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Biosilicate® and low-level laser therapy improve bone repair in osteoporotic rats

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Abstract

The aim of this study was to investigate the effects of a novel bioactive material (Biosilicate®) and low-level laser therapy (LLLT) on bone fracture consolidation in osteoporotic rats. Forty female Wistar rats were submitted to ovariectomy (OVX) to induce osteopenia. Eight weeks after surgery, the animals were randomly divided into four groups of 10 animals each: a bone defect control group (CG); a bone defect filled with Biosilicate group (BG); a bone defect filled with Biosilicate and irradiated with LLLT at 60 J/cm^2 group (BG60); and a bone defect filled with Biosilicate and irradiated with LLLT at 120 J/cm^2 group (BG120). Bone defects were surgically performed on both tibias. The size of particle used for Biosilicate was 180–212 μm. Histopathological analysis showed that bone defects were predominantly filled with the biomaterial in specimens treated with Biosilicate. LLLT with either 60 or 120 J/cm^2 was able to increase collagen, Cbfa-1, VGEF and COX-2 expression in the circumjacent cells of the biomaterial. A morphometric analysis revealed that the Biosilicate + laser groups showed a higher amount of newly formed bone. Our results indicate that laser therapy improves bone repair process in contact with Biosilicate as a result of increasing bone formation, as well as COX-2 and Cbfa-1 immunoexpression, angiogenesis and collagen deposition in osteoporotic rats.

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Keywords **Biosilicate®; low level laser therapy; bone repair; osteoporotic rats**

1. Introduction

Osteoporosis is defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (Anonymous, 1993). The decreased bone mass and bone mineral density due to osteoporosis probably lead to a delay in fracture healing rates and bone repair quality (Hollinger *et al*., 2008). Many authors suggest that, in osteoporotic people, there is a decreased proliferative activity of osteoblast progenitor cells and gene expression, an impairment in osteoblast function, a diminished osteoblast response to signalling and an imbalance between the coupling of bone formation and resorption (Kubo *et al*., 1999; Meyer *et al*., 2001). Consequently, bone healing in osteoporotic individuals may be delayed and new bone quality may be poor (Hollinger *et al*., 2008).

In this context, there is a critical need to develop technologies capable of treating osteoporotic fractures (Gauthier *et al*., 2005). One promising treatment is the use of bioglasses and polymers, which seem to induce osteogenesis and stimulate fracture healing (Clupper *et al*., 2003; Thomas *et al*., 2005). To date, a wide variety of biodegradable polymers, bioactive glasses and glass–ceramics have been used as grafts in the treatment of large bone defects, mainly due to their facility to adapt to defect shape, their potential to stimulate osteogenesis

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and their capability to influence bone bonding (Lu *et al.*, 2008). However, the success of the biomaterial implant and the improvement of the fracture consolidation are dependent on many characteristics of the material, such as composition, solubility and particle size (Vogell *et al.*, 2001).

One of the most common and studied bioactive glasses is Bioglass® 45S5, which has been reported as the bioactive material with the highest bioactivity index (Clupper *et al.*, 2003). 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 Application WO 2004/074 199; Fundação Universidade Federal de São Carlos, 2004) with improved properties. Biosilicate has showed a stimulatory effect on bone cell metabolism (Moura *et al.*, 2007). By comparing the growth of osteogenic cells on Biosilicate and Bioglass® 45S5 disks for a period of up to 17 days, Moura *et al.* (2007) found that although no significant differences were detected in terms of protein content and alkaline phosphatase activity at days 11 and 17, Biosilicate supported significantly larger areas of calcified matrix at day 17. The results indicate that full crystallization of some bioactive glasses in a range of compositions of the system P_2O_5–Na_2O–CaO–SiO_2 may promote enhancement of *in vitro* bone-like tissue formation in an osteogenic cell culture system.

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 fracture consolidation (Luger *et al.*, 1998; Ozawa *et al.*, 1998; Renno *et al.*, 2007). 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 (Renno *et al.*, 2009). These effects are known to increase the synthesis of DNA, RNA and cell-cycle regulatory proteins, therefore promoting cell proliferation (Renno *et al.*, 2007). In *vitro* studies using osteoblastic cells showed that LLLT is capable of increasing mitochondrial activity (Renno *et al.*, 2009), osteoblast DNA and RNA synthesis, bone nodule formation (Trelles and Mayayo 1987), osteocalcin and osteopontin gene expression and ALP activity (Oliveira *et al.*, 2007). Also, the LLLT has demonstrated to be able of accelerating the process of fracture repair in rabbits and rats, increasing the callus volume and bone mineral density (Stein *et al.*, 2005). However, little attention has been given to the effect of LLLT on bone with osteopenia or osteoporosis. Our group showed that LLLT had a positive effect on osteogenesis in rats (Matsumoto *et al.*, 2009). Moreover, Renno *et al.* (2006) demonstrated that the association of bisphosphonate and laser can also increase the trabecular bone volume in vertebraa in the osteopenic control group. It would be useful to know whether, and to what extent, the association between Biosilicate and laser therapy is able to improve bone repair in osteroporotic rats, particularly because there are no previous reports. In this context, the aim of this study was to investigate the effects of Biosilicate and laser therapy on bone fracture consolidation in osteoporotic rats.

2. Material and methods

This study was conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the Federal University of São Carlos (08/2098). The animals were maintained at 19–23 °C on a 12 : 12 h light–dark cycle in the Animal Experimentation Laboratory of the Federal University of São Carlos. They were housed in plastic cages and had free access to water and standard food.

A total of forty female Wistar rats (age 12 weeks, weight ±250 g) were submitted to ovariectomy (OVX) to induce osteopenia. This model is widely used as an experimental model of animal osteopenia and it is known to significantly decrease bone mass 8 weeks post-surgery (Kalu, 1991). Surgery was performed via bilateral translumbar incisions, under ketamine/xilazine anaesthesia (80/10 mg/kg). The uterine tubes were ligated (catgut, 4.0) and after removal of the ovaries the incisions were closed (catgut, 3.0). Eight weeks after the OVX, the animals were randomly divided into four groups of 10 animals each: a control bone defect group (CG) – the bone defects without any fillers; a Biosilicate group (BG) – the bone defects filled with Biosilicate; a Biosilicate group irradiated with LLLT at 60 J/cm^2 (BG60); and a Biosilicate group irradiated with LLLT at 120 J/cm^2 (BG120).

Bone defects were surgically performed on both tibias. The defect depth was guided until the rupture of cortical bone. The animals were anaesthetized with ketamine/xilazine (80/10 mg/g) and the mid-regions of the tibias were shaved and disinfected with povidone iodine. A dermo-periosteal incision was performed to expose the tibia. A 2 mm diameter cavity defect was made, using a spherical bur under copious irrigation with saline solution. 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 iodine. 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 the Biosilicate® parent glass. The chemicals were weighed and mixed for 30 min in a polyethylene bottle. Premixed batches were melted in a platinum crucible at a temperature range of 1250–1380 °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,

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SP, Brazil). Samples were cast into a 10 × 30 mm cylindrical graphite mould and annealed at 460 °C for 5 h. To obtain the fully crystallized Biosilicate glass–ceramic, 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 at 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 2004/074 199 (Fundação Universidade Federal de São Carlos, 2004). The biomaterial is presented as a particulate. The size of particles used was 180–212 μm.

2.2. 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, at 60 and 120 J/cm^2, with irradiation times of 17 and 34 s, were used in this study. Laser irradiation was initiated immediately after the osteotomy procedure and it was performed on days 2, 4, 6, 8, 10 and 12 post-surgery. On day 14 post-osteotomy, the rats were sacrificed with an intraperitoneal injection of general anaesthetic. The tibias were defleshed and the soft tissues were removed for analysis.

2.3. Histopathological analysis

For the histopathological analysis, the right tibiae were removed and fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 48 h, decalcified in 4% EDTA (Merck) and embedded in paraffin blocks. 5 μm serial sections were cut and stained with haematoxylin and eosin (H&E Merck).

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

2.4. Immunohistochemistry

Paraffin was removed with xylene from serial sections of •4 μm and the sections were rehydrated in a graded series of ethanols, then pretreated in a microwave with 0.01 M citric acid buffer, pH 6, for three cycles of 5 min each at 850 W for antigen retrieval. The material was pre-incubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) solution for 5 min for inactivation of endogenous peroxidase, and then blocked with 5% normal goat serum in PBS solution for 10 min. The specimens were then incubated with anti-Runx2 polyclonal primary antibody (Santa Cruz Biotechnology, USA) at a concentration of 1 : 200, anti-COX-2 polyclonal primary antibody (Santa Cruz Biotechnology) or anti-VEGF-2 monoclonal primary antibody (Santa Cruz Biotechnology) at a concentration of 1 : 200. Incubation was carried out overnight at 4 °C within the refrigerator. This was followed by two washes in PBS for 10 min. The sections were then incubated with biotin-conjugated secondary antibody anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1 : 200 in PBS for 1 h. The sections were washed twice with PBS, followed by the application of preformed avidin–biotin complex conjugated to peroxidase (Vector Laboratories) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3,3′-diaminobenzidine solution and counterstained with Harris haematoxylin. For control studies of the antibodies, the serial sections were treated with rabbit IgG (Vector Laboratories) at a concentration of 1 : 200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining batch.

2.5. Morphometric assessment

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 an experienced pathologist, using image analysis system Motican 5.0. Sections stained with Masson trichrome were observed; three areas of the cortical region of the defect were selected and named C1, C2 and C3, corresponding to the superior, inferior and central cortical areas 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 (Matsumoto *et al*., 2009).

2.6. Picrosirius polarization method

Histological sections stained by the Picrosirius polarization method were viewed under polarized light (Garavello-Freitas *et al*., 2009) to assess the structural changes in the neoforming trabecular matrix. This method allows an indirect evaluation of the stage of bone matrix organization, based on the birefringence of the collagen fibre bundles after staining with Picrosirius.

2.7. Biomechanical analysis

Biomechanical properties of the left tibia were determined by a three-point bending test with a 1 kN •load (• • • • •,

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USA, 4444 model, 1 KN load cell). Tibiae were placed on a 3.8 cm metal device, which provided a 1.8 cm distance between the two supports. The load cell was perpendicularly positioned in the anterior–posterior direction at the exact site of the bone defect. A 5 N preload was applied in order to avoid specimen sliding. Finally, the bending force was applied at a constant deformation rate of 0.5 cm/min until fracture occurred. Thus, the maximum load (N) was obtained.

2.8. 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. The Kruskal–Wallis test was performed for morphometric assessment. STATISTICA version 7.0 (data analysis software system, StatSoft Inc.) was used to carry out the statistical analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1. General findings

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

3.2. Histopathological analysis

Regarding the control group, all the defects were composed by woven bone inside the bone defect after 14 days (Figure 1A). Additionally, the defects were filled by medullar tissue and some bone fragments, possibly due to the surgical procedures (Figure 1A). No inflammatory process was noticed in any of the 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. No woven bone was noticed in the majority of specimens for this group (Figure 1B). In addition, granulation tissue was present in circumjacent areas to the wall of bone defect. 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 (Figure 1C). In the group exposed to laser 120 J/cm^2 and Biosilicate, a more pronounced effect was evidenced, in which woven bone was in apposition to the surface of the biomaterial in the majority of cases; granulation tissue was present as well (Figure 1D). Overall, our results indicate, by means of subjective morphological analysis, that laser therapy improves the bone repair process in contact with Biosilicate in osteoporotic rats, particularly exposure to the 120 J/cm^2 laser at 14 days after surgery.

3.3. Immunohistochemistry

COX-2 expression was detected predominantly in the cytoplasm. After 14 days of the surgery, COX-2 immunoreactivity could be seen in medullar tissue for the control

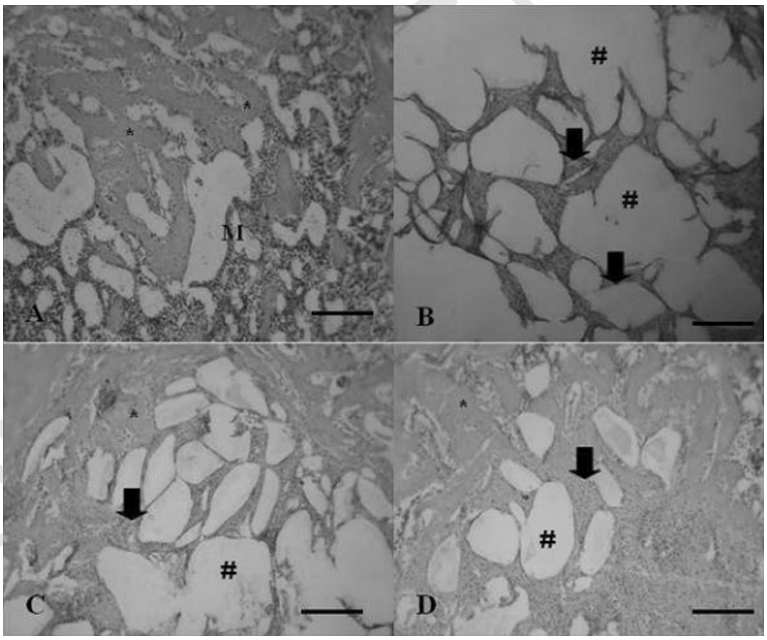

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Figure 1. Bone● defects from control group (A) displaying high celullarized woven bone inside the defect (*) and the medullar region (M). (B) Biosilicate group, showing biomaterial (#) and granulation tissue (arrow). (C) ●G2–Biosilicate + laser 60 J/cm^2 containing formed bone (*), granulation tissue (arrow) and the presence of biomaterial (#). (D) Biosilicate + laser 120 J/cm^2 showing woven bone (*), biomaterial (#) and granulation tissue (arrow). H&E stain; bar = 48 μm

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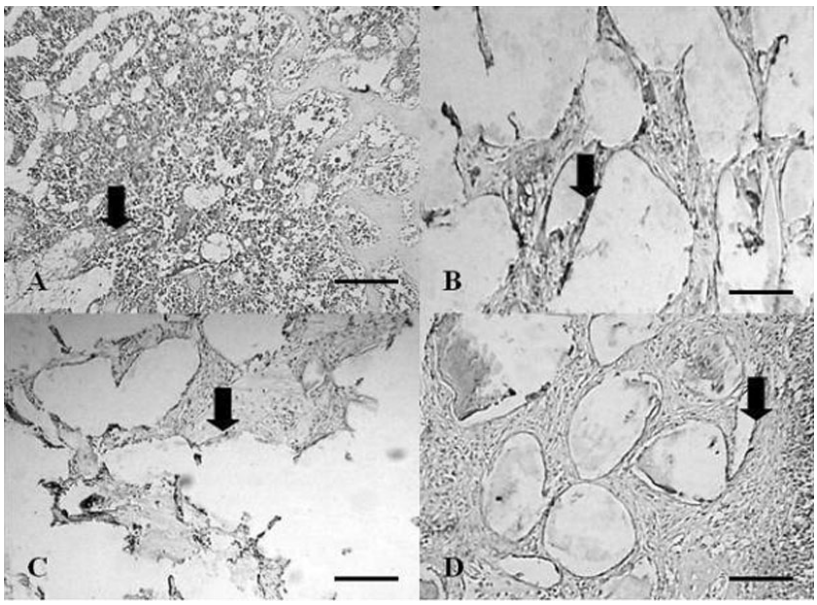

Figure 2. Immunohistochemistry for COX-2. (A) immunoexpression predominantly in the medullar tissue in the control group (arrow). (B) In group treated with Biosilicate, immunopositive cells were detected in contact with biomaterial (arrow); (C and D) in groups treated with laser, a strong immunoexpression was noticed to circumjacent cells or granulation tissue (arrow) at 14 days (B) after surgery. Immunohistochemistry stain; bar = 30 μm

group (Figure 2A). In the group exposed to Biosilicate, COX-2 immunoexpressivity was seen in cells circumjacent to the biomaterial with a weak pattern (Figure 2B). Interestingly, LLLT, either 60 or 120 J/cm^2, was able to increase COX-2 expression in the circumjacent tissue of the biomaterial as well as granulation tissue (Figure 2C, D).

Regarding Cbfa-1 immunohistochemistry, this could be detected in cells from medullar tissue in the control group (Figure 3A). Biosilicate-treated groups displayed expressivity for this immunomarker in areas circumjacent to the biomaterial (Figure 3B). In the groups treated with laser, either 60 or 120 J/cm^2, a similar pattern occurred, i.e. Cbfa-1 positive cells were noticed near to the Biosilicate and in the walls of the bone defect (Figure 3C, D, respectively).

VEGF expression was predominantly detected in cells from capillary walls, which are composed of endothelial cells; 14 days after surgery, VEGF immunoreactivity could be seen in the capillary walls for the control group

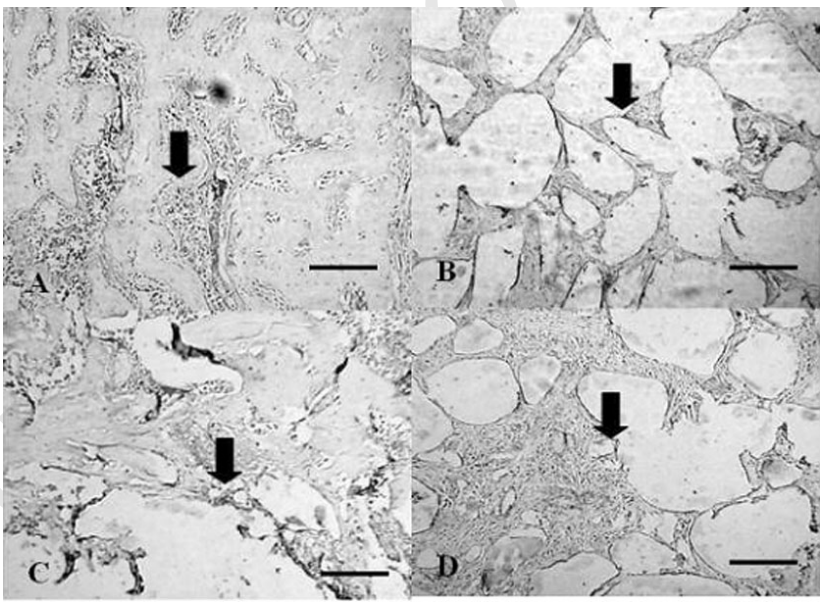

Figure 3. Immunohistochemistry for Cbfa-1. (A) Immunoexpression predominantly in the medullar tissue in the control group (arrow). (B) In group treated with Biosilicate, immunopositive cells were detected in contact with biomaterial (arrow). (C, D) The same picture occurred, i.e. positive cells were detected circumjacent to biomaterial (arrow) (C). Immunohistochemistry stain; bar = 30 μm

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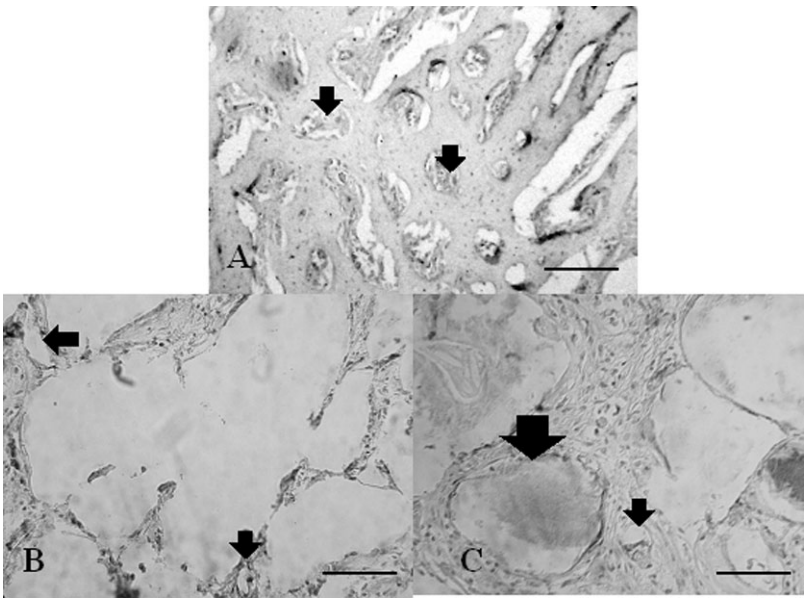

Figure 4. Immunohistochemistry for VEGF after 14 days of surgery. (A) Control group; (B) Biosilicate group; (C) Biosilicate and laser 120 J/cm^2. Arrow indicates VEGF-positive cells. Immunohistochemistry stain; bar = 56 μm

(Figure 4A). In the group exposed to Biosilicate, VEGF immunoexpressivity was seen in some capillaries present in the areas circumjacent to the biomaterial (Figure 4B). In groups treated with laser + Biosilicate, either 60 or 120 J/cm^2, a similar pattern occurred, i.e. VEGF-positive cells were noticed in the capillary walls of the bone defect (Figures 4C).

3.4. Morphometry

The area of neoformed bone tissue presented by the Biosilicate group was significantly smaller than in the other groups (Figure 5). No difference was found between the bone defect control group and the Biosilicate group treated with laser at 60 J/cm^2. Interestingly, the animals treated with Biosilicate and exposure to laser at 120 J/cm^2 showed a statistically significant increase in the area of neoformed bone, corroborating the qualitative analysis.

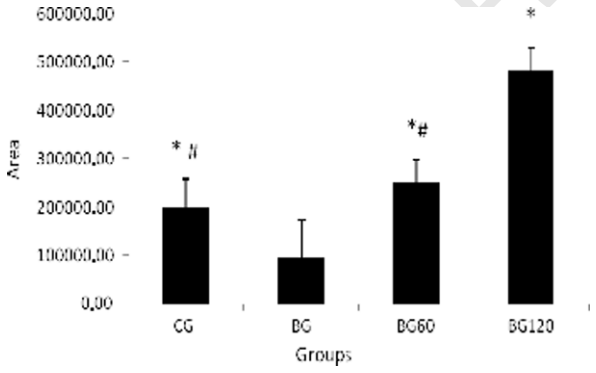

Figure 5. Area of neoformed bone in the defect area (μm^2). Results are expressed as mean ± SD. GC, fracture control; BG, Biosilicate; BG60, Biosilicate and laser 60 J/cm^2; BG120, Biosilicate and laser 120 J/cm^2. *$p < 0,05$ vs BC; $^{\#}p < 0.05$ vs BG120

3.5. Collagen assessment by Picrosirius

Figure 6 demonstrates the qualitative analysis for the collagen evaluation. It can be observed that the animals treated with Biosilicate only showed a smaller amount of collagen fibre deposition when compared to control group. Moreover, it seems that the animals exposed to laser therapy demonstrated a larger amount of collagen fibres when compared to the control. These findings corroborate those of the quantitative analysis of the collagen deposition. Figure 6 shows that the intensity of pixels demonstrated by the Biosilicate group was lower when compared to other groups. The groups treated with Biosilicate and irradiated with laser therapy showed a higher intensity of pixels, mainly at the dosage of 120 J/cm^2, indicating that these animals showed a higher amount of collagen fibre deposition (Figure 6).

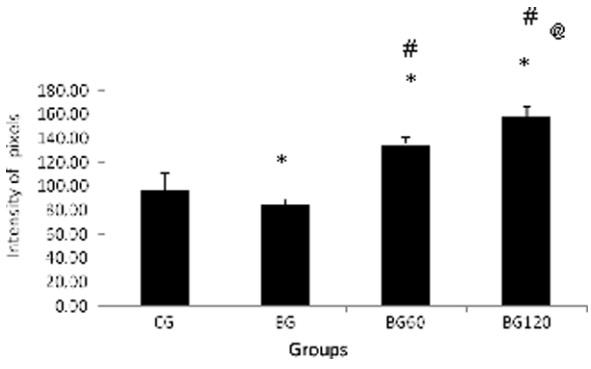

Figure 6. Mean and SD of the collagen assessment. CG, control group; BG, Biosilicate group; BG60, Biosilicate group plus laser at 60 J/cm^2; BG120, Biosilicate group plus laser at 120 J/cm^2. *vs. control; $^{\#}$vs. BG; $^{@}$vs. BG60

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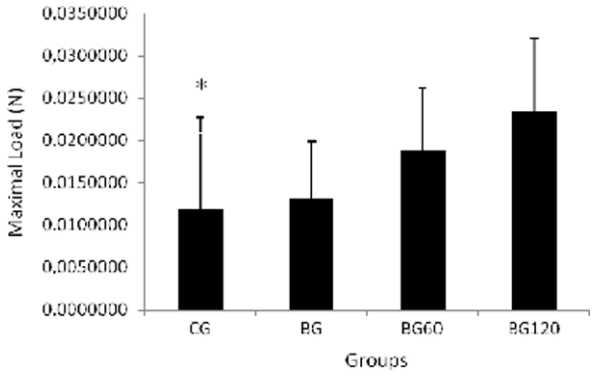

Figure 7. Biomechanical properties. CG, control group; BG, Biosilicate group; BG60, Biosilicate group plus laser at 60 J/cm^2; BG120, Biosilicate group plus laser at 120 J/cm^2. *vs. BG120, $p < 0.05$

3.6. Biomechanical analysis

Figure 7 shows the results found in the biomechanical analysis. The animals treated with Biosilicate and exposed to laser at 120 J/cm^2 presented a higher maximal load compared to the control group and the group treated with Biosilicate only. No other difference was found.

4. Discussion

Biological supplementation of traditional strategies needs to be exploited for osteoporotic and geriatric patients with poor bone density and compromised bone repair potential (Hollinger *et al*., 2008). Recently, many treatments have been proposed, such as ceramics, osteogenic bioglasses and laser therapy for recalcitrant tibial non-unions (Peter *et al*., 2006). In this context, the aim of this study was to evaluate whether concomitant administration of Biosilicate and LLLT are able to improve bone repair in osteoporotic rats. To the best of our knowledge, the approach has not been addressed previously.

Our results showed that, although the animals treated with Biosilicate presented smaller amounts of neoformed tissue, biomechanical properties and a lower amount of collagen, the qualitative histological analysis showed that, in most of the specimens of this group, the presence of woven bone in apposition to the surface of the biomaterial could be observed. This finding supports the principle of bioactive glasses or glass–ceramics, which is considered to be a rapid deposition of a layer of hydroxycarbonate apatite (HCA) on the surface of the silica gel, as a result of exchange between calcium and silica ions when bioactive glass comes into contact with living tissues (Hench and Polak, 2002). Entirely inorganic reactions, previously described by Hench and Polak (2002), lead to rapid growth of HCA bone mineral on the glass surface and to a rapid proliferation of new bone within a bone defect site, a process termed osteoproduction. It may be that the short time post-surgery used to perform the analysis was not enough to produce a proper interaction of the biomaterial isolated with bone metabolism, which avoids better responses. Probably if we extended the time of the experimental design, we could reach more positive responses. Despite these results, the osteogenic potential of Biosilicate needs to be highlighted.

This *in vivo* finding corroborates previous *in vitro* results (Moura *et al*., 2007) which showed that, although Biosilicate and Bioglass 45S5 discs supported osteogenesis, significantly larger areas of calcified matrix were detected for the fully-crystallized glass–ceramic (Biosilicate) after 17 days in an osteogenic culture system. Also, in an *in vivo* study, we showed that the Biosilicate was efficient to induce bone formation and to increase the biomechanical properties of the fracture callus, 20 days after the surgery, to induce tibial bone defects (Granito *et al*., 2009). It is worth noting that the special nucleation and growth thermal treatments developed by our research group to improve the mechanical properties of glasses were also useful for obtaining a fully crystallized glass–ceramic (Biosilicate) with the high bioactivity level and osteoproductive properties exhibited by glasses.

Interestingly, the group exposed to laser therapy (mainly at 120 J/cm^2) and Biosilicate showed a higher amount of newly formed bone when compared to the control, in which woven bone was in apposition to the surface of the biomaterial in the majority of cases. LLLT is a promising non-invasive method for stimulating osteogenesis and reducing the time of fracture consolidation through bioenergetic, bioelectrical, biochemical and biostimulatory effects on cells (Nicolau *et al*., 2003; da Silva *et al*., 2006; Ribeiro *et al*., 2008). Kazem Shakouri *et al*. (2010) showed that the use of laser therapy could enhance callus development in the early stage of the healing process in rabbits, with doubtful improvement in biomechanical properties of the healing bone; therefore, laser therapy may be recommended as an additional treatment in non-union fractures in humans. Also, LLLT has been demonstrated to stimulate fracture bone healing in osteoporotic rats (Renno *et al*., 2006).

Accumulating evidence suggests that inflammation plays an important role in connective tissue repair (Simon *et al*., 2002). Specifically, cyclo-oxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins, of which two isoforms, COX-1 and -2, have been identified during the inflammatory process. COX-1 is constitutively expressed in many tissues and mediates the synthesis of prostaglandins required for normal physiological function. COX-2 is normally undetectable in most tissues, but is rapidly induced by proinflammatory or mitogenic stimuli (Kargman *et al*., 1996). As demonstrated by Zhang *et al*. (2002), COX-2 enzyme acts on osteoblastogenesis, regulating osteoblastic differentiation genes such as *Cbfa1* and *osterix*. In this study we were able to evaluate COX-2 expression in this setting. Our results revealed that laser therapy promotes an upregulation of COX-2 expression, even when combined with Biosilicate, in osteoporotic rats. Since LLLT is able to increase DNA and RNA synthesis formation in the cell nucleus and consequently to increase

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cell proliferation and differentiation, we believe that the immunoexpression of COX-2 found in bone tissue could also be helpful for bone repair. This expectancy was confirmed in previous studies investigating the role of COX-2 during bone repair in rats (Ribeiro *et al*., 2008). In 1997, Sato *et al*. (1997) suggested that COX-2 could be involved in the early stage of osteogenesis, probably associated with the maturation of osteoblasts. More recently, it has been postulated that bone cells are able to produce COX-2 after mechanical trauma (Li *et al*., 2002), therefore being important to bone formation (Forwood, 1996). Our results agree with these previous studies.

To further elucidate the putative mechanisms of action involving Biosilicate and LLLT on bone repair in osteoporotic rats, we designed additional immunohistochemical experiments to observe the expression of Cbfa-1/RUNX2 (core binding factor protein-1), since some authors have assumed that that COX-2 enzyme acts on osteoblastogenesis, regulating osteoblastic differentiation genes such as *Cbfa-1* and *osterix* (Zhang *et al*., 2002). Interestingly, our results demonstrated a moderate Cbfa-1/RUNX2 expression in groups exposed to the biomaterial in combination or not with laser therapy. Taken as a whole, it seems that Biosilicate permits osteoblast differentiation during the process of bone repair in osteoporotic rats.

Accumulating evidence also suggests that neovascularization plays a crucial role in treating many diseases (e.g. coronary artery disease) and in virtually all approaches to tissue engineering (Boontheekul and Mooney, 2003). A lack of vascularization leads to insufficient nutrient delivery and waste removal, cell death and limited tissue development and tissue loss. Angiogenesis, new blood vessel formation by sprouting from the sides and ends of pre-existing microvascular vessels, has been widely studied to determine the rules guiding blood vessel formation in adult tissues (Boontheekul and Mooney, 2003). Numerous growth factors have so far been identified as regulators of this process. For example, vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis, as it is a potent mitogen for endothelial cells (ECs) and induces EC migration by upregulation of several endothelial integrin receptors. The VEGFs and their corresponding receptors are key regulators in a cascade of molecular and cellular events that ultimately lead to the development of the vascular system by angiogenesis (Ferrara *et al*., 2003). In the present study we were able to evaluate the VEGF expression in this setting. Our results revealed that Biosilicate was able to induce angiogenesis, as depicted by the positive immunoexpression found in this group. By comparison, Sojo *et al*. (2005) assumed that angiogenesis occurs predominantly before the onset of osteogenesis in bone lengthening in an osteodistraction model. These results are in line with our results, since positive angiogenesis was detected in early phases of bone repair after exposure to Biosilicate. Moreover, LLLT, either 60 or 120 J/cm^2, induced angiogenesis in a similar manner to the group exposed to Biosilicate only. The approach is new in the literature, and the results are difficult to discuss. Independent of its mechanism the action, we believe that the immunoexpression of VEGF found in these groups could also be helpful to bone repair in osteoporotic rats.

The Picrosirius polarization method showed that the association of Biosilicate and LLLT, mainly at a higher dosage, produced a higher concentration of collagen fibres and a better organization of the fibres, which can be attributed to the laser stimulation. Garavello-Freitas *et al*. (2009) also observed an increase of collagen deposition in bone defects irradiated with LLLT. The birefringent shades of yellow to red bands observed by these authors in the irradiated animals is indicative of type I collagen-containing collagen fibres areas of trabecular bone, which are typical of mature bone, and acquired a mature disposition in newly-formed bone matrix.

Moreover, the most used preclinical animal model used for osteoporosis research is the ovariectomized rat. This model mimics bone loss and compromised fracture repair prevalent in postmenopausal women who are oestrogen-deficient and prone to osteoporotic fractures; consequently, ovariectomized rats were used to determine the fracture-healing efficacy of the treatments LLLT and Biosilicate (Hollinger *et al*., 2003).

5. Conclusion

In summary, our findings indicate that LLLT laser therapy improves bone healing in tibial defects of osteoporotic rats (as a result of an upregulation of COX-2 expression in bone cells), even when associated with Biosilicate. Also, it is likely that Biosilicate induces osteoblastic differentiation following bone repair. These findings should be carefully addressed to elderly people suffering from osteoporosis and presenting trauma of the tibia, since they represent a new perspective for osteoporotic patients, although they do not solve the problem of general osteoporosis. Further studies would be welcome to elucidate this issue.

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