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Low level laser therapy does not modulate the outcomes of a highly bioactive glass–ceramic (Biosilicate®) on bone consolidation in rats

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Abstract The main purpose of the present work was to evaluate if low level laser therapy (LLLT) can improve the effects of novel fully-crystallized glass–ceramic (Biosilicate®) on bone consolidation in tibial defects of rats. Forty male Wistar rats with tibial bone defects were used. Animals were divided into four groups: group bone defect control (CG); group bone defect filled with Biosilicate® (BG); group bone defect filled with Biosilicate®, irradiated with LLLT, at 60 J cm^{-2} (BG 60) and group bone defect filled with Biosilicate®, irradiated with LLLT, at 120 J cm^{-2} (BG 120). A low-energy GaAlAs 830 nm, CW, 0.6 mm beam diameter, 100 W cm^{-2}, 60 and 120 J cm^{-2} was used in this study. Laser irradiation was initiated immediately after the surgery procedure and it was performed every 48 h for 14 days. Fourteen days post-surgery, the three-point bending test revealed that the structural stiffness of the groups CG and BG was higher than the values of the groups BG60 and BG120. Morphometric analysis revealed no differences between the control group and the Biosilcate® group. Interestingly, the groups treated with Biosilicate® and laser (BG 60 and BG120) showed statistically significant lower values of newly formed bone in the area of the defect when compared to negative control (CG) and bone defect group filled with Biosilicate (CB). Our findings suggest that although Biosilicate® exerts some osteogenic activity during bone repair, laser therapy is not able to modulate this process.

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1 Introduction

Fracture healing is a complex physiologic process in which bone heals for the purpose of transferring mechanical loads [1]. In fracture consolidation, the original tissue is regenerated and the properties of the preexisting tissue, in general, are restored. However, clinical situations involving great bone loss or the presence of wide variety of diseases or tumor resection can result in delayed fracture healing or even nonunion [2].

In this context, there is a critical need to know more about the biology of fracture healing to develop strategies for ensuring normal repair of the skeleton [3]. One promising treatment is the use of bioactive glasses as bone graft substitutes due to their ability to bond and integrate with living bone by forming a biologically active bonelike apatite layer on their surfaces [4, 5]. Moreover, surface reactions release critical concentrations of soluble silicon, calcium, phosphorus and sodium ions that stimulate the attachment, proliferation and differentiation of osteoblasts (bone-forming cells) [5]. Our research group has developed nucleation and growth thermal treatments to obtain a novel fully-crystallized bioactive glass–ceramic of the quaternary P_2O_5–Na_2O–CaO–SiO_2 system (Biosilicate®, patent application WO 2004/074199) [6]. In vitro experiments demonstrated that Biosilicate® promotes enhanced bone-like matrix formation in comparison to its parent glass and to Bioglass® 45S5 in an osteogenic cell culture system [7]. Recently, Granito et al. [8] found that the Biosilicate was capable of increasing the biomechanical properties of the bone callus of tibial defects in rats compared to the 45S5 (gold standard).

Also, the Biosilicate® produced a higher bone volume and higher number of osteoblasts in the callus area compared to the groups treated with Bioglass® 45S5. The authors concluded that the fully-crystallized Biosilicate® may enhance bone repair by increasing new bone formation, as well as improving bone mechanical properties.

Similarly, a significant body of evidence has now accumulated demonstrating that low-level laser therapy (LLLT) also has a positive effect on bone tissue metabolism and on fracture consolidation [9, 10]. The action of laser therapy is based on the absorption of the light by tissues, which will generate a series of modifications in cell metabolism. When laser is applied to tissue, the light is absorbed by chromophore photoreceptors located in the cells. Once absorbed, the light can modulate cell biochemical reactions and stimulate mitochondrial respiration, with the production of molecular oxygen and ATP synthesis [8, 11]. These effects are known to increase the synthesis of DNA, RNA and cell-cycle regulatory proteins, therefore promoting cell proliferation [11].

In bone repair, Nicola et al. [12] studied the activity in bone cells after LLLT (GaAlAs laser, 660 nm, 10 J cm^{-2}) close to the site of the bone injury. The histomorphometry analysis of the bone revealed higher activity in the irradiated bone and the investigators suggested that LLLT increases the activity in bone cells (resorption and formation) around the site of the repair.

Pinheiro et al. [13] showed that the 830 nm laser (830 nm, 10 mW, 4.8 J cm^{-2}) was capable of increasing the amount of mineralized bone tissue on femoral induced fractures in rats. Moreover, osteoblast DNA and RNA synthesis, bone nodule formation, osteocalcin and osteopontin gene expression were found to be enhanced after laser irradiation. Additionally, ALP activity, which is a marker of osteoblast differentiation and is expressed in pre and mature osteoblasts, appears to be increased with LLLT irradiation [9, 11, 14–16]. Faster callus formation and revascularization, promotion of bone formation, increased quantities of calcium, phosphorous, and collagen hydroxyproline, and denser trabecular networks have also been reported [10, 17].

Although the positive effects of Biosilicate® and the LLLT on bone cell proliferation and bone metabolism has been reported, the effects of the association of both treatments on fracture consolidation 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 studies in vivo to determine its safety and efficacy. In this context, we hypothesized that LLLT could improve the effects of Biosilicate® on bone consolidation in rats. In this context, this study aimed to investigate the effects of LLLT used at two different doses, on created-bone defects treated with Biosilicate®.

2 Methodology

2.1 Experimental design and surgical procedures

Forty male Wistar rats (aged 12 weeks and weighing 250–300 g) were used in this study. They were maintained under controlled temperature (24 ± 2°C), light–dark periods of 12 h, and with unrestricted access to water and commercial diet. All animal handling and surgical procedures were strictly conducted according the Guiding Principles for the Use of Laboratory Animals. This study was approved by the Animal Care Committee guidelines of the Federal University of São Carlos.

Rats were randomly distributed into four groups (ten animals each): Control bone defect group (CG): bone defects without any fillers; Biosilicate® group (BG): bone defects filled with Biosilicate®; Biosilicate® group irradiated with LLLT, at 60 J cm^{-2} (BG60) and Biosilicate® group irradiated with LLLT, at 120 J cm^{-2} (BG120).

Bilateral non-critical size bone defects were surgically created at the upper third of the tibia (10 mm distal of the knee joint). Surgery was performed under sterile conditions and general anesthesia induced by intra-peritoneal injection of Ketamine/Xylazine (80/10 mg kg^{-1}). The medial compartment of the tibia was exposed through a longitudinal incision on the shaved skin. A standardized 2.0-mm-diameter bone defect was created by using a motorized drill under copious irrigation with saline solution. Holes were compressed with gauze for 5 min. Immediately afterwards, bone cavities were completely filled with the corresponding biomaterial in the treated animal groups. After implantation, the cutaneous flap was replaced and sutured with resorbable polyglactin, and the skin was disinfected with povidone iodin. The health status of the rats was monitored daily.

2.2 Biomaterial

High purity silica and reagent grade calcium carbonate, sodium carbonate, and sodium phosphate were used to obtain glass compositions: Biosilicate® parent glass. The chemicals were weighed and mixed for 30 min in a polyethylene bottle. Premixed batches were melted in 'Pt' platinum crucible at a temperature range of 1,250–1,380°C for 3 h in an electric furnace (Rapid Temp 1710 BL, CM Furnaces Inc., Bloomfield, NJ, USA) at the Vitreous Materials Laboratory of the Federal University of São Carlos (São Carlos, SP, Brazil). Samples were cast into a 10 mm × 30 mm cylindrical graphite mold and annealed at 460°C for 5 h. To obtain the fully-crystallized Biosilicate® glass–ceramic, Biosilicate® parent glass cylinders underwent cycles of thermal treatment to promote their crystallization. The first thermal cycle was performed at a

relatively low temperature, just above the glass transition temperature to promote volumetric nucleation of crystals. Afterwards, the nucleated samples were submitted to further treatment for about 100°C above the nucleation temperatures. The detailed compositions and thermal treatment schedules to obtain the Biosilicate® glass–ceramic are described in the patent WO 2004/074199 [6].

2.3 Low level laser therapy

A low-energy GaAlAs (Teralaser, DMC® São Carlos, SP, Brazil), 830 nm, CW, 0.6 mm beam diameter, 100 W cm^{-2}, 60 and 120 J cm^{-2}, with a irradiation time of 17 and 34 s, respectively, was used in this study. Laser irradiation was initiated immediately after the surgery procedure and it was performed on days 2, 4, 6, 8, 10 and 12 post-surgery. On day 14 post-surgery, rats were sacrificed with an intra-peritoneal injection of general anesthetic. The tibias were defleshed and soft tissues were removed for analysis.

2.4 Mechanical test

Biomechanical properties of the left tibia were determined by a three-point bending test in an Instron® Universal Testing Machine (USA, 4444 model, 1 KN load cell).

Tibias were placed on a 3.8-cm metal device, which provides a 1.8-cm distance between the two supports. The load cell was perpendicularly positioned at the exact site of the bone defect, in the anterior–posterior direction. A 5-N pre-load was applied in order to avoid specimen sliding. Finally, the bending force was applied at a constant deformation rate of 0.5 cm min^{-1} until fracture occurred. From the load-deformation curve, the maximum load at failure (N), structural stiffness (N mm^{-1}) and energy absorption (J) were obtained.

2.5 Histopathological analysis

For the histopathological analysis, the right tibiae were removed, and then fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h and decalcified in 4% EDTA (Merck), and embedded in paraffin blocks. Five-micrometer slices were obtained in a serially sectioned pattern and stained with hematoxylin and eosin (H.E stain, Merck).

Histopathological evaluation was performed under a light microscope (Olympus, Optical Co. Ltd, Tokyo, Japan). Any changes in the bone defect, such as presence of woven bone, medullar tissue, inflammatory process as depicted by the presence of inflammatory cells such as neutrophils or macrophages, granulation tissue, or even tissues undergoing hyperplastic, metaplastic and/or dysplastic transformation were investigated per animal.

2.6 Morphometry analysis

The morphometry of the area of newly formed bone in the regions of bone repair previously indentified in the histopathological observation for each animal was measured in a blind fashion by one expert observer using an image analysis system Motican 5.0 (Meiji camera, USA). For slices stained with Masson tricromic, two areas of the cortical region of the defect were selected and named C1 and C2, corresponding to the superior and the inferior cortical area of the defect. The neoformed bone tissue presented in these regions was measured and the area registered at a magnification of 10×. After the registration, the areas were added, resulting in the total bone area of the defect. This analysis was established in a previous study conducted by our team [18].

2.7 Statistical analysis

The normality of all variables' distribution was verified using Shapiro–Wilk's W test. For the variable that exhibited normal distribution, comparisons among the groups were made using one-way analysis of variance (ANOVA), complemented by Tukey HSD post-test analysis. Kruskal–Wallis test were performed for morphometric assessment. STATISTICA version 7.0 (data analysis software system—StatSoft Inc.) was used to carry out the statistics analysis. Values of $P < 0.05$ were considered statistically significant.

3 Results

3.1 General findings

Neither postoperative complications nor behavioural changes were observed. The rats returned rapidly to their normal diet and showed no loss of weight during the experimentation. None of the animals died during the experiment.

3.2 Biomechanical analysis

Table 1 shows the means and SD of the biomechanical test of all groups. Statistical analysis showed that the structural stiffness of the group CG and BG was higher than the values found in the groups BG60 and BG120. No other difference was found in the variables Energy Absorption and Maximal Load.

3.3 Histopathological analysis

Regarding the control group, all the defects were composed of woven bone inside the bone defect after 14 days (Fig. 1a). Additionally, the defects were filled with fibrous

Table 1 Biomechanical properties

Group	Maximal load (KN)	Energy absorption (J)	Structural stiffness (N mm^{-1})
CG	0.064 ± 0.020[a]	0.029 ± 0.012	172.33 ± 54.83
BG	0.063 ± 0.014	0.033 ± 0.010	157.31 ± 33.54
BG60	0.061 ± 0.015	0.038 ± 0.016	115.59 ± 28.68*
BG120	0.049 ± 0.014	0.028 ± 0.007	110.20 ± 28.68*

CG fracture control group, *BG* Biosilicate group, *BG60* Biosilicate + laser 60 J cm^{-2}, *BG120* Biosilicate + laser 120 J cm^{-2}

* $P < 0.05$ (all groups versus CG and GB)

[a] Data are expressed as Mean + SD

connective tissue and some bone fragments possibly due to the surgical procedures (Fig. 1a). No inflammatory process was noticed in any specimens of this group, because no acute inflammatory cells were present. In specimens treated with Biosilicate® the bone defect was predominantly filled with the biomaterial (Fig. 1b). However, woven bone was in apposition to the surface of the biomaterial in some cases (Fig. 1b). In addition, granulation tissue was present in circumjacent areas to the wall of bone defect. Regarding the laser 60 J cm^{-2} and Biosilicate® group, the same picture occurred, i.e. there was the Biomaterial filled all bone defects containing woven bone and granulation tissue (Fig. 1c). In the group exposed to Laser 120 J cm^{-2} and Biosilicate®, similar findings are noticed as well. Such findings are displayed in the Fig. 1d.

3.4 Morphometry

The results showed no statistically significant differences ($P > 0.05$) between the control group and the Biosilcate® group after 14 days. Interestingly, the groups treated with Biosilicate® and laser, at the two dosages, showed significant statistical lower values of newly formed bone in the area of the defect when compared to negative control and BG ($P < 0.05$). Such findings are shown in Fig. 2.

4 Discussion

Since previous in vitro and in vivo studies have demonstrated the high osteogenic potential of Biosilicate® [7, 19], we hypothesized that the effect of this biomaterial could be improved by laser irradiation. However, results from the present work showed that bones which had the surgically created defects filled with Biosilicate® and exposed to laser irradiation presented a significant decrease in the amount of newly formed bone and in the biomechanical properties when compared to the Biosilicate® group and the control group.

Under histopathological examination, the rats exposed to Biosilicate® showed that bone defect was predominantly filled with biomaterial. However, woven bone was in apposition to the surface of the biomaterial in some cases. In specimens treated with Biosilicate® and Laser, the same picture occurred, i.e. there was the presence of biomaterial

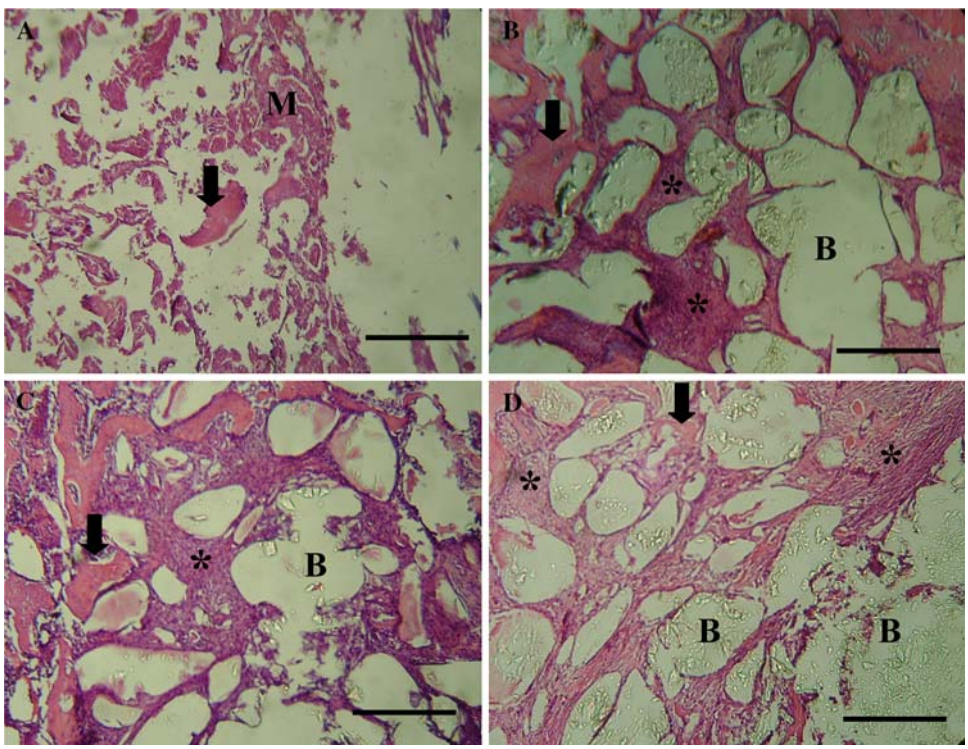

Fig. 1 Bone defects from control group (**a**) displaying medullar region (M) and bone fragments (*arrow*) inside the defect; **b** Biosilicate® group showing the presence of biomaterial (B), woven bone (*arrow*) and granulation tissue (*asterisk*); **c**, **d** Biosilicate® and Laser 60 and 120 J cm^{-2}, respectively displaying similar findings as those described by the Biosilicate® group only. H.E. stain, *Bar* = 36 μm

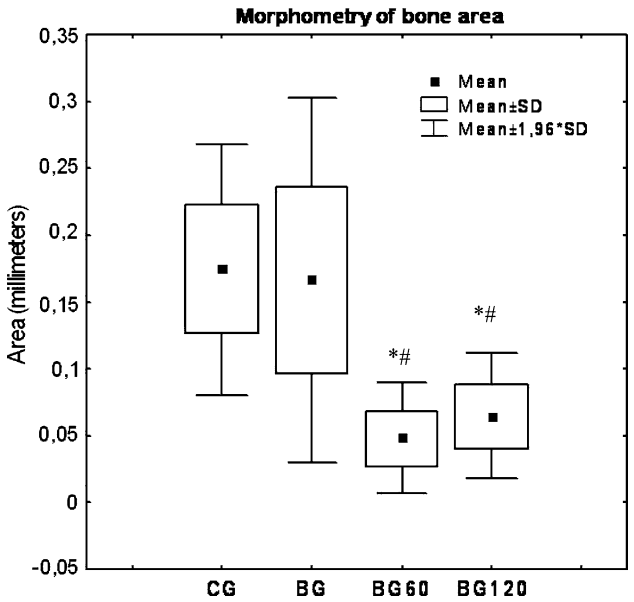

Fig. 2 Morphometric assessment of neoformed bone in the defect area. Results are expressed as Mean ± S.D. * $P < 0.05$ (all groups versus CG); # $P < 0.05$ (all groups versus BG). *CG* fracture control, *BG* Biosilicate, *BG60* Biosilicate + laser 60 J cm^{-2}, *BG120* Biosilicate + laser 120 J cm^{-2}. Areas are expressed in millimeters

filled inside the defect with granulation tissue and woven bone in circumjacent areas. This is new in biomaterials science and the mechanism involved to this process is unknown. By comparison, some authors have provided evidence that some bioactive glass particles induces new bone formation, and the degree of impairment resulted from a combination of factors such as type of material and phase of the reparation process [20]. Furthermore, others have revealed that full crystallization of Biosilicate® in a range of compositions of the system P(2)O(5)–Na(2)O–CaO–SiO(2) promoted enhancement of in vitro bone-like tissue formation in an osteogenic cell culture system [7]. Taken together, it seems that Biosilicate® is able to induce bone formation after 14 days of exposure. Nevertheless, laser therapy decreased bone formation exposed to Biosilicate®.

Also, Biosilicate® did not improve the biomechanical properties of the fracture callus. Bone mass, as well as, the quality and arrangement of its microstructural elements, influences bone mechanical properties [21]. Therefore, the lack of the improved load-bearing capacity and stiffness showed by the biomaterial treated group probably mirror the lack of difference in the amount and/or spatial distribution of newly formed bone into the defect site among the groups. Interestingly, the association of the biomaterial and the laser at the higher dosage produced a decrease in structural stiffness. It is unclear at this stage why these results have occurred however, as the Biosilicate® is composed of a glass–ceramic material, it may be influenced by the focal intensity and energy output of the laser light during the treatment duration. Such an effect may subsequently inhibit cellular migration and growth on the surface of the glass–ceramic composite.

These results are in agreement with the results found by our group in an in vitro study, investigating the effects of laser phototherapy at 830 nm (continuous; 10 J cm^{-2}) on osteoblast cell proliferation cultured on Biosilicate® scaffolds. We have demonstrated that osteoblastic MC3T3 cells were successfully grown on scaffolds composed by Biosilicate®, with cells presenting normal osteoblastic morphology and adhered, proliferated and migrated readily across disc surfaces. In contrast, laser irradiation at 830 nm produced a 13% decrease in osteoblast (MC3T3) proliferation on Biosilicate® glass–ceramic discs [22].

Some authors suggest that LLLT creates a number of environmental conditions that accelerate the healing of bone fractures and defects [23]. However, the exact mechanism of action is not fully understood. Probably, laser can stimulate mesenchymal cells or direct stimulation of osteoblasts, increasing bone mass deposition and secretion of components of the matrix [23, 24].

Despite the stimulatory effects of LLLT and biomaterials on the biostimulation of bone repair, there are few previous reports on the association of LLLT and implanted biomaterials [24]. Data in the literature showed that LLLT could result in an increase of hard tissue in new bone formation around hydroxyapatite implants in the bone [25]. Also, Gerbi et al. [26], investigated the influence of LLLT (4 J cm^{-2}, 40 mW, every 48 h for 15 days) on bone defect grafted with inorganic bovine material and observed that the repair of the irradiated bone was characterized by both increased bone formation and the amount of collagen fibers around the graft within the cavity.

However, it is still difficult to compare the studies on the action of LLLT on bone and implanted biomaterials because the experimental models, the materials used and duration of treatments are very distinct. In this context, clinical LLLT in the osseointegration of biomaterials cannot, as yet, be applied efficiently, as the mechanisms of action on bone have not been fully elucidated.

In summary, such findings suggest that although Biosilicate® exerts some osteogenic activity during bone repair, laser therapy is not able to modulate this process, at this stage. It seems that laser technology represents perhaps one of the most promising treatment modalities to improve biomaterials by enhancing osteoblast adhesion and vessel migration towards the surface, and to prepare an adequate implant site to reduce tissue damage [27, 28]. Considering this fact, further long-term studies should be developed to provide additional information concerning the late stages of the bone matrix synthesis and degradation induced by Biosilicate® and laser. These additional investigations should focus on the final aim of the induced-regeneration

of bone, which is the ability to restore the bone architecture with biological and mechanical properties similar to the uninjured one.

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