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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/