mitosis, and this increase was higher in an inflamed environment.

Keywords: Photobiomodulation, low-level laser, leukocytes, lymphocytes, neutrophils, dental implants, titanium.

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INTRODUCTION

In recent decades, dental implants have brought dental and oral rehabilitation to a higher level. The insertion of dental implants has proved to be the most conservative technique in oral rehabilitation, as it prevents the wear of adjacent teeth. [1]

Dental implants, as well as titanium screws, have been frequently used in dental and maxillofacial surgery, [2] because this material can integrate, allowing the placement of a masticatory load over them. [3,4,5]

On the other hand, implant surgery causes trauma in the alveolar bone that initiates an inflammatory process, the initial part of the reparative process. [6]

In the inflammatory phase, lymphocytes arrive at the site to recognize the offending agent or identify the extent of the trauma. They recruit and activate polymorphonuclear cells that release IL1, IL6, and TNF-$\boldsymbol{\alpha}$. For each type of aggression, chain reactions are initiated, the Pamps, for aggressive agents (bacteria and viruses), and the Damps for traumas. [7]

Placing an osseointegrated implant in the bone assumes an aseptic environment. When there is an infection in the site, inflammatory agents, first neutrophils and then lymphocytes, arrive to combat pathogens.

Following the chain caused by the trauma of implant placement, it is possible to identify neutrophils capable of phagocytizing invading agents, and cleaning up cell debris and damaged extracellular matrix. After cleaning the tissues, the reparative process begins, in which macrophages and neutrophils produce IL-10, and fibroblasts start the proliferation and formation of a collagen extracellular matrix**.** [8,9]

LLL is an ionizing radiation capable of causing changes in living tissues, accelerating the inflammatory phase and consequently the beginning of the repair process. [10] In addition, low-level laser irradiation accelerates the formation of type 1 collagen, significantly reducing the lesion. [11,12]

Laser beam

Biostimulatory effects of laser irradiation can be photochemical, photophysical, and photobiological. [13] The longer the wavelength, the deeper it produces its effects, so the red wavelength lasers (from 630 nm to 700 nm) reach more superficial tissues,

and the infrared wavelength lasers (from 700 nm to 904 nm) reach deeper tissues.[14] Photobiomodulation with red and infrared lasers accelerates the inflammatory phase and the beginning of the reparative process. [11]

In addition to the primary reactions that occur in the mitochondrial cytochrome c oxidase increasing energy acquisition by the cell, a cascade of secondary reactions occurs in the cytoplasm, membrane, and cell nucleus after photobiomodulation, altering the REDOX state, which stimulates cellular signaling and affects gene expression (via transcription factors and other effector molecules) by increasing RNA and amino acid synthesis [14,15] and causing cell differentiation and proliferation. [16,17,18]

Low-level lasers are applied directly to the injury site or various points on the body. [19, 20,21] produce analgesic, and anti-inflammatory effects, due to their ability to stimulate the release of endorphins, and oxidative stress, treating among other pathologies dermatitis, and autoimmune diseases, reducing swelling, bruises, and muscle pain, reducing edema, and local ischemia, improving bone and connective tissue remodeling and repair,[10,11,19,20,21]

Low-level lasers can accelerate tissue repair after tooth extraction, showing a more homogeneous bone trabecular configuration, [21] When applied before dental implant placement, it increases stability by peri-implant bone repair, bone-implant contact, and bone neoformation. [22] Moreover, it is capable of increasing the production of extracellular matrix after implant placement.[23]

Laser irradiation does not lead lymphocytes to a blastic transformation but activates mitochondria, leading to Ca^{2+} influx, increased RNA synthesis, extra ATP production, structural changes in chromatin and nucleolus, mitochondrial profiles increase, and functional activation of mitochondria during interphase. In the nucleolus, the laser causes an irregular fibrillar center (FC) appearance, indicating an increased level of transcription of r-genes; an increase in the area of the dense fibrillar component, improving the production of ribosomal pre-RNA; an increase in ribonucleoprotein granules, improving pre-RNA processing, pre-ribosomes production, and the appearance of vacuoles, stimulates RNA metabolism (synthesis, processing and transport), and increases DNA synthesis. [24,25]

LLL can reduce the number of neutrophils to the level of a non-inflamed tissue

when directly irradiating a lesion for a longer period with low power, reducing the levels of IL1 and IL6. Irradiating a lesion for a shorter period with high powers decreases the level of TNF-α.[26]

Neutrophils in an inflamed media increase the production of reactive oxygen species (ROS),[26] and are more active metabolically *in vivo* and *in vitro.* The kinetics proteins lead these cells to a more activated state increasing their viability and the number of cells that migrate to a damaged site, as well as their ability to synthesize proteins, and produce ROS.

OBJECTIVE

This work evaluated cell proliferation, cell cycle, and lipoperoxidation of leukocytes in contact with titanium discs in an inflamed environment irradiated with low-level laser AsGaAl 660 nm.

METHODS

Cell culture

All the experiments were conducted according to the Declaration of Helsinki, the norms of current legislation regarding the use of human cells for scientific purposes. This study was approved by the research ethics committee of “Plataforma Brasil”, protocol number 38655. The cells were obtained and treated at the Development and Innovation Laboratory of the Butantan Institute, under the supervision and guidance of Professor Dr. Durvanei Augusto Maria as follows: White cells, lymphocytes, and neutrophils were obtained from a 20 mL blood sample from a young, healthy male donor, and separated using histopaque 1077 and PBS (phosphate saline buffer) in laminar flow under sterile conditions. Briefly:

lymphocytes

10 mL of blood were incubated with the Cocktail of Rosettesep Tetrameric antibody complexes (Stem Cell Technologies) for 30 mins at room temperature, pipetted the Ficoll-Hypaque solution into a 50 mL conical centrifuge tube, using 2mL Ficoll-Hypaque per mL blood, mixed anticoagulated blood with an equal volume of PBS, slowly laid the diluted blood over the Ficoll-Hypaque solution by gently pipetting the diluted

blood down the side of the tube containing the Ficoll-Hypaque, centrifuged 30 min at 400 × *g*, 22°C, removed the mononuclear cells or desired non-aggregated mononuclear cell subset, located at the interface between the plasma (upper layer) and the Ficoll-Hypaque (bottom), transferred the aspirated mononuclear cells to a 15 mL conical tube, add 10 mL PBS or tissue culture medium and mixed thoroughly, centrifuged 10 min at 400 × *g*, 4°C, discarded the supernatant and repeated wash with PBS or tissue culture medium as needed**.**[28]

Neutrophils

At room temperature, 10.0 mL of neutrophil isolation media were collected in a centrifuge tube with 10.0 mL of blood, and centrifuged at 500 RCF for 35 min at 20-25°C, the blood was separated into 6 distinct bands: plasma, monocytes, isolation media, neutrophils, more isolation media, and the red blood cell pellet. Removed the top three layers (plasma, monocytes, and isolation media), pipetted the layer of neutrophils and all of the isolation media beneath the neutrophils, placed the solution into a clean centrifuge tube, diluted the neutrophil solution to 10 mL with HBSS without Ca^{2+}/Mg^{2+}. Inverted the tube a few times to suspend the cells, and centrifuged the neutrophil solution at 350 RCF for 10 minutes. The red pellet was formed by neutrophils and residual red blood cells (RBCs), and the supernatant was pipetted and discarded. To lyse the residual RBCs, 2 mL Red Cell Lysis Buffer was added to the tube, vortexed the vial at a setting of 3-4 to dissolve the pellet, centrifuged the tube at 250 RCF for 5 min, discarded the supernatant with a pipette, repeated the lysing process, added 500 µl HBSS without Ca^{2+}/Mg^{2+} to each tube, vortexed to resuspend the pellet at a setting of 3-4, diluted to 10 mL with HBSS without Ca^{2+}/Mg^{2+}, centrifuged the tubes at 250 RCF for 5 min. discarded the supernatant, and resuspended the pellet in 250 µl HBSS/HSA Solution (2% HSA). Cells were then counted and adjusted to the desired concentration.[29]

Study design

The cells were seeded at $1x10^5$ cells per well, in quintuplicate, and incubated at 5% CO_2 at 37°C, in 3 plates of 96 wells. The neutrophils were cultivated for 12 hs (due to their low confluence and life expectancy) and the lymphocytes were cultivated for 48 hs.

- Group 1 was the untreated control.

- Groups 2, 4, 6, and 8 were cultured with TNF-α (tumor necrosis factor-alpha) 10ng/ mL, obtained from Sigma Chemical Co. (St. Louis, MO, USA), to simulate an inflamed environment.
- Groups 3, 4, 6, and 8 were cultured with titanium discs (grade 4), produced by Conexão™ Brazil, ref. C0880199, lot.111021, in the format of disks of 4 mm in diameter, and 0,3mm in width, to evaluate the cell reactions in the presence of osseointegrated implants.
- Groups 5, 6, 7, and 8 had LLL irradiation to evaluate the effects of photobiomodulation. Neutrophils received 1 irradiation, and lymphocytes received 3 irradiations, (immediately after cell seed and every 24 hs). Laser parameters: 50mW, 660 nm AsGaAl red laser, DMC™, Brazil, for 50 s, 2.5 J, and 88.33 J/cm² per session, a holder positioned the laser tip at 2cm of the cells. (Table 1A, 1B)

Table 1A - LLL treatment parameters used to irradiate T-lymphocytes

Power output	0,05W
Walvelength	660nm
Laser beam	Continuous
Area of the laser beam	0,0283 cm^2
Time exposure per session	50 seconds
Energy per session	2,5 Joules
Irradiance	1,76 W/cm^2
Fluence	90 J/cm^2
Number of sessions	3
Total energy delivered	7,5 J

Table 1B - LLL treatment parameters used to irradiate Neutrophils

Power output	0,05W
Walvelength	660nm
Laser beam	Continuous
Area of the laser beam	0,0283 cm^2
Time exposure per session	50 seconds
Energy per session	2,5 Joules
Irradiance	1,76 W/cm^2
Fluence	90 J/cm^2
Number of sessions	1
Total energy delivered	2,5 J

Neutrophils and lymphocytes were seeded separately in three plates, and divided into 8 groups, as follows: (Figure 1)

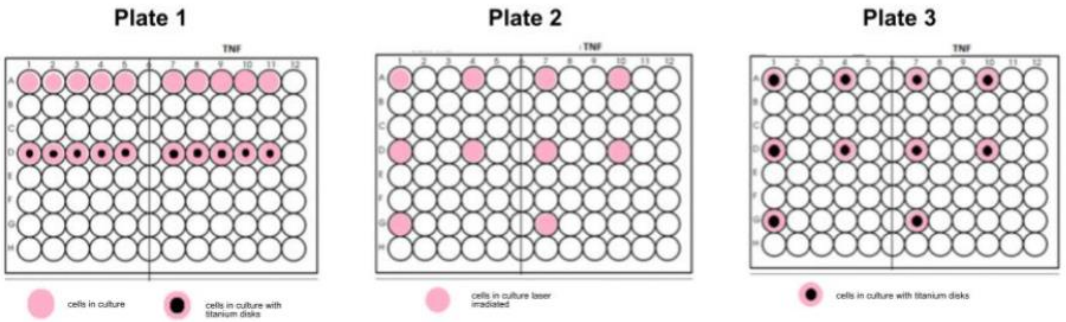


Figure 1 - Study design, division of groups of leukocytes (lymphocytes and neutrophils) according to treatment. Plate 1: Group 1 - untreated control. Group 2 - cells cultured with titanium disk. Group 3 - cells were cultured with TNF-***α*** alpha. Group 4 - cells were cultured with titanium disk and TNF-***α*** alpha. Plate 2: Group 5 - laser irradiated. Group 6 - laser irradiated with TNF-***α*** alpha. Plate 3: Group 7 - laser irradiated with titanium disk. Group 8 - laser irradiated with titanium disk and TNF-***α*** alpha.

Treatment diagram of neutrophils and lymphocytes

- The first plate (unirradiated):

- Group 1 - untreated Control (C),
- Group 2 - TNF-***α*** (TNF-***α***),
- Group 3 - titanium disk (TI),
- Group 4 - TNF-***α*** and titanium (TNF-***α*** - TI),

-Second plate (LLL irradiated):

- Group 5 - LLL (LLL),
- Group 6 - TNF-***α*** and LLL (TNF-***α*** - LLL),

-Third plate (LLL irradiated and titanium disks):

- Group 7 - titanium disk and LLL (TI - LLL),
- Group 8 - titanium disk, TNF-***α***, LLL (TI - TNF-***α*** - LLL).

Flow cytometry analyzed the cell cycle, lipid peroxidation was quantified, and statistical analysis was performed using GraphPad Prism™ 8.

Cell cycle

The cell cycle is a four-stage process in which the cell increases in size (gap 1, or G1, stage), copies its DNA (synthesis, or S, stage), prepares to divide (gap 2, or G2, stage) and divides (mitosis, or M, stage). The stages G1, S, and G2 comprise interphase, which accounts for the span between cell divisions.

Neutrophil and lymphocyte samples were placed in microtubes and centrifuged at 3000 rpm. The supernatant was used to determine the production of lipo-peroxidized free radicals (LPO). Propidium iodide (20mg/ mL, Sigma®), a fluorescent marker that binds to DNA to assess its quantity and integrity in the cell cycle phases was added to cell samples. Data was acquired using a Muse® Merck Millipore flow cytometer and the cycle phases (G0/G1, S phase, and G2/M) were analyzed using the GraphPrism™8 software.

After 6h in culture for neutrophils and 48 h in culture for lymphocytes, cell lines

were trypsinized and inactivated with FBS, centrifuged at 1,500 rpm for 10 min, and the supernatant was discarded. The pellets were resuspended in 5 mL of PBS in a concentration of 10^6 cells/ mL. To analyze intracytoplasmic and nuclear markers, cells were permeabilized with 5 µl of 0.1% Triton X-100 for 30 min before adding specific primary antibodies. The following markers were used to determine cell death pathways: Bax (Ab5714, Abcam Inc), Bad Ab32445, Abcam Inc), and Bcl-2 (Ab692, Abcam Inc). Antibodies for cyclin D1 (sc8396, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used to determine the proliferation index, and samples were analyzed by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA), the expression of cell proliferation and cell death markers were compared with parental control cells. Detection of the markers was followed by analysis of the cell cycle phases. In this step, the trypsinized cells were treated with 70% ice-cold ethanol containing 100 µg/ mL RNase. They were then washed and incubated in PBS at 37°C for 45 minutes. The labeling was performed in a solution containing propidium iodide (PI) at a concentration of 1.8 mg/ mL to assess the integrity and quantity of DNA in the cell cycle phases. The Annexin V FITC Apoptosis Detection Kit I (BD) was used to evaluate apoptosis. Cells were centrifuged and the cell pellet was suspended with a binding buffer (100 µl) and then incubated with Annexin V-FITC (2 µl) and PI (2 µl) for 15 minutes, at room temperature in the dark. After incubation, 400 µl of binding buffer was added and cells were analyzed in a FACScalibur (BD) using CellQuest software to determine the percentage of apoptotic cells. A minimum of 10,000 events was acquired for each sample.[30, 31]

Lipid peroxidation

This is a process of oxidation of lipids (polyunsaturated fatty acids, including those present in cell membranes or organelles) caused by the action of free radicals. Free radicals are ROS that do not have a complete valence layer (they have an unpaired electron) and thus can combine with the DNA of cells and alter their genetic code, causing uncontrolled multiplication in cells, in addition to altering the cell membrane and cell plasma, resulting in disturbances in its functions such as selectivity, transport, containment of cytoplasmic content, enzymes and other organelles. [32] Studies on the lipoperoxidation of lymphocytes and neutrophils after LLL photobiomodulation were motivated by studies that discuss the power of the laser in modulating oxidative stress.[33,34,35,36,37]

To analyze lipid peroxidation, 50uL of supernatant was removed from each sample and placed in 96-well plates, analyzed in a spectrophotometer with a wavelength of 330 nm. Data obtained from lipid peroxidation was calculated by subtracting the blank values of each series. The average absorbance of the series of 20% trichloroacetic acid (TCA) was obtained through the difference between TCA and 0.86% thiobarbituric acid (TBA), divided by the molar coefficient of malondialdehyde (MDA):

$$LPO = \frac{[(\text{MEAN ABSORBANCE TCA-BLANK}) - (\text{MEAN ABSORBANCE TCA/TBA-BLANK}) \times 5]}{(0.03212 - \text{MOLAR COEFFICIENT OF MDA})}$$

The oxidative stress on unsaturated lipids in cell membranes was evaluated by determining the amount of malondialdehyde (MDA), which is the final product of fatty-acid peroxidation that reacts with thiobarbituric acid (TBA) to form a colored complex. Thiobarbituric acid reactive substances (TBARS) were quantified by the spectrophotometric determination (LPO method).[35]

Statistical analysis

Data were statistically analyzed in the GraphPad Prism™ 8, (USA), using analysis of variance, ANOVA multiple comparisons, followed by the Brown-Forsythe test with a significance level of (****) $p < 0.0001$, $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*).

RESULTS

Cell cycle

Lymphocytes

Treatments changed the populations' distribution during the cell cycle. Untreated control lymphocytes present the larger part of the cells at the G0/G1 phase, the S, G2/M, and sub G0 Phases present few cells. Compared to treated cells; titanium-treated cells present the majority of cells at the G0/G1 phase, and sub-G0, S, and G2/M phases present few cells, the number of cells at the S phase increases, and the number of cells at the G2/M phase reduces significantly. More cells are still duplicating DNA and do not reach mitosis. This reduction in the G2/M phase indicates less proliferation. The

lymphocytes TNF-***α*** treated reduce the population at G0/G1 and significantly increase the sub-G0 population. More cells are starting apoptosis. The association of TNF-***α***-Titanium presents the greater part of cells at the G0/G1 phase, but while this population reduces, cells at the G2/M phase increase significantly compared to controls. Cell proliferation is significantly increasing. After laser irradiation, the larger part of the lymphocytes are still at G0/G1, the number of cells at the S phase increases, and the number of cells at the G2/M phase significantly reduces. More cells are replicating their DNA, and do not reach mitosis. This reduction in the G2/M indicates less proliferation. In the association of TNF-***α***-Irradiated, cells were better distributed, G0/G1 lymphocytes significantly reduced, and S and G/2M significantly increased, indicating that cells migrate from G0/G1 to S and G2/M phases, increasing lymphocyte proliferation. The major part of the cell population after titanium-irradiation migrates from Go/G1 to the S phase, indicating an increase in DNA synthesis but no significant lymphocyte proliferation. After the association of TNF-***α***-Titanium-laser Irradiation, G0/G1 phase cells reduced, and S and G2/M cells increased, raising DNA synthesis and lymphocyte proliferation. All the TNF-***α*** groups present lymphocytes with increased proliferation, whereas titanium and laser-irradiated lymphocytes present reduction. The TNF-***α*** group itself presented increased lymphocyte apoptosis. All the laser-irradiated cell groups and the titanium alone group present increased lymphocyte DNA synthesis. Laser irradiation, when associated with other treatments increased cells initiating the cell cycle, the titanium-irradiated group stayed at DNA synthesis and the laser associated with TNF-***α*** groups increased cell proliferation. (Figure2)

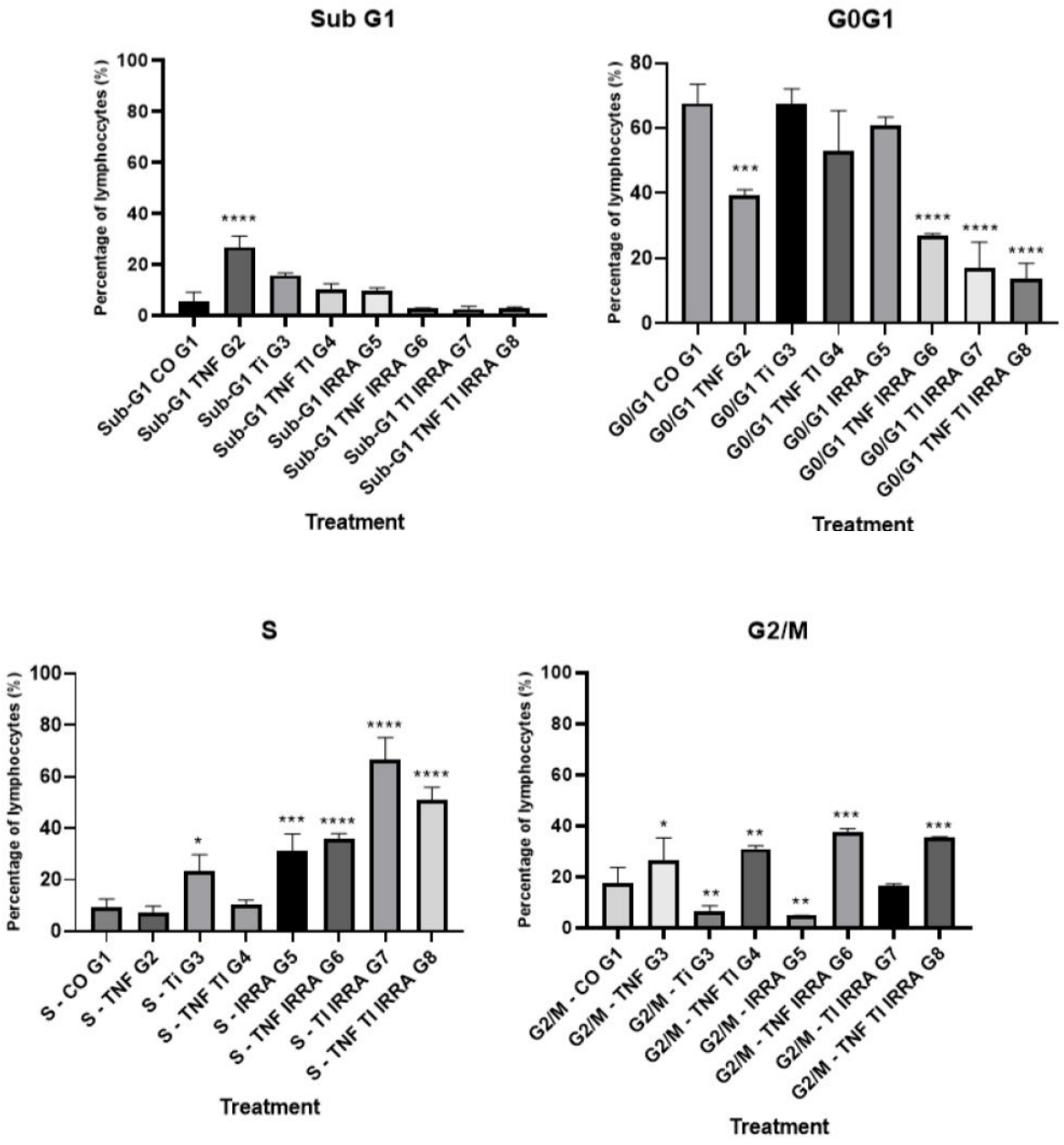
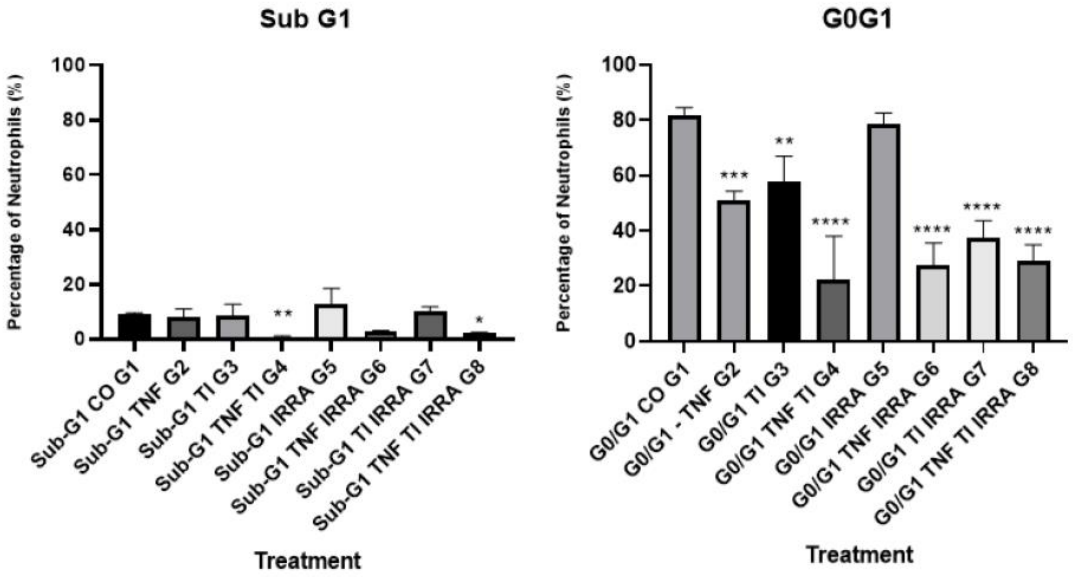


Figure 2 - Phases of the cell cycle of lymphocytes cells (Group 1 - untreated Control (C), Group 2 - TNF-***α*** (TNF-***α***), Group 3 - titanium disk (TI), Group 4 - TNF-***α*** and titanium (TNF-***α*** - TI), Group 5 - Low-Level Laser (LLL), Group 6 - TNF-***α*** and LLL (TNF-***α*** - LLL), Group 7 - titanium disk and LLL (TI - LLL), Group 8 - titanium disk, TNF-***α***, LLL (TI - TNF-***α*** - LLL)) 48 h after treatment, media and standard deviation of groups statistically evaluated compared to control group using ANOVA and TUKEY-KRAMER analysis, p values: p<0.0001 (******),** p<0.001 (***), p<0.01 (**), p<0.05 (*) represent significance compared to the control group.

Neutrophils

The greater part of untreated control neutrophils are at the G0/G1 phase, the other phases present few cells. Compared to treated cells, In the titanium group neutrophils migrated from G0/G1 to the G2/M phase, presenting more cell activity. Neutrophils from the TNF-***α*** group passed from G0/G1 to the G2/M phase, increasing

cell activity. The TNF-***α*** -Titanium group, had the majority of cells in the S phase, reduced cells at the G0/G1 phase, and increased cells at the G2/M phase, more cells are starting the cell cycle and the majority of them are replicating DNA. The LLL irradiated group without associated treatments did not present alterations compared to the untreated control, this treatment alone did not significantly change these cells without the association of other stimuli. The TNF-***α***-laser irradiated group presented the most similar cell distribution among groups, most cells started the cell cycle indicating neutrophil activation. The same occurred in the Titanium-irradiated group, there were more cells at the G2/M phase than at the S phase showing stage maturation. In the TNF-***α***-titanium-laser-irradiated group, there was no increase in the G2/M phase, but at the S phase, showing neutrophil activation. The combination of treatments: TNF-***α*** and titanium, TNF-***α*** and titanium and laser irradiation, reduced neutrophils apoptosis. TNF-***α*** alone and associated with other therapies increased the DNA synthesis of neutrophils. All the treatments present increased cellular activity, probably enzymatic or phagocytic function. (Figure 3)



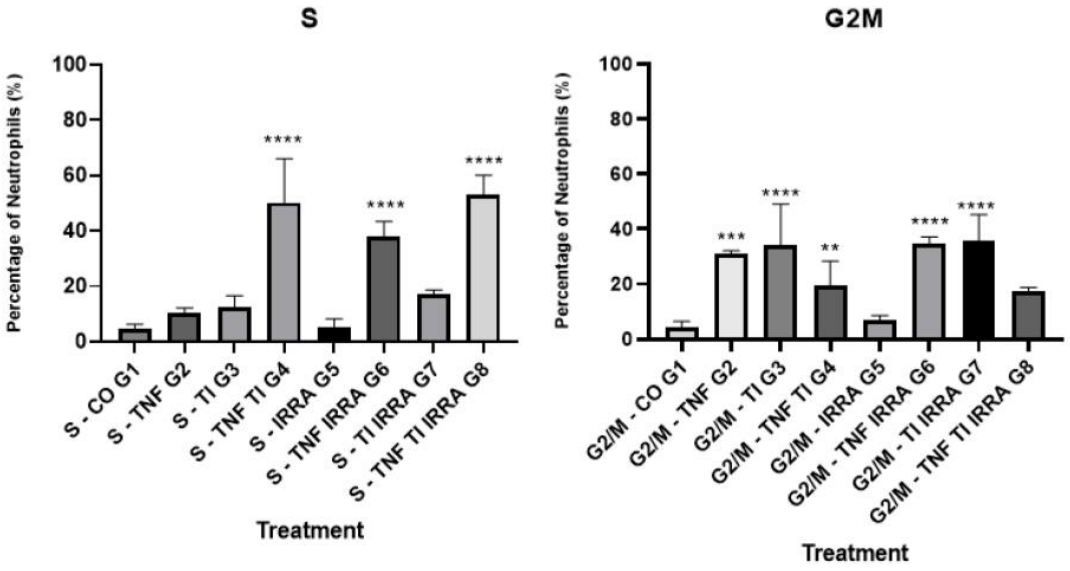


Figure 3 - Phases of the cell cycle of neutrophils 48 h after treatment, (Group 1 - untreated Control (C), Group 2 - TNF-***α*** (TNF-***α***), Group 3 - titanium disk (TI), Group 4 - TNF-***α*** and titanium (TNF-***α*** - TI), Group 5 - Low-Level Laser (LLL), Group 6 - TNF-***α*** and LLL (TNF-***α*** - LLL), Group 7 - titanium disk and LLL (TI - LLL), Group 8 - titanium disk, TNF-***α***, LLL (TI - TNF-***α*** - LLL)) 48 h after treatment, media and standard deviation of groups statistically evaluated compared to control group using ANOVA and TUKEY-KRAMER analysis, p values: p<0.0001 (******),** p<0.001 (***), p<0.01 (**), p<0.05 (*) represent significance compared to the control group.

Lipid Peroxidation

All lymphocyte groups were significantly affected by treatment, compared to the untreated control group, presenting increasing levels of malondialdehyde after 48 h in culture, indicating increasing cell activity. The groups cultured with the association of TNF-***α*** and titanium, and TNF-***α*** and laser irradiated were the least affected by lipid peroxidation. (Figure 4)

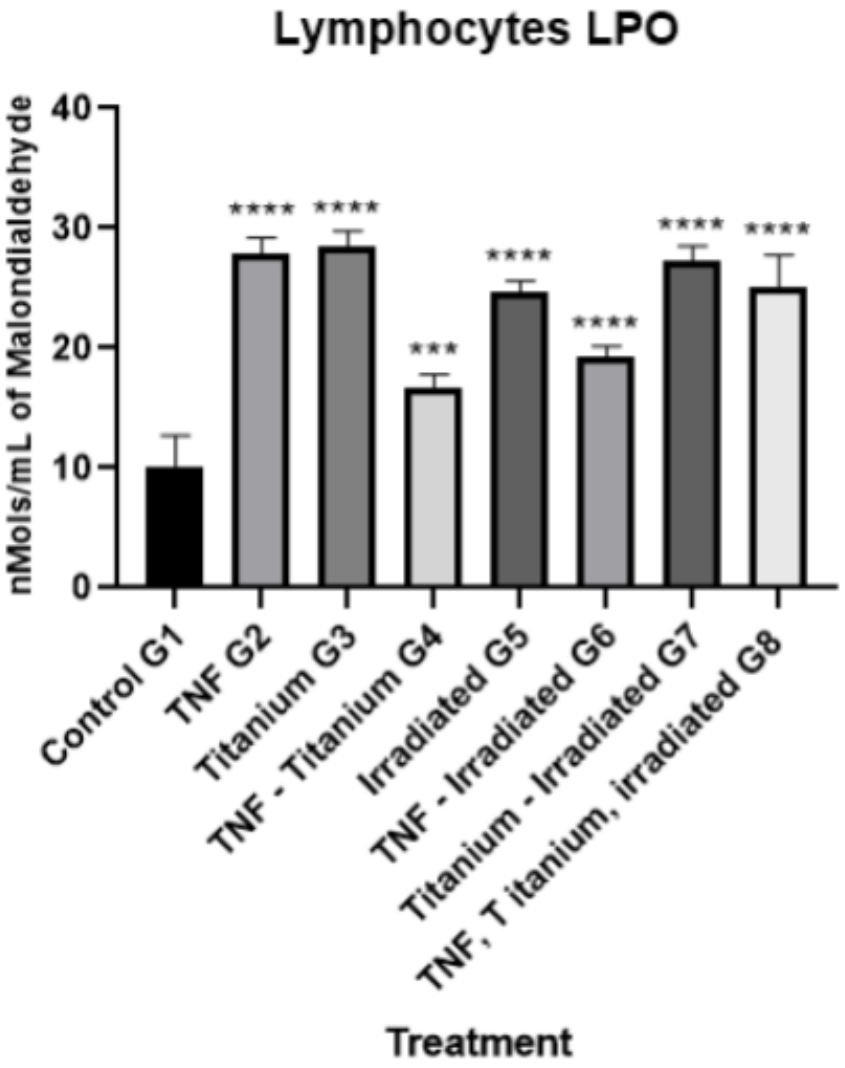


Figure 4 - Levels of malondialdehyde of lymphocytes 48 h after treatment, (Group 1 - untreated Control (C), Group 2 - TNF-***α*** (TNF-***α***), Group 3 - titanium disk (TI), Group 4 - TNF-***α*** and titanium (TNF-***α*** - TI), Group 5 - Low-Level Laser (LLL), Group 6 - TNF-***α*** and LLL (TNF-***α*** - LLL), Group 7 - titanium disk and LLL (TI - LLL), Group 8 - titanium disk, TNF-***α***, LLL (TI - TNF-***α*** - LLL)) 48 h after treatment, media and standard deviation of groups statistically evaluated compared to control group using ANOVA and TUKEY-KRAMER analysis, p values: p<0,0001 (******),** p<0,001 (***) represents significance compared to the control group.

All neutrophil groups were significantly affected by treatment, presenting increasing levels of malondialdehyde after 6 h in culture compared to the untreated control. The TNF-***α*** group showed the lowest lipid peroxidation production, while the Titanium group and the association of TNF-***α***, laser, and titanium presented the highest levels of malondialdehyde. These levels were similar to the values of the group laser irradiated. The group cultured with titanium laser irradiated presented reduced lipid peroxidation levels compared to the group of cells cultured just with titanium, indicating

that photobiomodulation reduces the increasing malondialdehyde levels. (Figure 5)

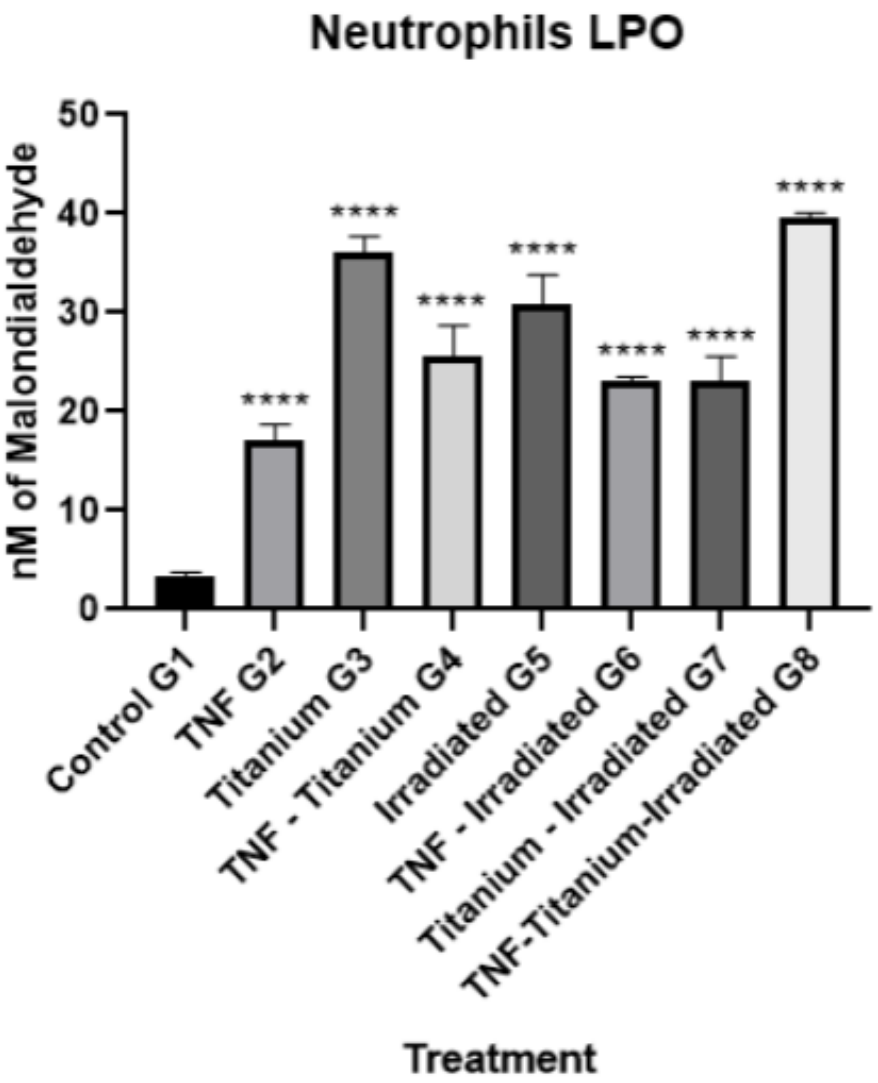


Figure 5 - Levels of malondialdehyde of neutrophils after 6 h in culture, (Group 1 - untreated Control (C), Group 2 - TNF-***α*** (TNF-***α***), Group 3 - titanium disk (TI), Group 4 - TNF-***α*** and titanium (TNF-***α*** - TI), Group 5 - Low-Level Laser (LLL), Group 6 - TNF-***α*** and LLL (TNF-***α*** - LLL), Group 7 - titanium disk and LLL (TI - LLL), Group 8 - titanium disk, TNF-***α***, LLL (TI - TNF-***α*** - LLL)) 48 h after treatment, media and standard deviation of groups statistically evaluated compared to the control group using ANOVA and TUKEY-KRAMER analysis, p values: p<0,0001 (********) represents significance compared to the control group.

DISCUSSION

Inflammation is part of the repair process after implant surgery, evolving to osseointegration after 8 weeks. Understanding how the Neutrophils and lymphocytes respond in contact with the titanium of an implant, in an inflamed environment, (simulated with TNF-***α***) and having this inflammation modulated using low-level laser, can provide elements to the professional to establish clinical protocols and decide on

the best treatment for the patient.

The cell cycle is the main event for cell growth, the correct progression of its four phases is vital to reach tissue reactions and self-renewal. It can be modulated by the activation or high expressions of specific cell cycle proteins, in addition to the production and release of growth factors interleukins and inflammatory cytokines. [31]

Unlike pharmaceutical agents, the effects of LLLT on cell proliferation, and metabolism, angiogenesis, apoptosis, and inflammation involve a wide range of parameters in terms of laser properties and dosage that are linked directly to the desired effects, underdosage results in poor cellular response but overdosage may paradoxically inhibit cell proliferation or induce apoptosis. These cellular responses also appear to be specific to the tissue type. [33,35,37,38,39]

Titanium is the main component of dental implants. This experiment used titanium disks to mimic implant placement and understand how lymphocytes and neutrophils react after surgery. Titanium is the metal of choice for oral and maxillofacial implants. It is a highly reactive metal that, in contact with the atmosphere oxygen, develops a layer of titanium dioxide (TiO2) over its surface. This TiO2 layer acts as a crucial interface between the implant and the biological milieu, ensuring biocompatibility by reducing material reactivity and partially preventing corrosion.[40,41]

Titanium enhances T-lymphocyte proliferation, the number of cells in the G0/G1 phase decreases and the number of cells in the S and G2/M phases increases. [41] In this experiment, using a titanium disk simulating an implant, the G0/G1 population of lymphocytes around a titanium disk does not change, the G2/M decreases and the S increases; some cells, despite DNA replication, do not reach mitosis.

TNF-α is a cell mediator present in inflamed tissues that can lead cells to apoptosis via stimulated p53 and NF-kB.[42] The TNF-α pathway plays as a double-edged sword that simultaneously regulates cell apoptosis and proliferation. Optimal levels of TNF-α signaling are required for tissue repair following the acute injury phase, increased levels of TNF-α signaling can trigger cytokine storms that lead to profound cell death during the phase of acute tissue injury.[42] This concentration of TNF-α mimics an inflamed environment, it reduces the G0/G1 phase and increases the sub-G0 phase, and these lymphocytes enter apoptosis. The majority of cells cultured in the association

of TNF-α and titanium are at G0/G1, but while this population reduces, cells at the G2/M phase increase significantly compared to controls, they are proliferating.

Low-level laser irradiation does not lead lymphocytes to a blastic transformation, it activates these defense cells, starting in mitochondria and extending to Ca^{2+} influx, increased RNA synthesis, extra ATP production, and structural changes in chromatin and nucleolus, mitochondrial profiles increasings, causing the functional activation of mitochondria during interphase. In the nucleolus, the laser causes an irregular fibrillar center (FC) appearance, indicating increased level of transcription of r-genes; an increase in the area of the dense fibrillar component, indicating improved production of ribosomal pre-RNA; increase in ribonucleoprotein granules which may be related to the improvement in pre-RNA processing and pre-ribosomes production; and the appearance of vacuoles, stimulates RNA metabolism (synthesis, processing and transport), and increases DNA synthesis.[24,25] Photobiomodulation with LLL can stimulate lymphocyte proliferation and cell viability,[37,38] reduce the peak of inflammation, stimulate repair, and increase ROS. [11] Lymphocyte proliferation is significantly higher in samples irradiated in the presence of whole blood than in lymphocytes irradiated after isolation from whole blood. [38] Lymphocyte cell cycle after laser photobiomodulation presents significantly more cells in the S phase than controls. [39] agreeing with our findings that despite the majority of the lymphocytes still at the G0/G1 phase, the amount of cells at the S phase increases, because the amount of cells at the G2/M phase significantly reduces, indicating that more cells are replicating their DNA, but fewer cells can reach mitosis.

Laser irradiation of lymphocytes cultured in an inflamed environment reduces G0/G1 cells and increases S and G2/M phase significantly, increasing DNA synthesis and mitosis, and consequently increasing proliferation. After laser irradiation of titanium cultured lymphocytes, the bigger part of the cell population passes from Go/G1 to the S phase, increasing DNA synthesis. LLL irradiation of titanium and TNF-α cultured lymphocytes presented a reduction of the G0/G1 cells and an increase in S and G2/M cells, increasing DNA synthesis, without an increase in mitosis.

Neutrophils are short-living white cells, with a half-life of 6-12hs, Neutrophil progenitors proliferate to increase cell population and are daily released by the bone

marrow.[43] Different biomaterials are widely used in many biomedical applications. They can cause side effects, but interactions between circulating neutrophils and an implanted titanium plate, in the inflammatory phase of the repair process, do not present excessive neutrophil activity nor systemic inflammatory response. [44] In this experiment, titanium reduced G0/G1 cells and arrested G2/M cells, stimulating inflammatory activation. TNF-α enhances neutrophil recruitment during inflammatory processes.[45] and promotes cell cycle-associated gene expression dose-dependent. [46] We notice G0/G1 migration to S and G2M phases, more neutrophils started and finished the cell cycle indicating cellular activity.

After the migration of many cells from the G0/G1 phase of the TNF-α-titanium group, the S phase presented the majority of cells, there were increasing cells replicating DNA.

LLL-irradiated neutrophils are more active metabolically *in vivo* and *in vitro*, and present kinetics protein production correlated to a more activated state, increasing the number and viability of the cells that migrate to a damaged site.[47] When irradiating a lesion for longer periods, LLL reduces the number of neutrophils to the level of a non-inflamed tissue, reducing the levels of IL1 and IL6, when irradiating it for shorter periods (higher powers), LLL decreases the level of TNF-α.[26] Although LLL may often work as an anti-inflammatory modality [22] it can, depending on the parameters, also trigger the activation of immune cells and pro-inflammatory pathways. [47] This dose of LLL does not stimulate untreated neutrophils. Cell distribution among groups is very similar, cells start the cell cycle and finish it after mitosis.

After laser irradiation of TNF-α cultured neutrophils, cell distribution among groups is very similar, more cells start the cell cycle and there are more cells at the G2M phase than at the S phase. The laser irradiation of TNF-α and titanium cultured neutrophils presented very similar cell distribution among groups, more cells start the cell cycle, and the greater the increase in this group was at the S phase, the more cells replicating DNA.

Oxidative stress is the term used for the imbalance between reducing and oxidizing species within cells, with a predominance of formation of ROS (oxidizing species). Their unstable particles, so-called free radicals, generate cellular oxidation,

which can damage healthy cells, degrade DNA, lipids, and proteins, and cause cellular aging. [26] ROS are generally produced in normal metabolic processes, but their excess can cause oxidative stress and trigger cell death.[48]

All the groups tested present significant ROS increases, indicating increased cellular activity. Titanium (TiO2) stimulates intracellular ROS production in lymphocyte cells[49] by the interaction with the cell membrane, and this increase is dose-dependent, the higher the concentration of titanium, the higher the levels of ROS generation.[50] The amount of TiO_2 present on the surface of the titanium disk used in this experiment increases the ROS levels of lymphocytes. Tumor necrosis factor alpha (TNF-α) is a pleiotropic inflammatory cytokine. Studies have shown that TNF-α is secreted, for example, by endothelial cells, inducing the formation of intracellular ROS. These observations highlight a potential mechanism by which TNF-α can activate and damage endothelial cells, resulting in endothelial dysfunction.[51] This concentration of TNF-α increases ROS levels. Free radical and lipid peroxide production increase significantly when samples are laser irradiated dose-dependent. Free radical and lipid peroxide production increase significantly when samples are irradiated with red blood cells. The mechanism for the photobiostimulation effect after irradiation at 660 nm is the reaction of light with hemoglobin, resulting in oxygen radical production. The effects of low-level laser irradiation at 660 nm on human lymphocytes were the proliferation of lymphocytes and the formation of free radicals and lipid peroxides. Lymphocyte proliferation was significantly higher ($P<0.05$) as expressed by a Stimulation Index in samples irradiated in the presence of whole blood compared with lymphocytes irradiated after isolation from whole blood. Free radical and lipid peroxide production increased significantly when samples were irradiated with red blood cells.[52]The dose of LLL used in this experiment increases ROS levels. All the groups tested present increasing levels of ROS, indicating increased cellular activity. Evidence supports the idea that TNF-α plays a role in chronic inflammation and migration of neutrophils to these lesions. Low-level lasers can modulate oxidative stress, [16,17] The desired effect on the redox state of Neutrophils can be achieved using different laser wavelengths, while 660 nm increases ROS production, 970 nm exerts an antioxidant activity. [52] In the presence of titanium, LLL reduces the release of free radicals by neutrophils.[53]

FINAL CONSIDERATIONS

Although this is an in vitro experiment, the association of treatments produced different results over lymphocytes and Neutrophils, and we can infer analyzing these results that the irradiation of the low-level laser, 660nm, 90J/cm³, and 50mW, after implant placement, that:

TNF-***α*** - irradiated lymphocytes and neutrophils present reduced G0/G1 phase, increased DNA synthesis, and lymphocyte proliferation, indicating that this dose of LLL increases cellular activity after a trauma or infection.

Titanium-irradiated lymphocytes and neutrophils present reduced G0/G1 phase, lymphocytes increase DNA synthesis, and neutrophils increase cellular activity, indicating that the first line of defense is active in contact with the titanium after LLL irradiation.

TNF-***α***-titanium irradiated lymphocytes and neutrophils present reduced inflammatory response, lymphocytes are increasing DNA synthesis and proliferation, and neutrophils are expanding the delivery of digestive enzyme granules, indicating that the second line of defense is active. Moreover, this dose of LLL accelerates the inflammation process.

The increasing ROS activity of these cells confirms these findings.

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