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João Pedro Gomes Faria

Dissertação conducente ao Grau de Mestre em

Medicina Dentária (Ciclo Integrado)

Gandra, 27 de setembro de 2020

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Trabalho realizado sob a Orientação de " Prof Doutor Júlio C. M. Souza" e Co-Orientação do Mestre Nuno Sampaio

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Resumo

O propósito deste trabalho foi avaliar o efeito da terapia laser de baixa intensidade em combinação com biomaterial xenógeno de origem bovina (DBBM) sobre a viabilidade e morfologia de fibroblastos em meio de cultura. Nos testes de cultura celular foram usados fibroblastos L929 em contacto com as partículas de DBBM de diâmetro entre 125 e 800 µm. Os aspetos morfológicos das partículas foram analisados por microscopia eletrónica de varrimento (FEGSEM) enquanto que a viabilidade celular foi analisada por ensaios de MTT conforme a norma ISO-10993. Um protocolo laser de baixa intensidade foi aplicado nos seguintes parâmetros: comprimento de onda a 1064nm, a 10 Hz e 0.25 W durante 30 s. As imagens obtidas das partículas por FEGSEM apresentaram poros em diferentes tamanhos variando de 234,9 nm e 667,2nm. Os testes MTT revelaram resultados positivos em ambos os tratamentos com DBBM ou terapia laser sendo que a combinação potenciou a viabilidade celular dos fibroblastos. A combinação da terapia laser em baixa intensidade e o uso de biomateriais xenógenos de origem bovina estimulam o crescimento celular de fibroblastos o que poderá acelerar o processo de reparo tecidual.

Palavras-chave: DBBM; MTT; low level laser therapy; tissue repair.

Abstract

The purpose of this study was to evaluate the effect of a low level laser therapy protocol in combination with deproteinized bovine bone mineral (DBBM) on the viability and morphologic aspects of fibroblasts. *In vitro* cell culture tests were carried with L929 fibroblasts in contact with DBBM particles ranging from 125 up to 800 μm in diameter. The cell morphologic aspects were evaluated by field emission guns scanning electron microscopy (FEGSEM) while the cell viability was assessed by MTT assays (ISO-10993). Fibroblast culture was stimulated by low level laser irradiation protocol at 1064 nm wavelength, at 10 Hz frequency, and at 0.25 W power for 30 s. FESEM images of DBBM showed different pores' size ranging from 234.9 to 667.2nm. MTT revealed positive effects in both treatments with DBBM or laser therapy and therefore the combined approached showed the highest cell viability data. Thus, the application of deproteinized bovine bone mineral under low level laser therapy stimulates the growth of fibroblasts that can enhance the tissue healing.

Key words: DBBM; MTT; low level laser therapy; tissue repair.

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1. Introduction

Nowadays, different bone graft materials can be used for bone repair such as xenografts, allografts, alloplasts, or autografts (1-3). In clinical cases of large bone defects, allografts and xenografts are used as bone graft materials (1-3). The porosity of the grafting materials provides conditions for an effective vascularization and bone fomation(2,3).

Considering the bone healing process, autogenous bone is the first choice to enhance osteconduction, osteoinduction, and angiogenesis (1-5). However, the use of autogenous bone graft has drawbacks such as limited supply and morbidity from bone harvest (4). Thus, xenografts are attractive options to overcome the limitations of autografts taking into account a well-controlled industrial processing prior to the placement in the surgical sites (5,6).

Deproteinized bovine bone material (DBBM) is commonly used as xenograft material regarding its chemical composition based on hydroxyapatite. Also, the natural morphological aspects comprises pores at the macro-, micro-, and nano-scale similar to the human bone structure (7,8). According to some studies, DBBM has an high porosity of around 70 and 80% and a surface area of approximately 100 m^2/g (2,3). The vast interconnecting pore system and porous architecture positively affects the deposition of osteogenic proteins and growth factors. Thus, the porous structure and hydroxyapatite-based microstructure serve as a bioactive scaffold for adhesion, proliferation and differentiation of osteogenic cells (2,3,9). The interconnecting porous network affords conditions for angiogenesis and migration of osteoblasts considering: oxygen and nutrients from fluids; removal of metabolic products; cell growth; and new-bone formation (10–12). A previous study reported 29.8% new bone after 8 months, 69.7% new bone for 20 months, and 86.7% new bone for 10 years. DBMM resorption rate in the first 2 years was recorded at 3.55% per month reducing down to 0.58% in the further 8 years (8). Furthermore, recent protocols have suggested the combination of DDBM with other biomaterials or alternative therapies to enhance the tissue healing and grafting resorption.

Recently, low level laser therapy (LLLT), also known as photobiomodulation process, has gathered attention by clinicians and scientists (13–15). Previous, studies showed a stimulant effect on cell metabolic processes that can result in enhanced tissue healing.

Some studies reported a photochemical effect on the cells' mitochondria that promote cell proliferation, leading to several chemical events (22,23). That affects mRNA synthesis leading to the modification of several cell-regulatory proteins (16,17), protein synthesis, and biomodulation in cytoskeletal organization (18). Therefore, LLLT has analgesic and, anti-inflammatory effects (19) although the exact mechanism of LLLT is not yet completely understood. Different protocols are currently used for tissue healing by varying laser type, power, and exposure time (13–15). For instance, a laser Gallium–Aluminum–Arsenide diode laser with a wavelength of 830 nm has been used at a frequency of 50 Hz and 62 or 150 mW power irradiation (0.50 cm^2) for 20 or 30 s (20,21). Nd:YAG laser with wavelength of 1064 nm is also used with a power of 70 mW, spot 400 μm spot diameter, and intensity of 0.5, 1, 1.5 (1-5 J), or 2 W/cm^2 (20,21).

Fibroblasts perform a significant role on gingival maintenance and tissue healing. In fact, a combination of LTT and DBBM could be a strategy to reduce the tissue healing time and to increase cell proliferation at the surgical site. Thus, the main objective of this study was to evaluate the combination of low level laser therapy and the xenograft deproteinized bovine bone material (DBBM) in a culture of fibroblasts. It was hypothesized that the combination of DBBM and LLLT enhances adhesion and proliferation of fibroblasts on to the biomaterial and surrounding environment.

2.Materials and Methods

2.1. Preparation of the biomaterial

Deproteinized bovine bone mineral (DBBM) (Biograft, Ossmed, Cantanhede, Portugal) was assessed in this study. DBBM was submitted to a thermal treatment up to 1200 ^{o}C to remove the organic compounds and maintain the hydroxyapatite network. Then, the biomaterial was milled to produce particles ranging maximum diameter from 125 up to 800 µm. DBMM particles were sterilized under gamma X-ray. The chemical composition of the DBBM comprised 32 C, 9.2 O, 8.1 Ca, 3.3P (wt%) within a Ca/P ratio at 2.45.

DBBM particles were sputter-coated with AuPd thin films for field emission gun scanning electron microscopy (FEGSEM). Morphological analyses were performed using FEGSEM (FEI 300 Quanta 3D, FEI Company, USA) at 5-15 kV for secondary (SE) and backscattered electrons (BSE) at high resolution.

2.2. Cell culture

Murine L929 fibroblast cell line (Sigma-Aldrich, USA) was used as a model of the human oral mucosa. Cells were cultured in MEM (minimum essential medium, Gibco, Life Technologies, USA) supplemented with 10% horse serum (Gibco, Life Technologies, USA) in humidified atmosphere at 37°C and 5% CO_2 to mimic the human body conditions. L929 cells were seeded on 96 well plates with the density of $5x10^4$ cells/ml on 0.05 g DBBM (test group) or on the polystyrene well free of DBBM (control group). The medium was renewed every two days. The experimental tests were performed in triplicates and were repeated at least two times.

2.3. Light irradiation parameters

On light irradiation, Neodymium: YAG laser (Nd:YAG, LightWalker, Fotona®, Slovenia) were evaluated regarding the cell growth behaviour (Figure 1). Nd: YAG has a wavelength at 1064 nm and it was applied at 10 Hz and 0.25 W on the cell culture medium (8 mm diameter spot) for 30 s.

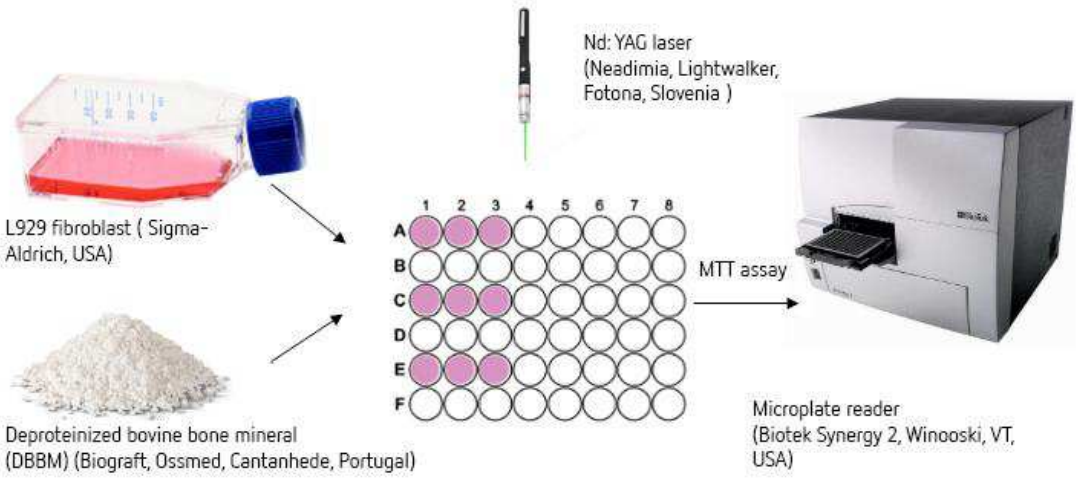

Figure 1. Schematics showing the placement of DBBM in contact with L929 fibroblast and MTT evaluation.

2.4. Cell viability assays

Cell viability was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)] (Promega, USA) at two different time points of cell incubation: 24 and 72 h. Then, cells were treated with paclitaxel (0e100 nM) for 48 h and therefore placed in fresh PBS medium containing 5mg/ml MTT reagent and incubated at 37 ^{o}C and 5% CO_2 for 4 h. The purple formazan crystals were solubilized in a detergent solution containing 89% (v/v) 2-Propanol, 10% (v/v) Triton X-100, and 1% (v/v) HCl for 2 h. Optical density was measured at 570 nm in a microplate reader (Biotek Synergy 2, Winooski, VT, USA) associated with a Gen5 software (version 1.07.5, Biotek, Winooski, VT, USA).

2.4 Statistical Analysis

The statistical analysis on MTT results was performed using normality test for normal distribution data by Shapiro-Wilk test. A factorial analysis of variance (ANOVA) followed by test Pos Hoc (Tukey) was used to assess the significant interactions between groups and therefore $p<0.01$ values were considered to be statistically significant. All analyses were performed using SPSS statistics 17.0 (SPSS, USA).

3. Results

3.1. Morphological analyses

FEGSEM images of the DBBM particles are shown in Figures 2 and 3.

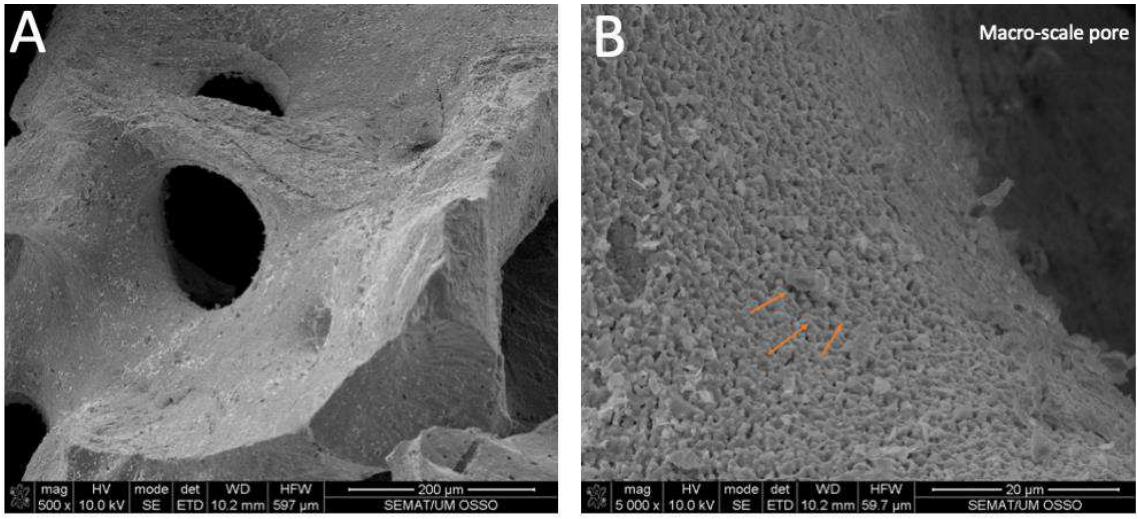

Figure 2. SEM images of a DBBM particle at two different magnification: (A) x500 and (B) x5,000.

As seen in Figure 2A, a DBBM particle has noticeable macro-scale pores when inspected at x500 magnification. Micro-scale features within a rough DBBM surface were noticed at higher magnification (x5000) as seen in Figure 2B.

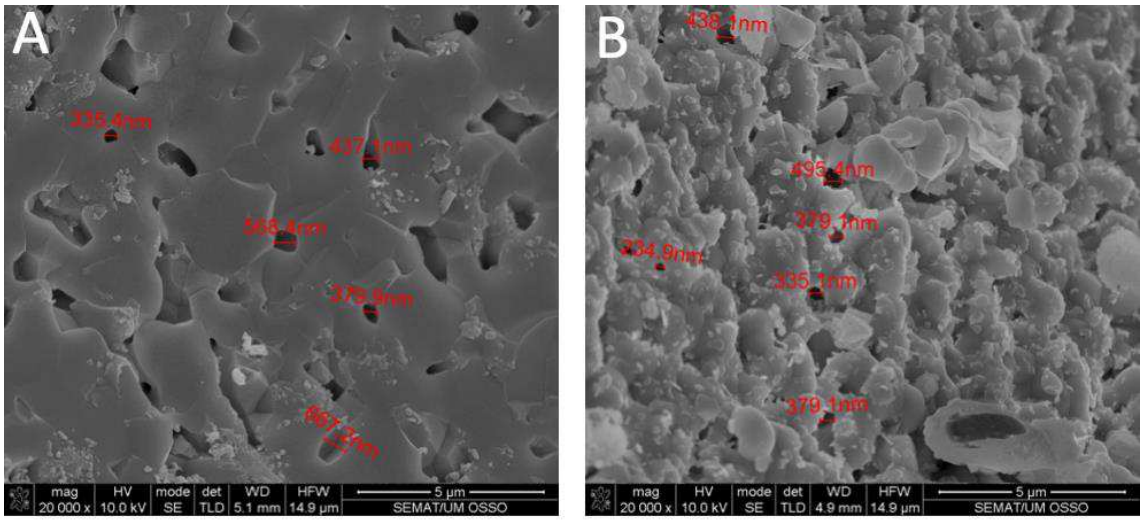

Figure 3. SEM images of different regions of the DBMM particles at same magnification: (A) and (B) x20,000.

Micro-scale pores were detected at x20,000 magnification (Fig. 3). In Figure 3A, the size of the pores ranged from 335.4 up to 667.2nm and therefore the mean size of the pores was around 437.1nm. In Figure 3B, the the pores' size varied from 234.9 up to 495.4nm and the mean value of the pores' size was at 379.1nm. The overall mean size of pores was recorded at 408 nm as shown in Figure 4.

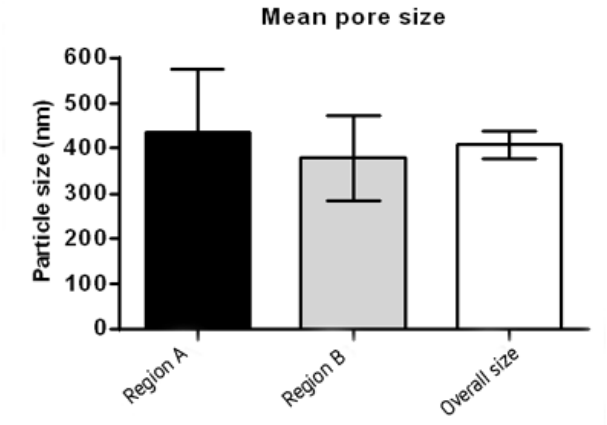

Figure 4. Mean size of DBBM pores at different regions.

3.2. Cell culture assays

Results from MTT assays in a fibroblast culture medium is shown in Figure 5. The fibroblasts' behavior was evaluated in contact or not with DDBM. Also, the effect of the low-level laser therapy (LLLT) was analyzed in the presence or not of DBBM particles.

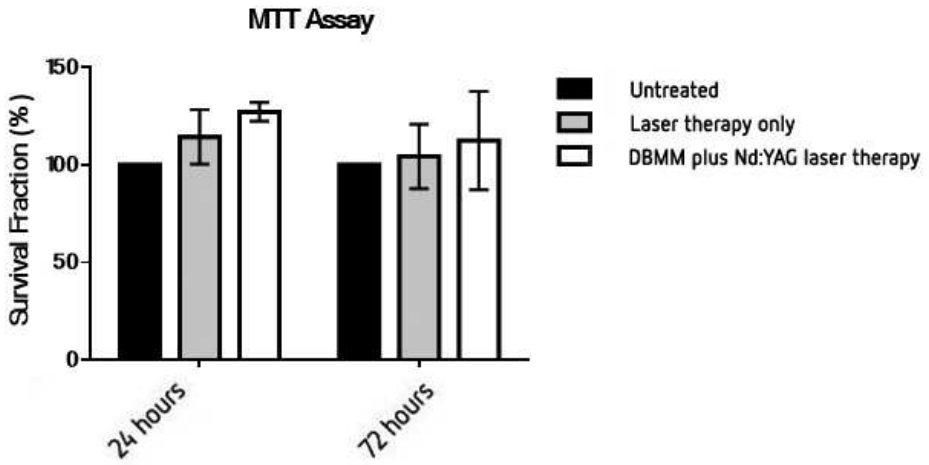

Figure 5. Cell viability recorded from MTT assays. Values are expressed in percentage (%) and as Mean ± standard deviation (s.d.). laser therapy only; DBBM plus Nd:YAG laser therapy;

Results from MTT assays are expressed as percentage (%) of survival fraction. After 24h, there was an increase in the fibroblast growth under Nd:YAG laser therapy. The survival fraction for fibroblasts under Nd:YAG laser irradiation was around 14.5% while the survival fraction for fibroblasts in contact with DBBM and under Nd:YAG laser irradiation was recorded at 27.4 %. After 72 h, the survival fraction under LLLT was around 4.4% while the survival fraction of fibroblasts in contact with DBBM and under Nd:YAG laser irradiation was at approximately 12.5%.

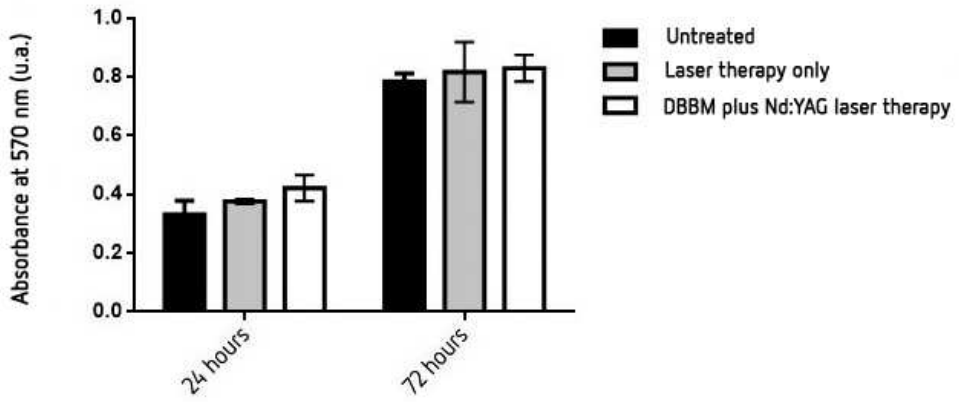

Figure 6. Absorbance values from MTT assays at 570 nm for 24 h and 72 h of fibroblast incubation in contact with DBBM submitted or not to Nd:YAG laser irradiation.

Results from MTT absorbance measurement are showed in Figure 6. For 24 h incubation, higher absorbance values were recorded for DBBM in contact with fibroblasts under Nd:YAG laser therapy. Abosrvance values increased over 72 h incubation although no statistically significant differences were noted between fibroblasts in contact with DBMM submitted or not to laser therapy.

4. Discussion

The results of this study validate the hypothesis that Low level laser Therapy (LLT) stimulates fibroblast cells' adhesion and proliferation on deproteinized bovine bone mineral (DBBM). Such results show a synergistic effect of the combination between LLT and DBBM on cell stimulation for tissue healing.

DBBM particles used in this procedure ranged from 125 a 800 µm with pores at macro-, micro-, and nano-scale as seen by scanning electron microscopy (Figure 2). Mean size of submicron-scale pores was recorded ranging from 234.9 to 667.2nm and macro-scale pores are also noticeable. Previous studies report that large pores (>100 µm) improve bone formation, by providing room for the adhesion of msenchymal and osteoblasts (22). The optimal size of macro-scale pores is reported ranging from 300 and 400 µm (23)(24)(25)(26). The connection among macro-scale pores promotes a network of channels for angiogenesis, nutrient exchange, and bone formation (27).Furthermore, the presence of submicron-, micro-, and nano-scale pores has also been reported to improving the adsorption of proteins and growth factors that stimulate the migration and differentiation of fibroblasts and osteogenic cells (1,28)(29).

Results of the present study on MTT assays revealed a stimulation of 14.5% cell growth under solely Nd:YAG irradiation within the first 24 h although that effect decreased to 4.4% for 72 h. In combination with DBBM, stimulative effect increased up to 27.4 % within the first 24 h and then a decrease down to 12.5% was noticed for 72h. Even though the laser effect decreased for 72 h, the first hours are crucial for cell migration and adhesion during the tissue healing process. Thus, any further stimulation of cell migration and proliferation can speed up the tissue healing to decrease the risks of failures on the bone grafting procedure. The results of this study corroborate with those reported by a previous studies assessing the same fibroblastic line and time points (30)(31). Another previous study reported similar results with a noticeable MG-63 osteoblastic like cells for 3 and 7 days (32) although the cell growth of L929 murine fibroblast cells showed a slightly decrease in cell viability from 24 up to 72 hours showing a small increase on the next time point 120 hours. An increase in the growth of human gingival-derived fibroblasts (HGF3-PI 53) from 24 to 72h was also reported by a previous study on gallium–aluminum–arsenide (Ga– Al–As) diode laser used with a power of 50mW for 32 s(33). On the other hand, a decrease of

human schwann cells' proliferation was reported in the first 24 and 72 hours during laser irradiation using gallium–aluminium–arsenic (Ga–Al–As) diode laser with output power of 50 mW. for 32 s period followed by an increase in cell proliferation after 96 h (18).

In short period of time, LLLT can also largely improve differentiation and proliferation of osteogenic cells when compared with non-irradiated cells (16). Recent studies showed an increase in MTT values in the first 24 h on osteoblastic cells isolated from bone irradiated at zero time point zero (immediately cultured) comparing with 30 min time point (14). Similar MTT values between the irradiated an non irradiated groups were only noted after 5 days (14). Moreover, other studies that combined LLLT with human osteoblast cells derived from bone had similar results to those reported in the present study for 24 h (14,15)(15)(15). Further addition of DBBM positively affected the proliferation and differentiation of ST2 stromal bone marrow cells for both 24 and 72 h cell growth (34). Also, similar results were found on proliferation and adhesion of C2C12 myoblastic cells over DBBM between 3 and 6 days cell growth (35). Other studies reported a low total amount of adherent osteogenic MC3T3-E1 cells on DBBM xenograft verified significant decrease of cell viability as recorded by MTT assays (36).

Being an in vitro study, is an experiment carried out of human body on cells, on controlled conditions that don´t happen in the human body, furthermore the time duration of this in vitro studies is to short comparing with conditions of oral mucosa healing.

5. Conclusions

Within the limitations of an *in vitro* study me main conclusions gathered from the present study can be drawn as follow:

- Deproteinized Bovine Bone Mineral granules has various scales of porosity ranging from macro to nano scale, thus DBBM porosity can promote angiogenesis, cell attachment, nutrient exchange improving cell growth.
- The combination of low laser therapy Nd: YAG with a power of 0.25 W for 30 s. and DBBM increased fibroblastic growth over DBBM for 24 h. Even though there is a decrease in the cell growth for 72h, the combined strategy has a strong potential to stimulate the proliferation and adhesion of fibroblasts:
- The low laser therapy with Nd: YAG laser with a power 0.25 W during 30 s generates a energy that stimulate the fibroblasts cells in a culture medium. However, the effect of tissue layers, proteins and other cells can influence the laser therapy effect.
- Nevertheless, future *in vitro* test with different laser parameters are recommended to evaluate the effect of different protocols and to establish a correlation among laser type, power, and irradiation time. Different cell lines should be assessed followed by simulated tissue healing assays.

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