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Biosilicate/PLGA osteogenic effects modulated by laser therapy: *In vitro* and *in vivo* studies

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ABSTRACT

The main purpose of the present work was to evaluate if low laser level therapy (LLLT) can improve the effects of Biosilicate®/PLGA (BS/PLGA) composites on cell viability and bone consolidation using a tibial defects of rats. The composites were characterized by scanning electron microscope (SEM) and reflection Fourier transform infrared spectrometer (FTIR). For the *in vitro* study, fibroblast and osteoblast cells were seeded in the extract of the composites irradiated or not with LLLT (Ga-Al-As, 808 nm, 10 J/cm^2) to assess cell viability after 24, 48 and 72 h. For the *in vivo* study, 80 Wistar rats with tibial bone defects were distributed into 4 groups (BS; BS + LLLT; BS/PLGA and BS/PLGA + LLLT) and euthanized after 2 and 6 weeks. Laser irradiation Ga-Al-As (808 nm, 30 J/cm^2) in the rats was performed 3 times a week. The SEM and FTIR results revealed that PLGA were successfully inserted into BS and the microparticles degraded over time. The *in vitro* findings demonstrated higher fibroblast viability in both BS/PLGA groups after 24 h and higher osteoblast viability in BS/PLGA + LLLT in all periods. As a conclusion, animals treated with BS/PLGA + LLLT demonstrated an improved material degradation and an increased amount of granulation tissue and newly formed bone.

1. Introduction

Nowadays, it is clear the tremendous need of the development of innovative therapeutic strategies for allowing and stimulating bone repair. Synthetic bone grafts have been emerging as very promising alternatives to stimulate bone tissue [1–3]. Among those, bioactive glasses and glass bioceramics are often used for bone substitutes due to their ability to bond and integrate with living bone by forming a biologically active bonelike apatite layer on their surfaces [4–7]. One of the most promising bioactive glass ceramic materials is the Biosilicate (BS) (patent application WO 2004/074199) [8]. It has been demonstrated that BS is capable of stimulating newly bone formation and increasing biomechanical properties of the bone callus in an experimental model of tibial bone defects [9–13]. Also, Fernandes et al. [14] demonstrated that composites of BS and poly(D,L-lactic-*co*-glycolic) acid (PLGA) accelerated degradation rates and improved biological performance of the material compared to samples of BS during the process of bone healing using an experimental model on bone defect.

Although, all the evidences of the positive effects of BS and BS/PLGA composites, there is a continuous search and growing interest for the development of interventions able of optimizing the properties of bone grafting [14,15]. In this context, one of the most promising alternatives is the low-level laser therapy (LLLT).

Many authors demonstrated that LLLT is able of stimulating bone tissue metabolism and accelerating fracture consolidation [16–19]. It has been demonstrated that LLLT is able of up-regulating the synthesis of genes and proteins related to bone cell proliferation and differentiation [14,16,20,21] producing a significant increase in the expression of osteogenic and collagen genes [22,23], modulating the inflammatory process and stimulating trabecular bone matrix and periosteal formation during the process of fracture healing [24,25]. Additionally, laser therapy is able to stimulate cell recruitment, proliferation and differentiation, as such as the increase of the angiogenesis [21].

Despite the positive results of the use of biomaterials and LLLT, few studies have evaluated the association of both therapeutic resources on the process of bone healing and their effects are still controversial. Pinheiro et al. [26] showed that hydroxyapatite associated with LLLT

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was effective in improving bone healing on fractured bones as a result of the increase of newly formed bone. Additionally, Fangel et al. [27] demonstrated that the laser therapy improves bone repair process in association with BS as a result of increasing bone formation as well as indentation biomechanical properties. However, Oliveira et al. [21] showed that Biosilicate associated to LLLT was not able to improve the bone repair in the rat tibia.

Although the positive effects of the composite BS/PLGA and LLLT on bone cell proliferation and bone metabolism, the effects of the association of the cited treatments on bone healing were not studied yet. Before both therapies can be used with confidence as a therapeutic modality in fractures, it is necessary to investigate the effects and dose-response characteristics of these treatments in *in vitro* and *in vivo* studies to determine its safety and efficacy. In this context, we hypothesized that LLLT could improve the effects of BS/PLGA composite on bone healing in rats. Thus, our aims are to investigate the effects of LLLT on fibroblast and osteoblast cell viability using *in vitro* studies and on created bone defects treated with BS and BS/PLGA in tibia of rats.

2. Methodology

2.1. Materials

Biosilicate parent glass (fully crystallized bioactive glass ceramic of the quaternary P_2O_5-Na_2O-CaO-SiO_2 system) was provided by Vitreous Materials Laboratory (LaMaV), Department of Materials Engineering, Federal University of São Carlos, São Carlos, São Paulo, Brazil (patent application WO 2004/074199) [8]. PLGA microspheres were prepared according to a previously described single emulsion technique [28–31]. For this technique, 0.2 g of poly(lactic-*co*-glycolic) acid (PLGA; Purasorb® 5002A, Purac, Gorinchem, The Netherlands) was dissolved in 2 mL of dichloromethane (DCM; analytical grade; Merck, Darmstadt, Germany) in a 20 mL glass tube. Then, this solution was transferred into a stirred beaker containing 100 mL of 0.3% polyvinyl alcohol solution (PVA; 88% hydrolyzed, M_w = 22.000, Acros, Geel, Belgium). Subsequently, 50 mL of 2% isopropanol (IPN; analytical grade; Merck, Darmstadt, Germany) was added and the solution was stirred for 1 h. The microspheres of PLGA were allowed to settle for 1 h and the clear solution was decanted. The clear solution on top was aspirated. The microspheres were washed and this process of decantation and aspiration was repeated three times. Finally, the solution with microspheres was lyophilized (Edwards, São Paulo, Brazil) for 24 h and stored at 20 °C until use [14,28–32].

2.2. Preparation of Composites

Composite samples were made by adding both BS and PLGA (in a ratio of 80% and 20%) in a syringe with 2% Na_2HPO_4 and mixing it for 20 s using a mixing apparatus (Silamat® S6, Ivoclar Vivadent, Amherst, USA). Immediately after mixing, the composites were injected into Teflon molds (8 mm in diameter and 2 mm thick for *in vitro* tests and 3 mm in diameter and 2 mm thick for *in vivo* tests). After overnight setting at room temperature, the composites were removed from the molds and sterilized by ethylene oxide (Acecil, Campinas, São Paulo, Brazil).

2.3. Physicochemical Characterization of Composites

Pre-set composites were first examined by scanning electron microscope (SEM, Le0 440, Carl Zeiss, Jena, Germany) operating with a 10 keV electron beam. The surface morphology was determined before incubation. Additionally, the degradation behavior in the surface of composites was evaluated after 4 weeks of incubation in phosphate buffered saline (PBS, 10 mM and pH = 7.4) at 37 °C in a water bath on a shaker table.

For the physicochemical characterization, infrared spectra of the BS, PLGA and BS/PLGA were obtained by a reflection Fourier transform infrared spectrometer (FTIR, Bomem Michelson Series at 400 a 4000 cm^{-1} interval with 4 cm^{-1} resolution).

Table 1
Laser parameters.

Parameters	*In vitro*	*In vivo*
Wavelength	808 nm (infrared)	808 nm (infrared)
Laser frequency	Continuous output	Continuous output
Optical output	30 mW	30 mW
Spot size	0.028 cm^2	0.028 cm^2
Power density	1.07 W/cm^2	1.07 W/cm^2
Dose	10 J/cm^2	30 J/cm^2
Energy	0.28 J	0.84 J
Time per point	9 s	28 s
Application mode	Stationary directly into the well	Stationary in skin contact mode

2.4. Cell Culture Experiments

In vitro experiments were conducted using rat calvarial osteoblast-like cells (Osteo-1 lineage) and murine fibroblasts (L929). These cells were maintained in growth medium (Dulbecco's Modified Eagle Medium - DMEM, Vitrocell, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (Vitrocell, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Vitrocell, Campinas, SP, Brazil) at 37 °C in a humidified atmosphere of 5% CO_2.

2.4.1. Low Level Laser Therapy

A laser equipment Ga-Al-As (Photon lase III, DMC Equipment, São Carlos, SP, Brazil) was used in this study. The laser parameters for the *in vitro* and *in vivo* studies are shown in Table 1. It is possible to find in the literature a wide range of wavelengths and fluencies of laser irradiation used on bone healing. However, the association of laser therapy and biomaterials of is still poorly investigated and the results are contradictory [11,21,27]. Therefore, the choice of the laser parameters used in this study was based on literature. Furthermore, a low laser dose was chosen to prevent the association of these types of treatments could cause excessive stimulation at the site of injury.

Cells were irradiated immediately upon the cells in the well plate and were applied directly into the well from the bottom plate [33]. Laser irradiation was initiated in the first day and performed every day with a 24 h interval between sessions, giving a total of one, two and three sessions for the cytotoxicity assay after 24, 48 and 72 h.

2.4.2. Cytotoxicity Assessment

Cytotoxicity of investigated materials against Osteo-1 and L929 was evaluated by indirect method using composite extracts. To obtain the extracts, the samples were thoroughly immersed and incubated in DMEM (50 mL/g) supplemented with 10% fetal bovine serum (Vitrocell, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Vitrocell, Campinas, SP, Brazil) at 37 °C in a humidified atmosphere of 5% CO_2 for 7 days. After this period, the samples were removed from DMEM and this liquid was used for the experiments *in vitro*. Osteoblasts and fibroblasts (1×10^3 cells/well) were cultivated with extract of BS and BS/PLGA 80/20 for 24, 48 and 72 h in 96-well plates. The MTT assay was performed to determine the activity of enzymes that reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide to formazan dyes according to Kido et al. [12]. Three independent experiments were performed in quadruplicate (n = 4).

2.5. Biocompatibility and Osteogenesis In Vivo

Eighty healthy male Wistar rats (12 weeks, weight 300–350 g) were randomly divided into 4 groups (BS; BS + LLLT; BS/PLGA and BS/

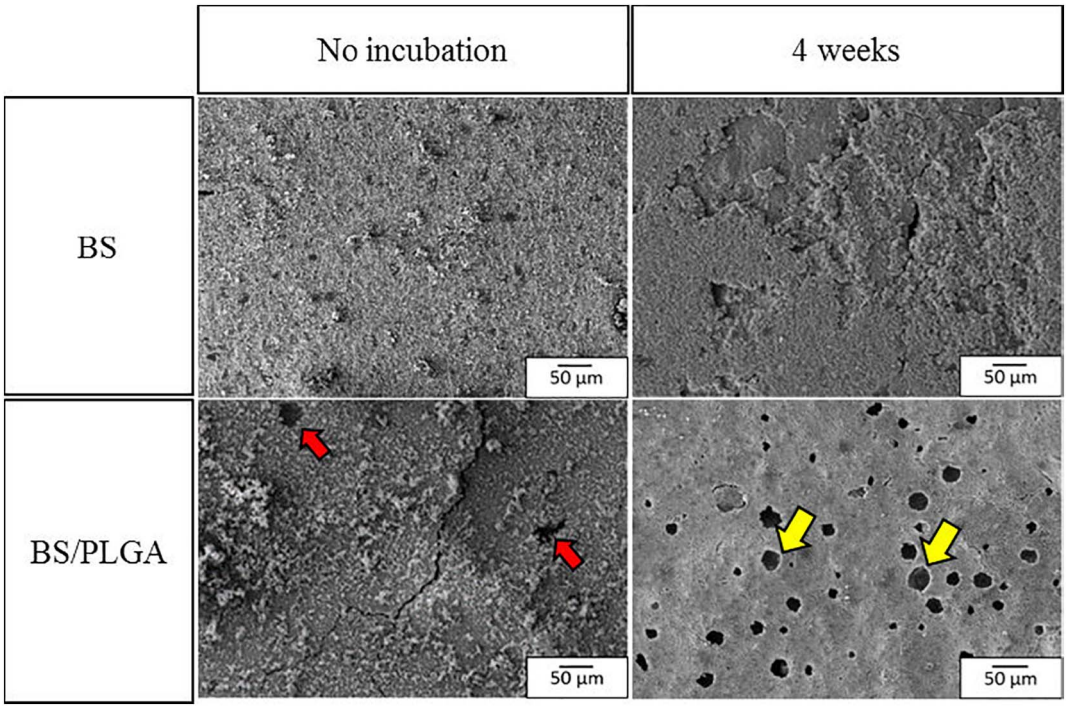

Fig. 1. Microscopic SEM micrographs of BS and BS/PLGA without incubation in PBS and after 4 weeks of incubation in PBS. PLGA microspheres (red arrows) and pores (yellow arrows) are indicated in the SEM micrographs. Bar represents 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PLGA + LLLT). Each group was divided into two subgroups, euthanized by carbon dioxide asphyxia after 2 and 6 weeks post-surgery (n = 10 for each subgroup). All rats received one surgical procedure during the course of this experiment to induce the unilateral noncritical size bone defects in tibia of all animals, which were filled with different composites. The animals were maintained under controlled temperature (22 ± 2 °C), light–dark periods of 12 h and had free access to water and standard food.

2.5.1. Surgery

All animal experiments were performed in accordance with protocols approved by Animal Care Committee Guidelines of the Federal University of São Paulo (2013/601498). Surgery was performed under sterile conditions and general anesthesia induced by intraperitoneal injection of 20 mg/kg xilazine (Anasedan; Sespo Industry and Trade Ltda., Jacareí, SP, Brazil) and 40 mg/kg ketamine (Dopalen; Sespo Industry and Trade Ltda., Jacareí, SP, Brazil). Then, the unilateral noncritical size bone defects (3 mm diameter) were created using motorized drill (Beltec®, Araraquara, SP, Brazil) under copious irrigation with saline solution at the upper third of the tibia (10 mm distal of the knee joint). The samples of composites were implanted into the prepared holes on the tibias of the rats and the animals received analgesia (i.m., 0.05 mg/kg buprenorphine (Temgesic; Reckitt Benckiser Health Care Limited, Schering Plough, Hoddesdon, United Kingdom)) and were returned to their cages. The animals were euthanized at 2 and 6 weeks post-surgery.

2.5.2. Low Level Laser Therapy

A laser equipment Ga-Al-As (Photon lase III, DMC Equipment, São Carlos, SP, Brazil) was used in this study. The laser parameters used in this protocol treatment are shown in Table 1. The irradiation was performed at one point, above the area of the created defect, by the punctual contact technique. Laser treatment was performed for 3 times per week, in non-consecutive days. The laser therapy was performed in both tibiae. Twenty-four hours after the last treatment session, rats were euthanized by CO_2 (carbon dioxide) suffocation and bones were removed for analysis.

2.5.3. Histopathological Analysis

The specimens were fixated in 10% formalin (Merck, Darmstadt, Germany) for 2 days, decalcified in 4% ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany), followed by dehydration in a graded series of ethanol and embedding in paraffin blocks, and histological sections were prepared. Therefore, thin sections (5 µm) were prepared using a microtome with a diamond blade (Leica Microsystems SP 1600, Nussloch, Germany). At least three sections of each specimen were stained with hematoxylin and eosin (Merck, Darmstadt, Germany) and examined using light microscopy (Leica Microsystems AG, Wetzlar, Germany, Darmstadt-Germany). Any changes in the bone defect, such as presence of woven bone, bone marrow, inflammatory process, granulation tissue, or even tissues undergoing hyperplastic, metaplastic, and/or dysplastic transformation were investigated per animal [14,21,34]. The analysis was performed by two observers (KRF and HWK), in a blinded way.

2.5.4. Biomechanical Test

For the biomechanical test, it was used a three-point bending test in an Instron® Universal Testing Machine (Instron® Worldwide Headquarters, Norwood, MA, USA), 3342 model and 500 N load cell. Tibiae were placed on a device (3.8 cm), which provides a 1.8 cm distant double support on the diaphysis. The load cell was perpendicularly positioned at the middle point of the tibiae. First, pre-load (5 N) was applied and the bending force was used at a constant deformation rate (0.5 cm/min) until the fracture occurred. Finally, the load-deformation curve was obtained and the maximum load (N), resilience (J) and force of fracture (N) was measured [17,35].

3. Results

3.1. Physicochemical Characterization of Composites

The degradation process of BS and PLGA microspheres was monitored using scanning electron microscopy. Fig. 1 illustrates the morphological structure of BS and BS/PLGA in two moments: without incubation and after 4 weeks of incubation in PBS. BS composites showed

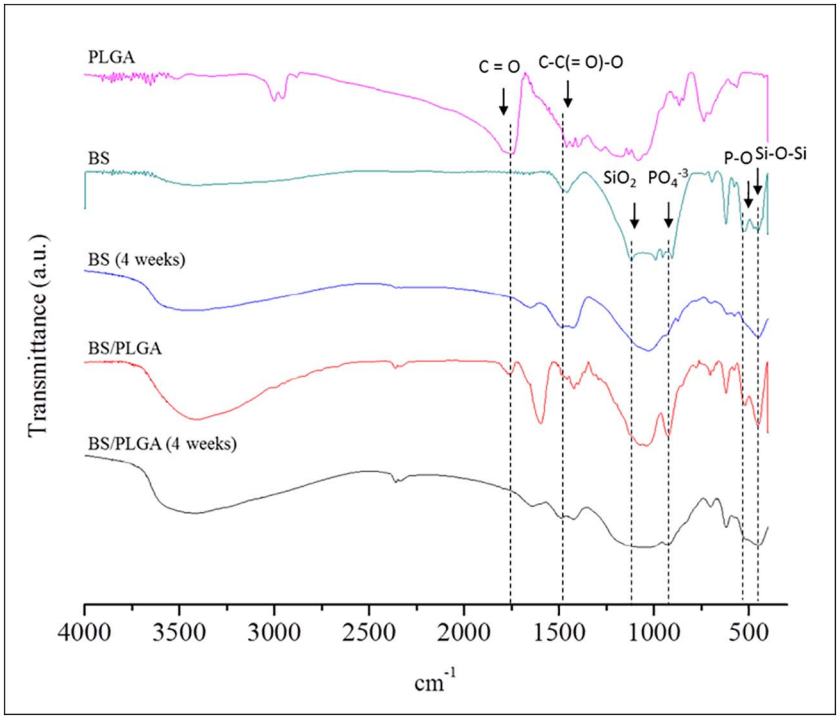

Fig. 2. FTIR spectra of BS and BS/PLGA without incubation in PBS and after 4 weeks of incubation in PBS.

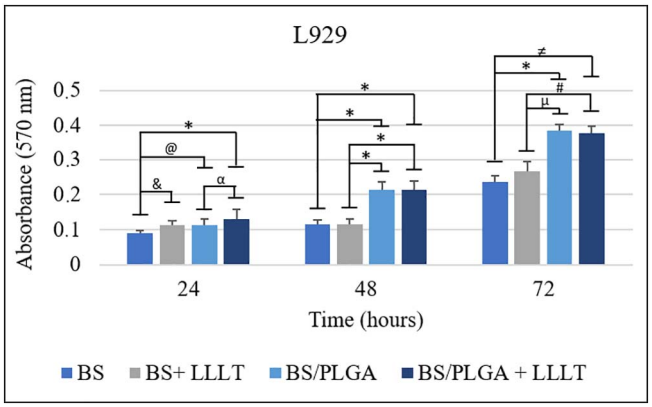

Fig. 3. Viability of fibroblasts cell line grown for all the experimental groups. BS; BS + LLLT; BS/PLGA; BS/PLGA + LLLT composites after 24, 48 and 72 h. *$p < 0.0001$; $^{\&}p = 0.0175$; $^{@}p = 0.0223$; $^{\alpha}p = 0.0471$; $^{\neq}p = 0.0001$; $^{\mu}p = 0.0007$ and $^{\#}p = 0.0064$.

no apparent modification in the material surface and no signs of degradation, before incubation and after 4 weeks of incubation. The morphological examination using SEM revealed that BS/PLGA without incubation, presented PLGA microsphere into BS. Four weeks after incubation, initial PLGA microspheres degradation was seen, resulting in pore formation into the composite, with a spherical appearance (Fig. 1).

3.2. FTIR

The changes in the chemical compositions of the composite before and after incubation in PBS can be observed in the FTIR spectra shown in Fig. 2. PLGA spectrum showed an intense band in the region of characteristic carbonyl (C═O), between 1850 and 1650 cm^{-1}. In this same spectrum, can also be observed a band between 1300 and 1150 cm^{-1} characteristic of the C−C(═O)−O. BS spectrum showed a band in the region 1100–900 cm^{-1}, which represent the SiO_2 and PO_4^{-3}. In the BS spectrum, the bands between 500 and 610 cm^{-1} represent the PO and Si−O−Si bonds. In the BS spectra after 4 weeks of incubation, it is possible to observe that the BS bands become less intense and narrow, showing the BS degradation.

The BS/PLGA composites were made by adding both BS and PLGA (in a ratio of 80% and 20%). In view of this, it is evident that the BS/PLGA spectrum presents the characteristic bands of BS and PLGA in the proportions (80/20). Therefore, the characteristic bands of PLGA (carbonyl (C═O), between 1850 and 1650 cm^{-1} and C−C(═O)−O, between 1300 and 1150 cm^{-1}) are less intense than the bands characteristic of BS (SiO_2 and PO_4^{-3} (1100–900 cm^{-1}) and PO and Si−O−Si bonds (500–610 cm^{-1})). After 4 weeks of incubation in PBS, in the BS/PLGA spectrum is evident the disappearance of the carbonyl band (1850 and 1650 cm^{-1}) and C−C(═O)−O (1300 and 1150 cm^{-1}). These bands are characteristic of PLGA, thus evidencing the PLGA degradation over time in this composite. However, it is possible to observe the presence of the characteristic bands of BS with less intensity and narrowing. In this way, in the BS/PLGA spectrum it is possible to evidence the degradation of BS and mainly of PLGA (Fig. 2).

3.3. Cytotoxicity Assessment

The cell viability of fibroblast and osteoblast cells on different extracts of BS and BS/PLGA, associated or not with LLLT, was assessed by MTT assay after 24, 48 and 72 h (Figs. 3 and 4).

For fibroblast cell viability, 24 h after seeding, a significantly higher value were observed in BS + LLLT compared to BS ($p = 0.0175$). Additionally, the BS/PLGA + LLLT showed a higher fibroblast viability compared to the same group without laser treatment (BS/PLGA) ($p = 0.0471$). Furthermore, BS/PLGA and BS/PLGA + LLLT presented a higher cell viability compared to BS ($p = 0.0223$ and $p < 0.0001$, respectively). In the second period analyzed (48 h), BS/PLGA showed a higher cell viability compared to BS ($p < 0.0001$) and BS + LLLT ($p < 0.0001$). Additionally, in the same period, BS/PLGA + LLLT demonstrated higher values of cell viability compared to BS ($p < 0.0001$) and BS + LLLT ($p < 0.0001$). These differences found after 48 h were also found after 72 h. BS/PLGA showed a higher cell viability compared to BS without or with laser treatment ($p < 0.000$ and $p = 0.0007$, respectively). Still, in the same period, BS/PLGA + LLLT presented higher values of cell viability compared to BS ($p = 0.0001$) and BS + LLLT ($p < 0.0064$) (Fig. 3).

For osteoblast cells, BS/PLGA + LLLT showed higher viability

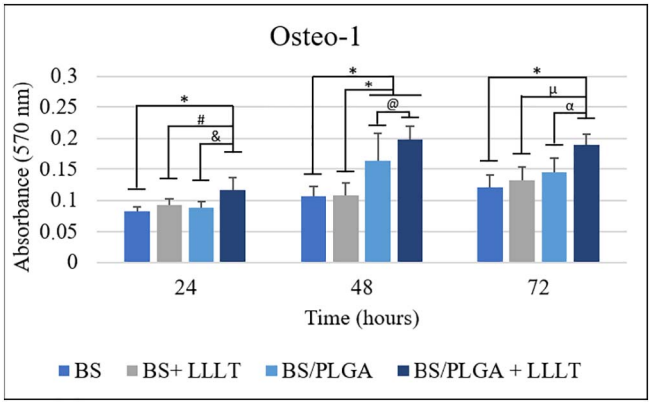

Fig. 4. Viability of osteoblastic cell line grown for all the experimental groups. BS; BS + LLLT; BS/PLGA and BS/PLGA + LLLT composites after 24, 48 and 72 h. *$p < 0.0001$; $^{\#}p = 0.0386$; $^{=}p = 0.0024$; $^{@}p = 0.0239$; $^{\mu}p = 0.0013$ and $^{\alpha}p = 0.0155$.

compared to BS ($p < 0.0001$), BS/PLGA ($p = 0.0386$), BS/PLGA + LLLT ($p = 0.0024$) in the first period analyzed (24 h) (Fig. 4). After 48 h, BS/PLGA demonstrated higher cell viability compared to BS ($p < 0.0001$) and BS + LLLT ($p < 0.0001$). In addition, BS/PLGA + LLLT showed substantial increase in the cell viability compared to BS ($p < 0.0001$), BS + LLLT ($p < 0.0001$) and BS/PLGA ($p = 0.0239$). For the last experimental period, BS/PLGA + LLLT presented the higher cell viability compared to BS ($p < 0.0001$), BS + LLLT ($p = 0.0013$) and BS/PLGA ($p = 0.0155$) (Fig. 4).

3.4. Histopathological Analysis

Representative histological sections of all groups analyzed n this study is depicted in the Fig. 5.

Two weeks after implantation, histopathological evaluation of BS showed that the bone defect area was almost filled with the biomaterial. Some degradation of the material was observed at the periphery, with some granulation tissue and newly formed bone. Animals treated with BS and irradiated with LLLT demonstrated similar findings, with, with some signs of degradation only at the border of the defect, filled with granulation tissue and newly formed bone. For BS/PLGA, in the same experimental period, initial biomaterial degradation and the presence of some pores, filled with granulation tissue can be observed at the edges of the defect. For BS/PLGA + LLLT, a larger extent of material degradation was noticed. Moreover, a more intense amount of granulation tissue and newly formed bone were present in the region of the bone defect.

Six weeks post-surgery, for BS and BS + LLLT, the material degradation had continued, with an increase in the amount of granulation tissue and newly formed bone. For BS/PLGA animals, the degradation of the biomaterial and pore formation continued, allowing granulation and newly formed bone tissue in growth in the spaces occupied before by the material. For BS/PLGA associated to LLLT, an intense material degradation and pore formation were observed, with the ingrowth of granulation tissue in the center of the defect. Additionally, the edges were filled with newly formed bone.

3.5. Biomechanical Test

Table 2 shows the means and standard errors of the biomechanical test of all groups in the two experimental periods (2 and 6 weeks post-surgery). No statistically significant difference was observed among groups for toughness (J), modulus of elasticity (MPa) and force of fracture (N).

4. Discussion

The present study aimed to evaluate the *in vitro* and *in vivo* tissue response in the presence of BS/PLGA irradiated with LLLT. It was hypothesized that laser therapy would positively affect the viability of fibroblast and osteoblast cells seeded into BS/PLGA composites. Moreover, the hypothesis that, the association of both therapies would be more effective in stimulating bone formation in an experimental model of tibial defect was raised. The SEM and FTIR results revealed that PLGA microspheres were successfully inserted into BS and the microparticles degraded over time, leaving pores into the material. The main *in vitro* findings demonstrated higher fibroblast viability in BS/PLGA + LLLT after 24, 48 and 72 h and higher osteoblast viability in BS/PLGA irradiated with LLLT, in all periods analyzed in this study. Furthermore, animals treated with BS/PLGA and irradiated with LLLT demonstrated an improved material degradation and an increased amount of granulation tissue and newly formed bone in the region of the defect.

It is well known that the inclusion of PLGA microspheres as a porogen material is a promising strategy for bone tissue engineering due to capacity of creating macroporosity and accelerating the degradation of ceramic materials and bioglasses, which are crucial for the adequate performance of bone graft into the tissue [14,28–32,34]. Moreover, in the present study, the infrared spectra of the materials allowed the detection of specifics peaks of PLGA and BS. These peaks have become sharper and less intense after the immersion in PBS, demonstrating the degradation of PLGA microspheres and BS, resulting in pore formation. Additionally, it is well known that PLGA degradation releases lactic and glycolic acid monomers and creates an acidic environment, which culminates in the acceleration of BS degradation [31].

The *in vitro* studies demonstrated that the lower values of fibroblast viability were found for BS and BS + LLLT groups, suggesting that the stimulus offered by BS alone or the association with LLLT was not enough to influence cell metabolism. Interestingly, both BS/PLGA groups presented higher values of fibroblast viability. The improved cell performance may be related to the more physiological pH related to the presence of BS and PLGA. It is well known that PLGA degradation results in an acidic environment due to the release of monomers lactic and glycolic acids [28,29]. Conversely, the ions released from BS increases pH [14,36]. In this context, the combination of PLGA and BS may counteract excessive acidification and in a more homeostatic environment, culminating in an increase of cell viability [30,37]. For fibroblast cells, LLLT associated with materials produced an increased viability only in the first period analyzed (no additional effect was observed in the other experimental periods). It is unclear at this stage why these results have occurred however, they may be related to the laser parameters used.

Increased osteoblast cell viability was observed with the association of the material composites and LLLT, indicating that both therapies were efficient to stimulate cell metabolism. These positive effects may be explained by the counteract pH produced by the presence of BS and PLGA associated to the cell laser stimulation, culminating the increasing of osteoblast cell metabolism. The distinctive nature of the findings for both osteoblast and fibroblast cells may be due to a range of factors, including cell line specificity, cellular physiology and/or specificity of cell lineage to the laser wavelengths employed. The findings further support the notion of cell/tissue and dose/wavelength specificities, possibly indicating that osteoblast cell line respond better to infra-red laser wavelength.

Furthermore, to reach success in bone tissue engineering, bone graft needs to be degraded and replaced newly bone tissue [14,37,38]. In this study, animals treated with BS/PLGA irradiated with LLLT presented the higher rate of degradation and the increased amount of newly bone tissue at the site of the defect. It is well known that the association of PLGA microparticles and BS stimulate bone matrix deposition and

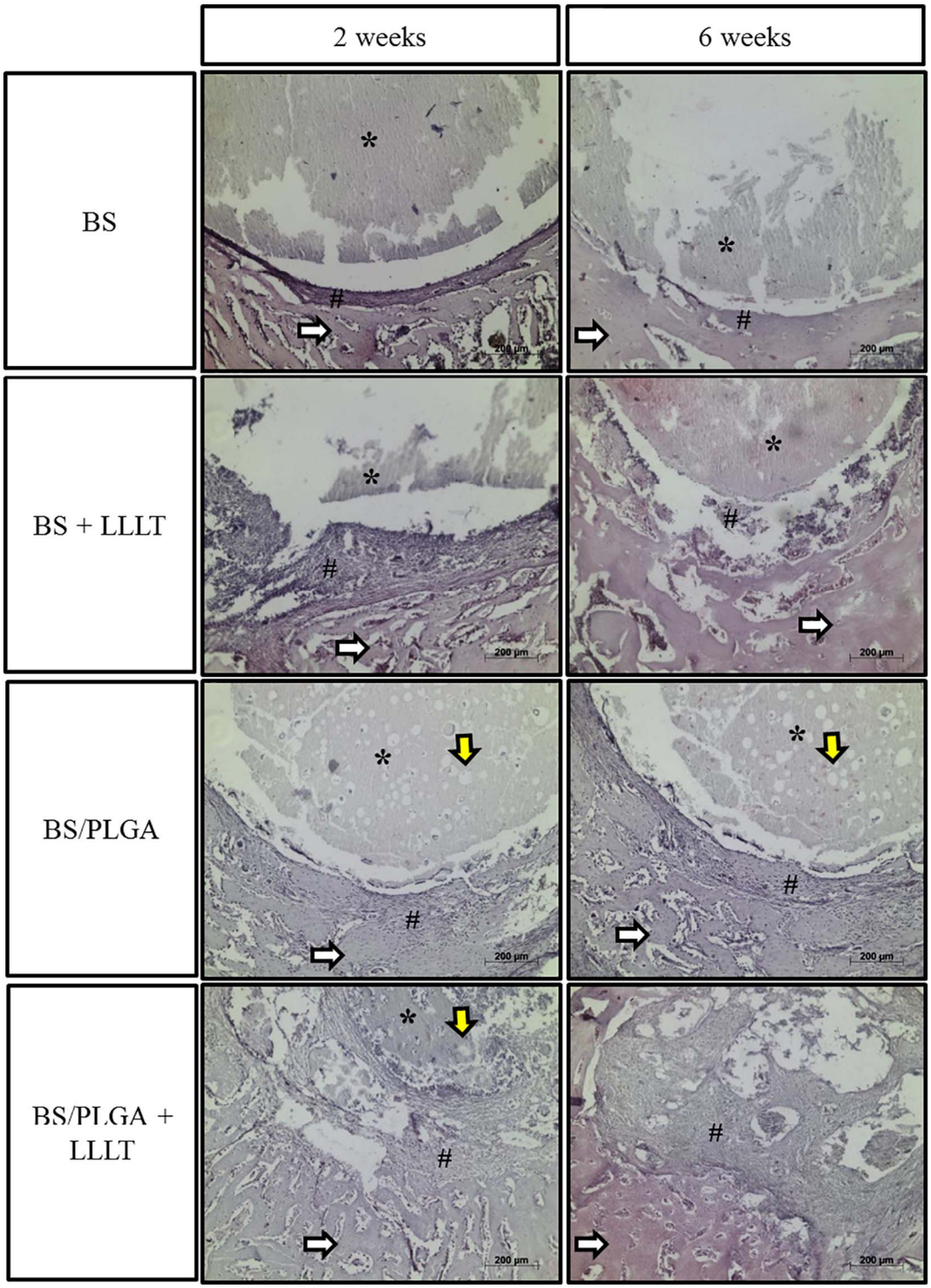

Fig. 5. Representative histological sections of bone defect for all the experimental groups. BS; BS + LLLT; BS/PLGA; BS/PLGA + LLLT after 2 and 6 weeks. (*) Biomaterial, (yellow arrows) pores, (#) granulation tissue and (white arrows) newly formed bone. Hematoxylin and eosin stain. Bar represent 200 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accelerate the healing process [30]. This phenomenon may be due to the release of acidic monomers from PLGA, producing an acidic environment that acts as a stimulus for BS dissolution [37,39]. The increased BS degradation resulted in a lower amount of remaining implant material, leaving more space for tissue ingrowth. In addition, BS ions dissolution after implantation beneficially affects osteogenesis by formation of a silica layer which directs new bone formation [7,30].

Furthermore, the histological findings demonstrated that LLLT improved the stimulatory effect of BS/PLGA composite, presenting a higher amount of newly formed bone. It has been reported that LLLT has an osteogenic and angiogenic potential, being able of increasing the deposition of newly bone and neovascularization at the area of the bone defect [20,22,34], which together with the osteopromotive properties of BS might further influence bone formation.

The biomechanical evaluation did not show any difference among groups indicating that the increased bone deposition in the defects of laser irradiated and BS/PLGA treated animals did not culminate in an increase in bone callus strength. Possibly, the period of 6 weeks was not

Table 2
Means and standard errors for biomechanical analysis 2 and 6 weeks post-surgery.

		Toughness (J)	Modulus of elasticity (MPa)	Force of fracture (N)
2 weeks	BS	0.0226 ± 0.0047	0.0878 ± 0.0046	33.2681 ± 4.5611
	B S + LLLT	0.0230 ± 0.0044	0.0947 ± 0.0067	36.6819 ± 3.4676
	BSIPLGA	0.0379 ± 0.0100	0.0948 ± 0.0046	42.4194 ± 3.1394
	BSIPLGA + LLLT	0.0183 ± 0.0054	0.0804 ± 0.0096	27.3406 ± 3.6124
6 weeks	BS	0.0316 ± 0.0077	0.1471 ± 0.0247	42.9025 ± 1.6035
	BS + LLLT	0.0365 + 0.0090	0.1171 + 0.0071	42.2118 + 7.1363
	BSIPLGA	0.0374 + 0.0098	0.1371 + 0.0122	49.0412 + 4.7529
	BSIPLGA + LLLT	0.0343 ± 0.0130	0.1351 ± 0.0183	37.8448 ± 5.8787

enough to produce the mineralization of the bone callus to produce changes in biomechanical properties.

The outcomes of the current study confirmed the hypothesis that laser irradiation can improve the biological performance of BS/PLGA in osteoblast cells and during the process of bone healing in a tibial bone defect model. Although the promising results showed by our data related to the stimulation of bone tissue by LLLT, our experimental design was limited to a healthy rat. Further, studies are required to investigate the effects of BS/PLGA and laser therapy under compromised conditions remains to be provided.

5. Conclusion

Summarizing, this study suggests that the irradiating BS/PLGA with laser therapy increased osteoblast viability, accelerated material degradation and increased bone formation in a tibial bone defect in rats. Consequently, these data highlight the potential of the combination of BS/PLGA and LLLT as an effective tool in bone tissue engineering. Further long-term studies should be carried out to provide additional information concerning the late stages of bone healing.

References

[1] R. Yunus Basha, T.S. Sampath Kumar, M. Doble, Design of biocomposite materials for bone tissue regeneration, Mater. Sci. Eng. C 57 (2015) 452–463.

[2] J. Li, Q. Xu, B. Teng, C. Yu, J. Li, L. Song, Y.X. Lai, J. Zhang, W. Zheng, P.G. Ren, Investigation of angiogenesis in bioactive 3-dimensional poly(D,L-lactide-*co*-glycolide)/nano-hydroxyapatite scaffolds by in vivo multiphoton microscopy in murine calvarial critical bone defect, Acta Biomater. 42 (2016) 389–399.

[3] H. Alan, E. Farahani, S. Tunik, G. Kavak, The histological study of osseous regeneration following implantation of various bone graft biomaterials, Niger. J. Clin. Pract. 19 (2016) 517–522.

[4] L.L. Hench, J.M. Polak, Third-generation biomedical materials, Science 295 (2002) 1014–1017.

[5] O. Peitl, E.D. Zanotto, F.C. Serbena, L.L. Hench, Compositional and microstructural design of highly bioactive P_2O_5-Na_2O-CaO-SiO_2 glass-ceramics, Acta Biomater. 8 (2012) 321–332.

[6] J.R. Jones, Review of bioactive glass: from Hench to hybrids, Acta Biomater. 9 (2013) 4457–4486.

[7] J.R. Jones, Reprint of: review of bioactive glass: from Hench to hybrids, Acta Biomater. 23 (Suppl) (2015) S53–S82.

[8] E.D. Zanotto, C. Ravagnani, O. Peitl, H. Panzeri, E.H. Lara, Process and Compositions for Preparing Particulate, Bioactive or Resorbable Biosilicates for Use in the Treatment of Oral Ailments, Universidade Federal de Sao Carlos, Universidade de Sao Paulo, 2004 International Classification C03C10/00. (WO 2004/074199 (INPI 03006441)).

[9] R.N. Granito, A.C. Renno, C. Ravagnani, P.S. Bossini, D. Mochiuti, V. Jorgetti, P. Driusso, O. Peitl, E.D. Zanotto, N.A. Parizotto, J. Oishi, In vivo biological performance of a novel highly bioactive glass-ceramic (Biosilicate®): a biomechanical and histomorphometric study in rat tibial defects, J. Biomed. Mater. Res. B Appl. Biomater. 97 (2011) 139–147.

[10] M.A. Matsumoto, G. Caviquioli, C.C. Biguetti, A. Holgado Lde, P.P. Saraiva, A.C. Renno, R.Y. Kawakami, A novel bioactive vitroceramic presents similar biological responses as autogenous bone grafts, J. Mater. Sci. Mater. Med. 23 (2012) 1447–1456.

[11] K.N. Pinto, C.R. Tim, M.C. Crovace, M.A. Matsumoto, N.A. Parizotto, E.D. Zanotto, O. Peitl, A.C. Renno, Effects of Biosilicate® scaffolds and low-level laser therapy on the process of bone healing, Photomed. Laser Surg. 31 (2013) 252–260.

[12] H.W. Kido, P. Oliveira, N.A. Parizotto, M.C. Crovace, E.D. Zanotto, O. Peitl-Filho, K.P. Fernandes, R.A. Mesquita-Ferrari, D.A. Ribeiro, A.C. Renno, Histopathological, cytotoxicity and genotoxicity evaluation of Biosilicate® glass-ceramic scaffolds, J. Biomed. Mater. Res. A 101 (2013) 667–673.

[13] M.R. Azenha, S.A. de Lacerda, H.F. Marao, O.P. Filho, O.M. Filho, Evaluation of crystallized biosilicate in the reconstruction of calvarial defects, J. Maxillofac. Oral Surg. 14 (2015) 659–665.

[14] K.R. Fernandes, A. Magri, H.W. Kido, F. Ueno, L. Assis, K. Fernandes, R.A. Mesquita-Ferrari, V.C. Martins, A.M. Plepis, E.D. Zanotto, O. Peitl, D. Ribeiro, J.J. van den Beucken, A. Renno, Characterization and biological evaluation of the introduction of PLGA into Biosilicate®, J. Biomed. Mater. Res. B Appl. Biomater. (2016).

[15] S.A. Danesh-Sani, S.P. Engebretson, M.N. Janal, Histomorphometric results of different grafting materials and effect of healing time on bone maturation after sinus floor augmentation: a systematic review and meta-analysis, J. Periodontal Res. 52 (3) (2017) 301–312.

[16] H.O. Kazancioglu, S. Ezirganli, M.S. Aydin, Effects of laser and ozone therapies on bone healing in the calvarial defects, J. Craniofac. Surg. 24 (2013) 2141–2146.

[17] A.M. Magri, K.R. Fernandes, L. Assis, N.A. Mendes, A.L. da Silva Santos, E. de Oliveira Dantas, A.C. Renno, Photobiomodulation and bone healing in diabetic rats: evaluation of bone response using a tibial defect experimental model, Lasers Med. Sci. 30 (2015) 1949–1957.

[18] A.H. Acar, U. Yolcu, S. Altindis, M. Gul, H. Alan, S. Malkoc, Bone regeneration by low-level laser therapy and low-intensity pulsed ultrasound therapy in the rabbit calvarium, Arch. Oral Biol. 61 (2016) 60–65.

[19] C.C. Medalha, A.L. Santos, O. Veronez Sde, K.R. Fernandes, A.M. Magri, A.C. Renno, Low level laser therapy accelerates bone healing in spinal cord injured rats, J. Photochem. Photobiol. B 159 (2016) 179–185.

[20] E. Favaro-Pipi, S.M. Feitosa, D.A. Ribeiro, P. Bossini, P. Oliveira, N.A. Parizotto, A.C. Renno, Comparative study of the effects of low-intensity pulsed ultrasound and low-level laser therapy on bone defects in tibias of rats, Lasers Med. Sci. 25 (2010) 727–732.

[21] F.A. Oliveira, A.A. Matos, M.R. Santesso, C.K. Tokuhara, A.L. Leite, V.S. Bagnato, M.A. Machado, C. Peres-Buzalaf, R.C. Oliveira, Low intensity lasers differently induce primary human osteoblast proliferation and differentiation, J. Photochem. Photobiol. B 163 (2016) 14–21.

[22] C.R. Tim, P.S. Bossini, H.W. Kido, I. Malavazi, M.R. von Zeska Kress, M.F. Carazzolle, N.A. Parizotto, A.C. Renno, Effects of low-level laser therapy on the expression of osteogenic genes during the initial stages of bone healing in rats: a microarray analysis, Lasers Med. Sci. 30 (2015) 2325–2333.

[23] C.R. Tim, P.S. Bossini, H.W. Kido, I. Malavazi, M.R. von Zeska Kress, M.F. Carazzolle, A.C. Renno, N.A. Parizotto, Low-level laser therapy induces an upregulation of collagen gene expression during the initial process of bone healing: a microarray analysis, J. Biomed. Opt. 21 (2016) 88001.

[24] Y. Sela, M.E. Baratz, Distal humerus fractures in the elderly population, J. Hand. Surg. [Am.] 40 (2015) 599–601.

[25] M. Briteno-Vazquez, G. Santillan-Diaz, M. Gonzalez-Perez, T. Gallego-Izquierdo, D. Pecos-Martin, G. Plaza-Manzano, N. Romero-Franco, Low power laser stimulation of the bone consolidation in tibial fractures of rats: a radiologic and histopathological analysis, Lasers Med. Sci. 30 (2015) 333–338.

[26] A.L. Pinheiro, N.R. Santos, P.C. Oliveira, G.T. Aciole, T.A. Ramos, T.A. Gonzalez, L.N. da Silva, A.F. Barbosa, L. Silveira Jr., The efficacy of the use of IR laser phototherapy associated to biphasic ceramic graft and guided bone regeneration on surgical fractures treated with miniplates: a Raman spectral study on rabbits, Lasers Med. Sci. 28 (2013) 513–518.

[27] R. Fangel, P.S. Bossini, A.C. Renno, D.A. Ribeiro, C.C. Wang, R.L. Toma, K.O. Nonaka, P. Driusso, N.A. Parizotto, J. Oishi, Low-level laser therapy, at 60 J/cm^2 associated with a Biosilicate® increase in bone deposition and indentation biomechanical properties of callus in osteopenic rats, J. Biomed. Opt. 16 (2011) 078001.

[28] R.P. Felix Lanao, S.C. Leeuwenburgh, J.G. Wolke, J.A. Jansen, In vitro degradation rate of apatitic calcium phosphate cement with incorporated PLGA microspheres, Acta Biomater. 7 (2011) 3459–3468.

[29] R.P. Felix Lanao, S.C. Leeuwenburgh, J.G. Wolke, J.A. Jansen, Bone response to fast-degrading, injectable calcium phosphate cements containing PLGA microparticles, Biomaterials 32 (2011) 8839–8847.

[30] A.C. Renno, P.S. Bossini, M.C. Crovace, A.C. Rodrigues, E.D. Zanotto, N.A. Parizotto, Characterization and in vivo biological performance of biosilicate, Biomed. Res. Int. 2013 (2013) 141427.

[31] A.C. Renno, M.R. Nejadnik, F.C. van de Watering, M.C. Crovace, E.D. Zanotto, J.P. Hoefnagels, J.G. Wolke, J.A. Jansen, J.J. van den Beucken, Incorporation of bioactive glass in calcium phosphate cement: material characterization and in vitro degradation, J. Biomed. Mater. Res. A 101 (2013) 2365–2373.

[32] H.W. Kido, P. Brassolatti, C.R. Tim, P.R. Gabbai-Armelin, A.M. Magri, K.R. Fernandes, P.S. Bossini, N.A. Parizotto, M.C. Crovace, I. Malavazi, A.F. da

Cunha, A.M. Plepis, F.F. Anibal, A.C. Renno, Porous poly(D,L-lactide-*co*-glycolide) acid/Biosilicate® composite scaffolds for bone tissue engineering, J. Biomed. Mater. Res. B Appl. Biomater. 105 (2017) 63–71.

[33] L.M. Silva, C.A. Silva, A. Silva, R.P. Vieira, R.A. Mesquita-Ferrari, J.C. Cogo, S.R. Zamuner, Photobiomodulation protects and promotes differentiation of C2C12 myoblast cells exposed to snake venom, PLoS One 11 (2016) e0152890.

[34] K.R. Fernandes, D.A. Ribeiro, N.C. Rodrigues, C. Tim, A.A. Santos, N.A. Parizotto, H.S. de Araujo, P. Driusso, A.C. Renno, Effects of low-level laser therapy on the expression of osteogenic genes related in the initial stages of bone defects in rats, J. Biomed. Opt. 18 (2013) 038002.

[35] Y.Y. Huang, S.K. Sharma, J. Carroll, M.R. Hamblin, Biphasic dose response in low level light therapy - an update, Dose-Response 9 (2011) 602–618.

[36] L.L. Hench, I.D. Xynos, J.M. Polak, Bioactive glasses for in situ tissue regeneration, J. Biomater. Sci. Polym. Ed. 15 (2004) 543–562.

[37] H.H. Lu, A. Tang, S.C. Oh, J.P. Spalazzi, K. Dionisio, Compositional effects on the formation of a calcium phosphate layer and the response of osteoblast-like cells on polymer-bioactive glass composites, Biomaterials 26 (2005) 6323–6334.

[38] X. Qi, J. Ye, Y. Wang, Improved injectability and in vitro degradation of a calcium phosphate cement containing poly(lactide-*co*-glycolide) microspheres, Acta Biomater. 4 (2008) 1837–1845.

[39] A. Weizbauer, M. Kieke, M.I. Rahim, G.L. Angrisani, E. Willbold, J. Diekmann, T. Florkemeier, H. Windhagen, P.P. Muller, P. Behrens, S. Budde, Magnesium-containing layered double hydroxides as orthopaedic implant coating materials—an in vitro and in vivo study, J. Biomed. Mater. Res. B Appl. Biomater. 104 (2016) 525–531.