PHOTOBIOMODULATORY EFFECT OF LOW-INTENSITY LASER RADION ON MULTICELLULAR SPHEROIDS

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RESUMO: Os efeitos biomoduladores da radiação laser de baixa intensidade (RLBI) em células cultivadas em condição bidimensional padrão já estão bem estabelecidos. Em contrapartida, as características desse efeito em cultivos tridimensionais (3D), atualmente recomendados devido à maior similaridade com o comportamento celular in vivo, ainda não foram amplamente investigados. O objetivo deste trabalho foi analisar o efeito biomodulador da RLBI, no comprimento de onda (λ) de 685 nm, no processo de constituição e na viabilidade de células cultivadas como esferoides multicelulares (EMs). Para tal, moldes de agarose contendo micropoços foram semeados ($2x10^5$ células/ml) com células precursoras osteogênicas (CPOs - MC3T3-E1) e mantidas sob condições ideais de cultivo. Os moldes foram irradiados por cinco dias consecutivos com doses de 0.5, 1.0 e 1.5 J/cm², sendo a primeira irradiação realizada imediatamente após a semeadura. Foram analisadas o processo de constituição dos EMs e os cultivos foram submetidas ao ensaio de viabilidade celular. Os resultados demonstraram que a RLBI no λ 685 nm exerceu um efeito biomodulador dose-dependente sobre o metabolismo celular e sobre o processo de constituição dos EMs de CPOs. Estes resultados evidenciam a potencialidade da fotobiomodulação em contribuir com o processo de constituição dos EMs, podendo ser explorado nas estratégias de terapia celular utilizadas em medicina regenerativa e bioimpressão.

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Photobiomodulatory effects of low-intensity laser radiation (LILR) in cells cultured in standard, two-dimensional conditions are well established. Conversely, the characteristics of this effect in three-dimensional (3D) cultures, which are currently recommended due to the greater similarity with cellular behavior *in vivo*, have not yet been widely investigated. The objective of this work was to analyze the biomodulator effect of LILR, on the wavelength (λ) of 685 nm, on the constitution process and on the viability of cells cultured as multicellular spheroid (MSs). For this, agarose molds containing microwells were seeded ($2x10^5$ cell/ml) with osteogenic precursor cells (OPCs - MC3T3-E1) and kept under ideal culture conditions. The molds were irradiated for five consecutive days with doses of 0.5, 1.0 and 1.5 J/cm², the first irradiation being performed immediately after sowing. The process of constitution of MSs was analyzed and the cultures were submitted to the cell viability test. The results demonstrated that the LILR at λ 685 nm exerted a dose-dependent biomodulatory effect on cell metabolism and on the process of constituting the MSs of OPCs. These results demonstrate the potential of photobiomodulation to contribute to the process of constituting MSs, which can be explored in the strategies of multicellular spheroids therapy used in regenerative medicine and bioprinting.

Introduction

Cell culture experiments represent a valuable resource for scientific research. They encompass a set of techniques that involve the isolation and cultivation of cells in an artificial (*in vitro*) and highly controlled environment (*in vitro*), enabling the analysis of biochemical, biophysical and molecular mechanisms in the most diverse experimental conditions. The results obtained from *in vitro* experiments have become indispensable for scientific and technological advancement in the areas of pharmacology, biotechnology, biomedicine, regenerative medicine and tissue engineering (FRESHNEY, 2006; MOLINARO *et al.*, 2010).

In a standard *in vitro* experiment, cells are grown by being adhered to the surfaces of bottles or plate wells, specially treated to facilitate the interaction of the cell with the substrate. In this model, the cells adhere to these surfaces, acquire a spread out two-dimensional (2D) conformation and, during the expansion phase of the cultivation, they start to proliferate and occupy the available area constituting a monolayer. The morphological and physiological characteristics resulting from 2D cultivation directly influence the organization of the cytoskeleton, the shape of its nucleus and access to nutrients, with significant repercussions on the profile of gene expression and the capacity for cell proliferation and differentiation (SIMIAN; BISSELL, 2016; LASCHKE; MENGER, 2017).

Although 2D culture has been a valuable method, its limitations are being increasingly recognized cells in tissues and organs in vivo (FENNEMA *et al.*, 2013; EDMONDSON *et al.*, 2014). The development of methods and techniques for conducting *in vitro* experiments with three-dimensional cell cultures (3D culture) began in the 1970s, with the aim of obtaining a physiologically more relevant model that would mimic, more faithfully, the microenvironment experienced when cultivated in three-dimensional models, as cells establish cell-to-cell and cell-matrix relationships more compatible with the microenvironment found in biological tissues (ACHILLI *et al.*, 2012; WANG *et al.*, 2014).

Among the most used 3D cultivation models, multicellular clusters, also called multicellular spheres (MS), stand out. In this model, the cells are directed, by depriving contact with a substrate, to the agglomeration and constitution of cell-to-cell interactions, resulting in the formation of a spherical conformation of cells. Currently, MSs have been widely used in trials as models for studies of diseases such as cancer (LABARBERA *et al.*, 2012; THOMA *et al.*, 2014; NATH; DEVI, 2016), for drug development and testing (KIJANSKA; KELM, 2004; MENG, 2010) and for 3D bioprinting (3D-BP), considering the concept of building-blocks in tissue engineering (MOLDOVAN *et al.*, 2017; ONG *et al.*, 2017). The proposal to use MSs in tissue engineering created the possibility of its use as a cell release system for cell therapy, with potential clinical application in regenerative medicine (MATSUSAKI *et al.*, 2014).

Regenerative medicine (RM) consists of applied interdisciplinary science aimed at developing products, methods and techniques in order to improve the efficiency of the intrinsic repair process in the structural and functional restoration of tissues and organs. Its approach is based on the use of biological supports, biomolecules and/or therapy with autologous cells, as isolated or combined strategies, in order to reduce the time and/or improve the quality of the newly formed tissue to restore, as much as possible, its structure and function (SAMPOGNA; GURAYA; FORGIONE, 2015; DOLAN *et al.*, 2018).

The proposal for cell therapy in RM and 3D-BP, preferably using mesenchymal stem cells, is justified by the possibility of differentiation in specific tissue cells and by the potential for biochemical modulation of the repair microenvironment. In theory, such influences potentiate the cellular phenomena of the self-repair process (ZHANG *et al.*, 2017; SHOJAEI *et al.*, 2019). Studies show that, although promising, the technique has limitations that can compromise its therapeutic efficacy, including the guarantee of preservation of cell viability after implantation at a rate compatible with the determination of the desired effects (CANADAS *et al.*, 2018; CSETE, 2019). In order to maximize the potential attributed to cell therapy in RM, several studies are being conducted seeking to assess the influence of biomodulatory resources even during the pre-implantation cell cultivation phase (IMURA *et al.*, 2019; O'SULLIVAN *et al.*, 2019;

SHOJAEI *et al.*, 2019).

Low-intensity LASER radiation (LILR) is a therapeutic resource known to be capable of exerting a modulatory effect (photobiomodulation) on eukaryotic cells and, consequently, stimulating its function in repairing various types of biological tissues. It is an athermic and photobiochemic stimulus, resulting from the interaction of electromagnetic radiation, in specific wavelengths (λ), with metalloproteic constituents of intracellular biochemical cascades with photoreceptor properties (ZHU *et al.*, 2017; PASSARELLA; KARU, 2008; OLIVEIRA *et al.*, 2016). This process can result, under ideal conditions, in physiological benefits such as stimulating cell viability, proliferation and differentiation (TSAI; HAMBLIN, 2017).

The establishment and characterization of the LILR biostimulatory influence on the cellular viability of MS, mimicking the stage of pre-implantation in vitro culture, is necessary to determine the real potential of using this resource in order to enhance the results desired with the cell therapy in RM. On the other hand, research for this purpose had not yet been carried out.

Objective

The purpose of this study was to analyze the influence of LILR of 685 nm, on the constitution process and viability of osteogenic precursor cells (OPCs) in 3D cultures such as MSs.

Methodology
Cell cultivation

OPCs from MC3T3-E1 mice were used in the experimental procedure. The cells were grown in bottles with Dulbecco's modified Eagle's culture medium (DMEM - Vitrocell), supplemented with 10% fetal bovine serum (FBS - Nutricell), Penicillin (100 U/ml - Vitrocell) and Streptomycin (100μg / ml - Vitrocell), and kept in a cell culture oven (Panasonic - Co² incubator MOC -19 AIC-UV) at 37° C, in a humidified atmosphere containing 5% CO_2 and 95% atmospheric air.

Multicellular spheroid constitution

For the constitution of MSs, a protocol adapted from Napolitano and collaborators (2007) was used, based on the principle of cultivation in microwells with a non-adherent surface. For this, agarose molds were made from a preheated (60ºC) solution of agarose (20 mg/ml in phosphate buffer - PBS) aliquoted (2000 μl) inside a countermold for further solidification. The countermold used (positive), produced by additive manufacturing and supplied by the Renato Archer Technology and Information Center, generates molds consisting of 164 microwells with an individual diameter of 500 μm.

For cellularization, the molds, previously sterilized by immersion in 70% alcohol followed by ultraviolet irradiation (30 minutes), were initially placed in cultivation plate wells (12 wells/KASVI). The cultured cells were trypsinized, resuspended in a DMEM medium (10^6 cells/ml), quantified and subsequently transferred to each mold ($2x10^5$ cells/mold). Finally, the wells received an additional volume of 800 μl of DMEM medium and the plates returned to ideal culture conditions. Three molds were used (n = 3) in the four experimental groups. The monitoring of the culture during the stage of constitution of the MSs was carried out through an inverted microscope (Nikon Eclipse TS100) using open field microscopy with phase contrast, and the analysis of the images was performed by a blind examiner.

Irradiation procedure

the parameters used for LASER irradiation are shown in table 1. The equipment used was Thera Laser® (DMC, São Carlos-SP, Brazil), previously subjected to measurement and calibration in order to guarantee the planned dosimetric parameters.

In order to enable the irradiation process, a support device consisting of a base and a vertical rod was

developed. The base surface, which is black, in order to reduce the reflection of radiation that passes through the mold, contains a guide with markings intended to guide the positioning of the plate to guarantee the incidence of the radiation beam on the area of each mold. The rod is designed to hold the probe and provides ideal positioning and stability during irradiation.

The irradiations were carried out inside the laminar flow hood in a sterile environment. During the irradiation procedures, the plate covers were removed and the flow light was turned off. The irradiation protocol consisted of 5 applications, the first of which was performed immediately after sowing the molds and with an interval of 24 hours between each irradiation. Four experimental groups were established, being 1 group (CG) and 3 groups of LASER irradiation, with the respective doses of 0.5 J/cm² (G-0.5), 1.0 J/cm² (G-1.0) and 1.5 J / cm² (G-1.5). The CG molds were subjected to the same manipulation procedures as the irradiated groups, but with the equipment turned off.

Table 1 - Parameters used in the MSs irradiation procedures.

Active medium	Gallium Aluminum Arsenide (GaAlAs)
Wavelength	685 nm
Output power	35 mW
Emission mode	Continuous
Irradiation area	3.85 cm²
Power density	9 mW/cm²
Energy density	0.5 J/cm² / 1.0 J/cm² / 1.5 J/cm²
Exposure time	55 s /110 s /165 s
Energy deposited	1.9 J / 3.8 J / 5.7 J

Source: Prepared by the authors.

Cell viability test

Cell viability was performed by fluorescence assay using the resazurin-rezofurin method, according to the protocol described by Pagé and collaborators (1993). The test was carried out on the fifth day after the formation of the MS without removing them from the molds. For this, the culture medium of the molds was replaced by a standard medium supplemented with resazurin (100 μg/ml) and the plates were again conditioned in an oven for a period of four hours. After this period, a 100μl aliquot was removed from the culture medium, transferred to wells in a 96-well plate and subsequently subjected to fluorescence analysis in a plate reader (Biotek®), with excitation and detection in the lengths of 530 and 590 nm, respectively. The results of cell viability were expressed as a percentage in relation to the values obtained in the CG.

Statistical analysis

The data referring to the viability values were tabulated and submitted to statistical analysis using the GraphPad Prisma 7.01 software. The ANOVA One-Way parametric test was used, followed by the Tukey post-test. The results obtained were expressed as Mean ± Standard Deviation (SD) and the level of significance adopted was 5% ($p < 0.05$).

RESULTS

CONSTITUTION OF MSS

The constitution of the MSs was observed through the microphotographs obtained during the process. On the day the molds were cellularized (day 0), the were, immediately after sowing, dispersed in the microwell. After 24 hours, the occurrence of the cell agglomeration phase was identified. After 48 hours, the start of the MSs compaction process was identified, which progressed until the 4th day of cultivation (Figure 1), ending the constitution process.

Figure 1 - Time sequence of the process of constituting the MSs in the Control group.
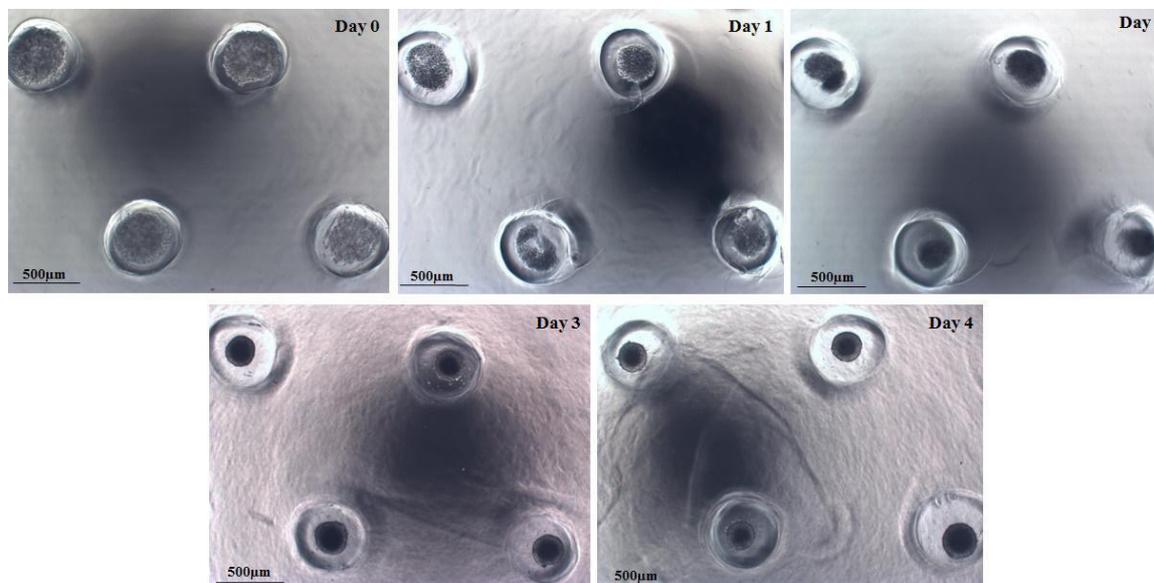
Source: Prepared by the authors.

Figure 2 illustrates, in greater detail, the organization of the MSs of the CG on day 4, presenting spherical conformation and organized in three different regions. A superficial region characterized by a thin and dense layer, an intermediate region in the form of a ring with less density and a central region can be observed, determining a dense spherical nucleus.

The comparative analysis of the morphology of the MSs between the experimental groups allows us to observe a difference in the pattern corresponding to the CG previously described (Figure 3). The G-1.0 MSs presented a similar morphology to the CG, with the 3 regions appearing very evidently and with a diameter of approximately 200 μm. The MSs of the G-0.5 group had a spherical conformation, but only showed the middle and central regions. In addition, its average diameter was smaller, at approximately 150 μm. In the G-1.5 group, the MSs did not present the standard morphology. In addition to the spherical conformation not being identified, they did not show the organization in regions as seen in the previous groups. These results suggest a possible interference of radiation in the process of constituting MSs in these groups.

Figure 2 - Microphotography of the multicellular organization of a spheroid corresponding to the CG. A superficial region (A) is observed in the most peripheral portion of the MS, an intermediate region in the shape of a ring (B) and a central region (C) constituting the nucleus of the MS.
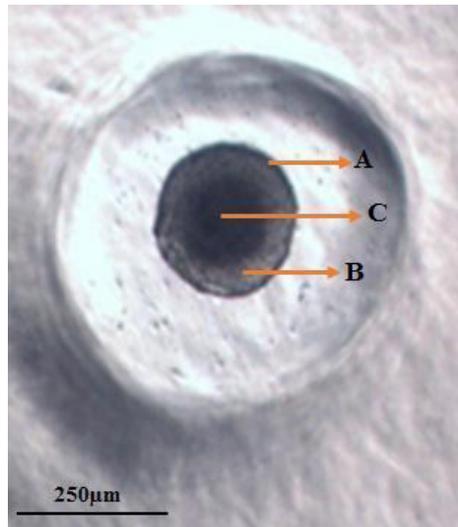
Source: Prepared by the authors.

Figure 3 - Micrographs referring to the morphology of the MSs of the experimental groups on the fourth day of cultivation. In the groups CG and G-1.0, the same morphology is observed with 3 very evident regions and similar diameters. The G-0.5 group appears to have only 2 regions and with smaller diameters. And the G-1.5 did not exhibit the same organization as the MSs of previous groups.
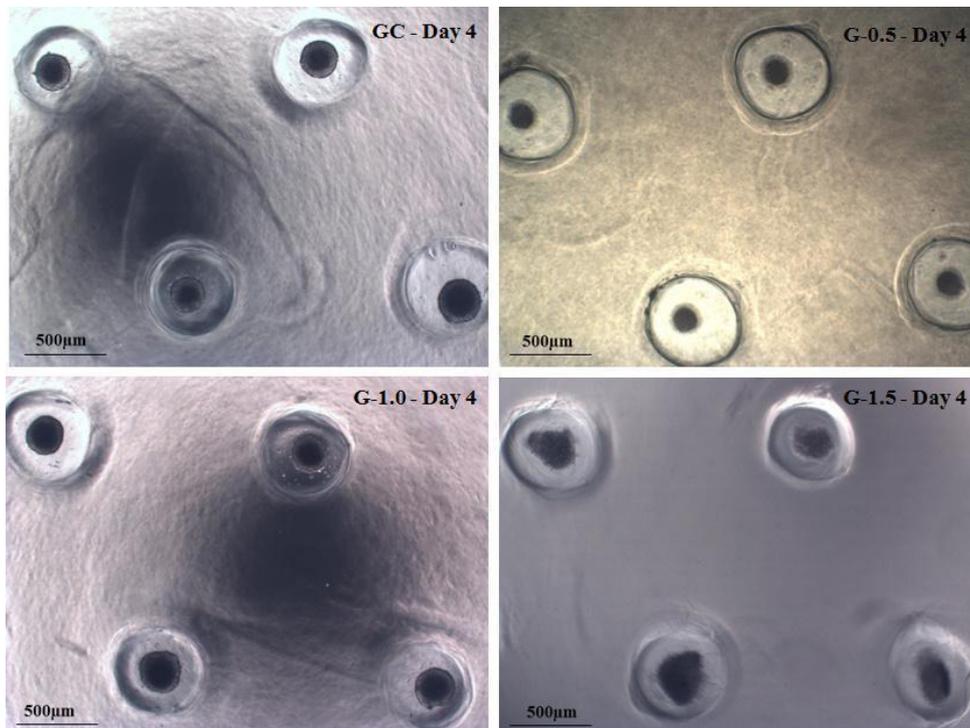
Source: Prepared by the authors.

CELL VIABILITY

The results obtained in the feasibility test demonstrated that there was a statistically significant difference between the G-1.0 and the other experimental groups. The increase in cell viability in relation to the CG, G-0.5 and G-1.5 groups was 79, 73 and 84%, respectively (Figure 4). The statistical analysis shows that there is no difference between the other experimental groups.

Figure 4 - Cell viability (M ± SD) in OPCs grown in 10% FSB associated or not with the influence of 685 nm LASER radiation (* $p < 0.05$).
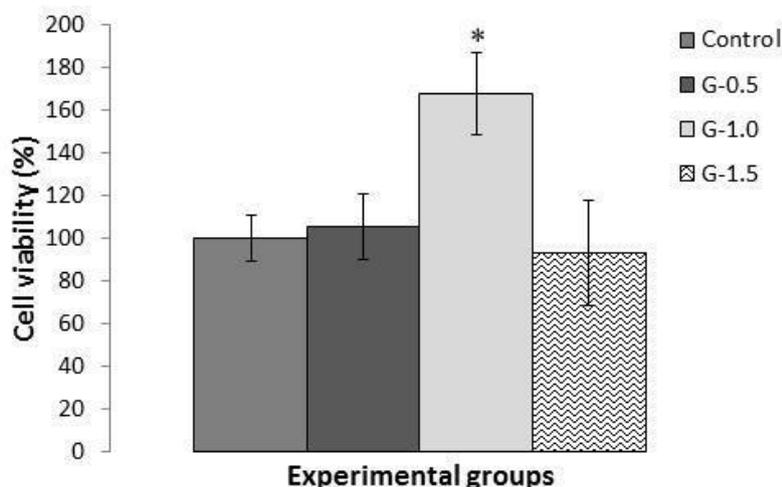
Source: Prepared by the authors.

DISCUSSION

In vitro research based on 2D culture has given rise to relevant conceptual advances in cell biology, such as an understanding of the function of cells and tissues and the development of disease models for therapeutic screening. On the other hand, its limitations are being increasingly recognized and 3D cultivation has shown to mimic the *in vivo* microenvironment in a more realistic way, providing the acquisition of a cellular morphology and metabolic conditions (generation of gradient for nutrients, gases, factors of growth and other signaling molecules) typical of biological tissues, under physiological and pathological conditions (XU *et al.*, 2011; ACHILLI; MEYER; MORGAN, 2012; HUANG; GAO, 2018; TOMASI *et al.*, 2020).

MSs have a complex morphological structure, characterized primarily by cell-cell interactions, in their initial constitution phase, complemented, later, by the additional constitution of cell-matrix interactions. This morphology was first described by Sutherland and collaborators (1971), subsequently, researchers such as Zanoni and collaborators (2016) and Vadivelu and collaborators (2017) more accurately demonstrated the organizational characteristics of MSs. The process of formation of MSs, according to these authors, consists of the following phases: initial phase of cell grouping; phase of self-organization or cellular consolidation; cell growth phase; and, finally, the stationary phase.

Regardless of the method used for constituting MSs, these researchers described a standard morphology, depending on the cell type used, characterized by the formation of a spherical structure organized in 3 regions: the peripheral or proliferative region, formed by metabolically active cells and with high potential for proliferation; intermediate or quiescent region formed by cells with more compacted membranes and reduced metabolic activity; and the central or necrotic region made up of dead cells (SATO *et al.*, 2016; SCHMIDT *et al.*, 2016). The average diameter size of MSs identified in the scientific research is between

200 and 500 μm and is directly related to the sowing density and the characteristics of the constitution model. It is noteworthy that, the larger the size, the greater the difficulty of diffusing nutrients and oxygen to the center of MSs and the greater the degree of necrosis in its central region (MARQUES *et al.*, 2016).

The results obtained, referring to the aspects of the constitution of MS grown on non-adherent surface in the CG, allowed to identify the temporal evolution of the constitution process, considering the protocol established in this research. On the day the molds were cellularized (day 0), the OPCs were, after sowing, dispersed in the microwells. After 24 hours, the cells already exhibited the typical pattern of the cell agglomeration phase. After 48 hours, the start of the MS compaction process was identified, which progressed until the 4th day of cultivation, ending the constitution process. This dynamic was also observed in groups G-0.5 and G-1.0. On the other hand, group G-1.5 showed evolution only up to the cell agglomeration process, suggesting some degree of inhibitory influence of this radiation dose in the compacting phase of the MSs.

Regarding the morphological pattern, in the groups CG and G-1.0, MSs with dimensions of approximately 200 μm and presenting the 3 regions are quite evident. The G-0.5 group presented MSs with a spherical conformation, but exhibited a significant difference in their morphology, with average diameters of approximately 150 μm and with the identification of only the intermediate and central regions. It is believed that, due to the biomodulatory influence of this dose of LILR, these spheroids had a smaller number of cells, due to a lower proliferation rate during the constitution process, and/or a greater degree of agglomeration and compacting may have occurred in the final stages of the process. Changes due to the smaller diameter on the diffusion profile of nutrients and oxygen may be the cause of the absence, or not identification, of the peripheral region. The G-1.5 group presented, at the end of the predicted period of constitution, a cell agglomeration with characteristics incompatible with those of a standard MS, suggesting a bioinhibitory influence of this radiation dose on the biochemical regulatory mechanisms of the spheroid constitution process. Bioinhibitory influence of LILR is widely described in the scientific literature, being associated, primarily, with the highest doses of irradiation. This conception integrates the Arndt-Schulz theory also applicable to photobiomodulatory mechanisms, which determines biostimulatory effects provided by doses within an ideal range and bioinhibitory in proportionally higher doses (MARQUES *et al.*, 2016).

The results regarding cell viability demonstrated that the G-0.5 group presented a similar pattern to that shown by the CG. Since they have MSs with a smaller diameter, and considering the hypothesis of a smaller cell quantity, it is suggested that the LILR has exerted a photobiostimulation action, resulting in an increase in the rate of cellular metabolism. On the other hand, considering a smaller diameter due to a higher degree of cellular compaction, the cellular metabolic rate would not have been influenced by this radiation dose, but would have influenced some cellular function related to the cell-to-cell interaction process, determining a greater degree of compaction of MSs. The G-1.0 group showed a significant increase in cell viability, demonstrating a biostimulating influence of LILR at this dose. Since the MSs had the same diameter as the CG, suggesting the same number of cells, it is believed that the 79% increase in cell viability results from the increase in the metabolic rate of OPCs. The G-1.5 group also showed no significant difference in cell viability when compared to the CG. Even though the group has a supposed bioinhibitory influence on the formation of MSs, radiation does not seem to have influenced cell viability at this dose. A general analysis, based on the results of the present research, allows us to consider that the LILR, at wavelength of 685 nm, exerted a dose-dependent biomodulator effect on the metabolic rate and the cellular mechanisms involved in the process of constituting MSs.

The mechanism that underlies the biomodulatory influence of LILR on cell metabolism, widely described in the scientific literature, is of a photochemical nature, initiated by the absorption of photons, in specific wavelength, by the photoreceptors present in cells (chromophores). The participation of these

chromophores in biochemical cascades allows their metastable state of electronic excitation induced by radiation to alter the kinetics of intracellular biochemical reactions, concomitantly influencing signaling mechanisms and cellular metabolism (KARU, 2008; FREITAS; HAMBLIN, 2016).

Wavelength, power density and energy density are parameters that must be considered when the goal is biomodulation. 2D *in vitro* studies have been effectively contributing to the characterization of the influence of these parameters and guiding preclinical and clinical research based on the photobiomodulation process. On the other hand, the results of the present research show that the type of cultivation also needs to be considered to understand the photobiomodulatory mechanisms, especially in the strategies of translational research. Asai and collaborators (2014) demonstrated that the 1.5 J/cm² dose stimulated cell proliferation in OPCs irradiated for 5 days in 2D culture. The results presented in our research demonstrated that the respective dose, in a similar irradiation protocol (5 days), did not influence the viability of the OPCs and exerted a bioinhibitory influence on the process of constituting the MSs. Further research needs to be conducted to expand understanding of the extent of this inhibitory action and its consequence for preimplantation stimulation strategies for use in RM or 3D-BP.

The results presented in this research reinforce the thesis that the real influence of photobiomodulation is dependent on the metabolic profile and cell expression, which in turn are directly influenced by the morphology and type of interaction that this cell establishes in its cell niche. Therefore, the need to expand 3D cultivation strategies in preclinical research involving photobiomodulation becomes evident, considering the greater mimicry between this type of culture and the cellular environment *in vivo*.

Conclusion

The results demonstrated that the LILR, at wavelength of 685 nm, exerted a dose-dependent biomodulator effect on cell metabolism and on the process of constituting the MSs of OPCs. The results indicate, in a preliminary way, that the dose of 1.0 J/cm² exerted a biostimulating influence on the viability of OPCs cultivated as MSs, and can be used, in the pre-implantation phase of cultivation, to benefit the cell therapy strategies used by RM. In contrast, the evidence of a bioinhibitory influence, as demonstrated by the dose of 1.5 J/cm², highlights the need for further research to broaden the understanding of the characteristics of this biomodulatory influence and identification of the molecular mechanisms related to such influence.

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