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Abstract. We investigate the effects of a novel bioactive material (Biosilicate®) and low-level laser therapy (LLLT), at 60 J/cm^2, on bone-fracture consolidation in osteoporotic rats. Forty female Wistar rats are submitted to the ovariectomy, to induce osteopenia. Eight weeks after the ovariectomy, the animals are randomly divided into four groups, with 10 animals each: bone defect control group; bone defect filled with Biosilicate group; bone defect irradiated with laser at 60 J/cm^2 group; bone defect filled with Biosilicate and irradiated with LLLT, at 60 J/cm^2 group. Laser irradiation is initiated immediately after surgery and performed every 48 h for 14 days. Histopathological analysis points out that bone defects are predominantly filled with the biomaterial in specimens treated with Biosilicate. In the 60-J/cm^2 laser plus Biosilicate group, the biomaterial fills all bone defects, which also contained woven bone and granulation tissue. Also, the biomechanical properties are increased in the animals treated with Biosilicate associated to lasertherapy. Our results indicate that laser therapy improves bone repair process in contact with Biosilicate as a result of increasing bone formation as well as indentation biomechanical properties. [DOI: 10.1117/1.3598847]

Keywords: low-level lasertherapy; biomaterials; osteoporosis; bone healing; biomechanical properties.

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1 Introduction

Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural disturbance in bone tissue, with a consequent increase in bone fragility and susceptibility to fracture.[1] Altered bone microarchitecture and diminished bone mineral density ultimately lead to greater bone fragility and increased susceptibility to pathologic fracture.[2] In the United States, there are approximately 7×10^5 osteoporotic vertebral compression fractures annually.[1]

In this context, there is a critical need to develop technologies for treating osteoporotic fractures.[3] One promising treatment is the use of bioglasses and polymers, which seem to induce osteogenesis and stimulate fracture healing.[4,5] To date, a wide variety of biodegradable polymers, bioactive glasses, and glass ceramics have been used as a graft in the treatment of large bone defects,[6] mainly to their facility to adapt to the shape of the defects, their potential to stimulate osteogenesis, and their capability to influence bone bonding.[7] Recently, our research group has developed a novel fully crystallized bioactive glass ceramic of the quaternary P_2O_5–Na_2O–CaO–SiO_2 system (Biosilicate®, patent pending[8]). This biomaterial has shown a stimulatory effect on osteoblast cell metabolism[9,10] observed, in an *in vivo* study, that Biosilicate produced a higher amount of neoformed bone in tibial bone defects in rats when compared to Bioglass 45S5 treated animals.

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.[11,12] *In vitro* studies using osteoblastic cells showed that LLLT is capable of increasing mitochondrial activity,[13] bone nodule formation,[14] osteocalcin and osteopontin gene expression, and alkaline phosphate (ALP) activity.[12,15] Also, the LLLT has demonstrated of the ability to accelerate the process of fracture repair in rabbits and rats, increasing the callus volume and bone mineral density.[16] However, little attention has been given to the effect of LLLT on osteoporotic animals.[17] Our group showed that LLLT had a positive effect on osteogenesis in osteopenic rats, increasing femora strength, calcium content, and bone density.[18] Moreover, Diniz *et al*. demonstrated that the association of bisphosphonate and LLLT can also increase the trabecular bone volume in vertebra in the osteopenic control group.[19]

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Although the positive effects of Biosilicate and the LLLT on bone cell proliferation have been shown, their effects on the process of bone consolidation in osteoporotic animals were not studied yet. Before both therapies can be used with confidence as a therapeutic modality, it is necessary to investigate the effects and dose-response characteristics of these treatments in studies *in vivo* to determine its safety and efficacy. We hypothesized that rats with established osteopenia would be responsible for a delay in bone healing response and in the formation of the callus, leading to a decrease in the bone biomechanical properties. Then, we expected that both treatments may accelerate bone metabolism at the site of the bone defect in the osteopenic rats.

In this context, this study had the aim of investigating the effects of Biosilicate and 830 nm laser therapy, at 60 J/cm^2, on the biomechanical properties of the bone callus through the Indentation test and on the histology modifications in osteoporotic rats.

2 Material and Methods

Female Wistar rats (weighing 300 ± 20 g, 12–13 weeks, $N = 40$) were assigned randomly to one of the four groups ($n = 10$): osteoporotic control bone defect group (OC), osteoporotic bone defect treated with Biosilicate group (B), osteoporotic bone defect treated with lasertherapy, at 60 J/cm^2 (L60), and osteoporotic bone defect treated Biosilicate and irradiated with laser therapy, at 60 J/cm^2 (BL60). Animals were maintained under a controlled temperature (22 ± 2°C), light-dark periods of 12 h, and with free access to water and a 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.

Animals were submitted to the ovariectomy (OVX) to induce osteopenia.[19] This is the most common experimental model used for experimental osteoporosis research,[19] and it mimics bone loss and compromised fracture repair prevalent in postmenopausal women who are estrogen deficient and prone to osteoporotic fractures. Surgery was performed via bilateral translumbar incisions, under general anesthesia induced by intraperitoneal injection of xilazin (Syntec®, 20 mg/kg, IP) and ketamin (Agener®, at 40 mg/kg, IP). The uterine tubes were ligated (Catgut 4.0), the ovaries were removed, and the incisions were closed (Catgut 3.0). After the surgery, all animals were conditioned for a period of eight weeks for the purpose of inducing osteopenia.[20]

Eight weeks after OVX, bone defects were surgically performed on the right tibia. The animals were anesthetized with ketamine/xilazine anesthesia (80/10 mg/Kg), and the mid region of the tibias was shaved and disinfected with povidone iodin. A dermoperiostal incision was performed to expose the tibia. A 2-mm-diam cavity defect was made, using an espherical bur under copious irrigation with saline solution. A new drill was used for each animal. In the Biosilicate-treated animals, the cavities were carefully filled with the corresponding biomaterial. 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.

2.1 *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 crucible at a temperature range of 1250–1380 °C for 3 h in an electric furnace (Rapid Temp 1710 BL, CM Furnaces Inc., Bloomfield, New Jersey) at the Vitreous Materials Laboratory of the Federal University of São Carlos (São Carlos, Brazil). Samples were cast into a 10 × 30mm 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. Afterward, the nucleated samples were submitted to further treatment at ∼100 °C above the nucleation temperatures. The detailed compositions and thermal treatment schedules to obtain the Biosilicate glass ceramic are described in Ref. 8.

2.2 *Low-Level Laser Therapy*

A low-energy GaAlAs (Teralaser, DMC®, São Carlos, Brazil), 830 nm, CW, 0.028 cm^2 beam, 100 W cm^2, 60 J/cm^2 (1.7 J) with an irradiation time of 17 s was used in this study. Laser irradiation was initiated immediately after the bone-defect procedure and was performed on days 2, 4, 6, 8, 10, and 12 postsurgery. On day 14 after the injury, rats were sacrificed with an intraperitoneal injection of general anesthetic. The tibias were defleshed and removed for analysis.

2.3 *Histopathological Analysis*

For the histopathological analysis, the left tibiae were removed, fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h, 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) for the qualitative analysis. 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, granulation tissue, or even tissues undergoing hyperplastic, metaplastic, and/or dysplastic transformation were investigated per animal.

2.4 *Biomechanical Test*

The indentation test was used to measure the biomechanical properties of the right tibia [Instron® Universal Testing Machine (Instron, Canton, Massachusetts, model 4444)]. Before the test, bones were thawed at room temperature. To perform the indentation test, an indenter was used to test the mechanical properties of the bone callus. A cylindrical indenter of 2.0 mm diam was applied to the center of the bone callus on the face of the tibia at a constant displacement velocity of 1 mm/min. A 1 – N preload was applied in order to avoid specimen sliding. A special device was used to locate the tibias, prior to submitting their medial surface (repair area) to penetration. The indenter

was allowed to penetrate the cavity to a depth of 1.5 mm. From the load-penetration curve, the maximal load (*KN*) and energy absorption (*J*) were obtained to a depth of 0.5 and 1.0 mm.

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, complemented by Tukey Honestly Significantly Different (HSD) posttest analysis. Kruskal–Wallis test were performed for biomechanical analysis. STATISTICA version 7.0 (data analysis software system, StatSoft Inc., Tulsa, Oklahoma) Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 *General Findings*

Neither postoperative complications nor behavioral changes were observed in the animals. None of the animals died during the experiment, and no infection in the surgical site was observed.

3.2 *Histopathological Analysis*

Regarding the control group, all defects were composed by woven bone inside the bone defect after 14 days [Fig. 1(a)]. Additionally, the defects were filled by medullar tissue and some bone fragments possibly due to the surgical procedures [Fig. 1(a)]. No inflammatory process was noted in any of this group's specimens. In specimens treated with Biosilicate, the bone defect was predominantly filled with the biomaterial. No woven bone was noted in the majority of this group's specimens [Fig. 1(b)]. In addition, granulation tissue was present in circumjacent areas to the wall of a bone defect. In the irradiated animals, newly formed bone as well as granulation tissue can be observed [Fig. 1(c)]. Regarding the 60-J/cm^2 laser and Biosilicate group, we observed the presence of the biomaterial filling all bone defects, associated with the presence of woven bone and granulation tissue [Fig. 1(d)]. Overall, our results indicate that the association of 60-J/cm^2 laser therapy and Biosilicate improves the bone repair process in osteoporotic rats at 14 days of surgery by means of subjective morphological analysis.

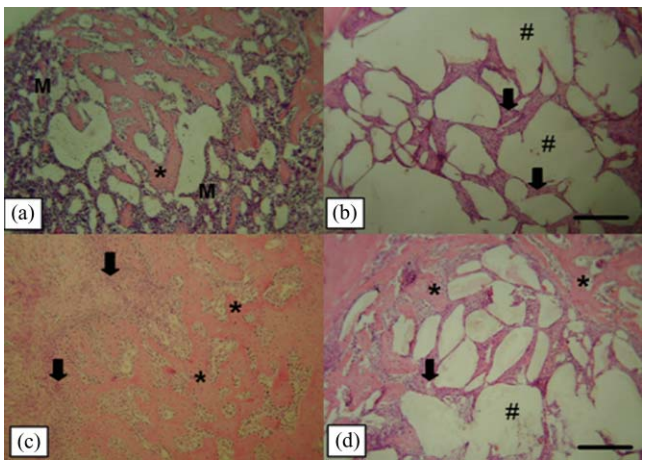

Fig. 1 Bone defects from control group (a) displaying high cellularized woven bone inside the defect (asterisk) and medullar region (M); (b) Biosilicate group showing granulation tissue (arrow); (c) Laser 60 J/cm^2 containing formed bone (asterisk), granulation tissue (arrow); and (d) Biosilicate + laser 60 J/cm^2 showing woven bone (asterisk), biomaterial (#), and granulation tissue (arrow). H.E. stain, Bar = 36 mm.

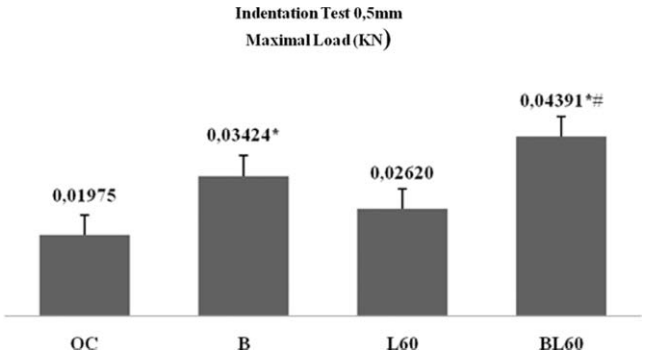

Fig. 2 Changes in the maximal load of the indentation test 0.5 mm depth $p < 0.05$, * versus group OC, # versus group L60.

3.3 *Indentation Test*

Figure 2 shows the values found in the evaluation of the maximal load at 0.5 mm of the four groups. It can be observed that control group showed statistically significant lower values compared to the Biosilicate and Biosilicate-irradiated animals. No difference was found between control group and laser-irradiated group. Also, the values for the BL60 were significantly higher compared to L60.

Energy absorption at 0.5 mm depth of the control group was statistically significant lower compared to BL60 animals. No other difference was found among control, Biosilicate- and laser-treated animals. Also, BL60 animals demonstrated significant higher values compared to L60 (Fig. 3).

Indentation's test at 1.0 mm depth showed that B and BL60 groups demonstrated significant higher values when compared to the control and irradiated animals. No other difference was found (Fig. 4).

Figure 5 shows the values of the energy of absorption at 1.0 mm found in the indentation test for all experimental groups. Animals treated with Biosilicate and Biosilicate and laser showed the higher values compared to the control. Also, BL60 group showed statistically significant higher values compared to the L60 group.

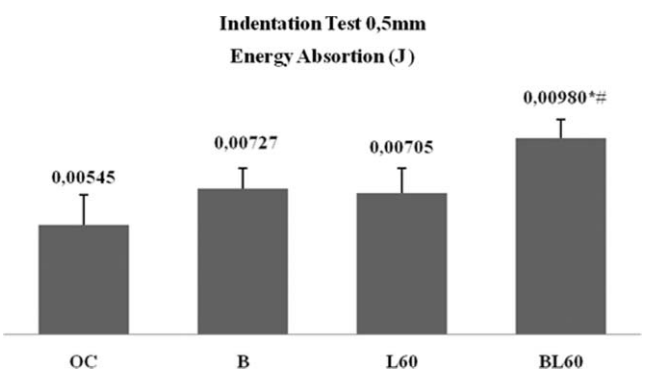

Fig. 3 Changes in the energy's absorption from indentation test 0.5 mm $p < 0.05$, * versus group OC, # versis group L60.

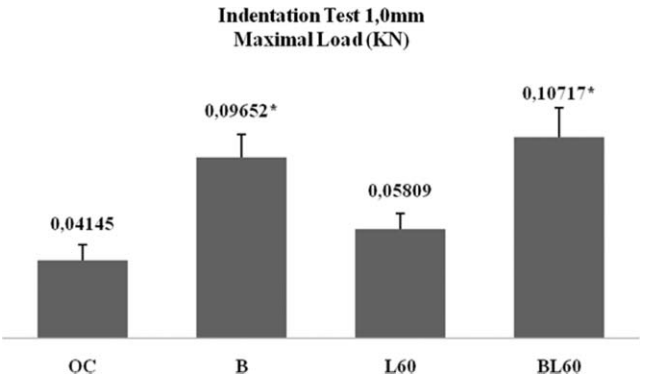

Fig. 4 Changes in the maximal load from indentation test 1.0 mm $p < 0.05$, * versus group OC.

4 Discussion

The goal of this study was to investigate the histopathological and biomechanical changes after LLLT, at 60 J/cm^2 irradiation and Biosilicate on bone healing in tibias of osteopenic rats. We observed that 14 days after the surgery the bone defects treated with Biosilicate presented in most species, woven bone in apposition to the surface of the biomaterial. Laserterapy, at 60 J/cm^2, produced a good deal of newly formed bone and granulation tissue. Interestingly, the group exposed to laser at 60 J/cm^2 and Biosilicate, showed a higher amount of newly formed bone. Also, it was observed an increase in the indentation biomechanical properties in the animals treated only with Biosilicate and the ones treated with the biomaterial and irradiated with laser compared to other groups. Also, it was observed that LLLT was not able of improving the values of the energy of absorption and maximal load.

The osteogenic effects of the Biosilicate was demonstrated in an *in vitro* study that showed that this new biomaterial supported significantly larger areas of calcified matrix at day 17 postseeding in osteoblasts cells.[9] Moreover, in an *in vivo* study, we showed that Biosilicate was efficient to induce bone formation and to increase the biomechanical properties of fracture callus 20 days after a surgery that induced tibial bone defects.[10]

In this study, LLLT had a positive effect on bone mass deposition but did not interfere in biomechanical properties. Low-level laser therapy is a promising noninvasive method for stimulating osteogenesis and reducing the time of fracture consolidation through bioenergetic, bioelectrical, biochemical, and biostimulatory effects on cells.[11,12] It seems that this therapy is capable of stimulating ostoblast proliferation and osteocalcin and osteopontin gene expression, which reflects in the osteoblastic activity.[16] Also, LLLT has been effective in accelerating fracture consolidation.[11,16]

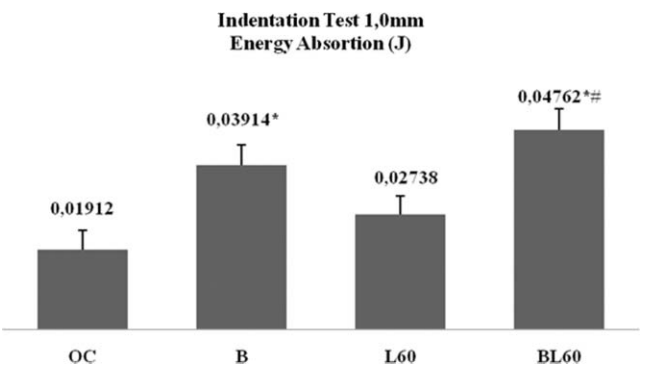

Fig. 5 Changes in the energy's absorption from indentation 1.0 mm $p < 0.05$, * versus group OC, # versus group L60.

In addition, we investigated the effects of the association of lasertherapy, at 60 J/cm^2 with Biosilicate on bone metabolism. Our results clearly demonstrated that both treatments together resulted in induction of bone formation at 14 days after surgery. 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.[21] 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.[21] Also, Gerbi *et al*. investigated the influence of LLLT (4 J/cm2, 40 mW, every 48 h for 15 days) on a 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.[22]

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, because the mechanisms of action on bone have not been fully elucidated.

We consider the methodology used in this work very appropriate. The ovariectomy is a reliable and widely used experimental model to induce osteopenia in rats, conducting a decrease of bone mass and an increase of bone fragility.[23] Also, the indentation test has been widely used in the literature with the aim of measuring the biomechanical properties of bone in different experimental conditions, including fracture bone consolidation.[11] Also, filling limited bone defects with particulate biomaterials was an efficient model. The difficulty of placing and retaining the biomaterial granules in the defect site was overcome by the cohesive mass formed when Biosilicate particles were placed in bleeding sites. Consequently, the particles could easily pack into the bone defect site and stay in place. The same phenomenon was reported by Oonishi *et al*. using Bioglass 45S5 particles to fill noncritical bone defects in femoral condyles of rabbits.[24] According to Oonishi *et al*., the cohesive mass is formed because rapid reactions on the material surface lead to the formation of a gel layer and, consequently, sufficient hemostasis is reached.

A limitation of our work should be pointed out. We investigated only one period postsurgery, which corresponded to an intermediary time of bone repair in the experimental model used. The development of experiments investigating the different bone tissue responses in earlier and later periods of bone consolidation after laser treatment and in the presence of Biosilicate seems very interesting.

In spite of this limitation, the results of this work highlight the stimulatory effects of both laser therapy and Biosilicate on bone healing. Such findings would allow us to obtain preliminary data on the potential safety and efficacy of both therapies as effective treatments for bone injuries. Our preliminary studies of exploring the effects of LLLT and Biosilicate on bone defects

in osteoporotic rats would also allow us to design future research strategies using human experiments.

In summary, our findings indicate that low-level laser therapy, at the fluence of 60 J/cm^2, associated to the Biosilicate, improved bone healing in a tibial defect of osteoporotic rats (as a result of an higher deposition of new bone tissue and a significant increase in biomechanical properties). Although further long-term studies and clinical trials are required, the findings of this study point to a promising utilization of such therapeutic modalities for tissue repair.

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