Introduction

The association of Bioglass (BG) and Magnesium (Mg) in stimulating bone tissue has been highlighted by many authors, based on the osteogenic activity of BG and the increased mechanical properties of magnesium (Mg) \(^1\). Recently, Mg has received much attention, mainly due to its similar mechanical properties to those of bone tissue \(^2, 3\). Furthermore, Mg implants would be intended to fully degrade, making it a very suitable material for orthopedic applications \(^4\). Many BG and Mg composites have been manufactured and, \textit{in vitro} cytotoxicity tests showed that their ionic products released during degradation was able of supporting the osteoblastic proliferation and differentiation \(^5\).

Although the positive effects of BG/Mg on the process of bone healing, there is a continuous search for the development of optimized therapeutic interventions to be used as bone grafts. In this context, the introduction of an organic component (such as collagen) to the composites is expected to enhance its osteoinductive and osteoconductive properties. In this line, collagen has been extensively used as an osteoconductive material due to its biological properties and bioactivity. Additionally, Collagen (Col) is a common and efficient approach for bone tissue engineering applications toward cell proliferation. Recently, studies demonstrated that BG/Col/Mg composites presented proper mechanical properties and were non-cytotoxic. Although the osteogenic potential of BG/Col/Mg composites, in specific situations, biomaterials may not be capable of stimulating bone tissue. Therefore, combining biomaterial matrices and effective post-operative therapies (such as low level laser therapy; LLLT) may be necessary to appropriately stimulate bone tissue. In this context, the aim of this study was to develop intra- and extra-operatively bone regenerative therapeutical strategies, based on the association of Col-enriched BG/Col composites with LLLT.

Materials and Methods: Thereby, an \textit{in vivo} study, using tibial defect in Wistar rats, was performed in order to investigate the bone regenerative capacity. LLLT treatment (Ga-Al-As laser 808 nm, 30 mW, 2.8 J, 94 s) was performed 3 times a week, in non-consecutive days. Histology, histomorphometry, immunohistochemical analysis and mechanical test were done after 15 and 45 days post-implantation.

Results: The results showed that Col could be successfully introduced into BG/Mg and the association of BG/Mg/Col and LLLT constituted an optimized treatment for accelerating material degradation and increasing bone deposition. Additionally, mechanical tests showed an increased maximal load for BG/Mg + LLLT compared to other groups.

Conclusions: These results lead us to conclude that the Col enriched BG/Mg composites irradiated with LLLT presented superior biological and mechanical properties, demonstrating to be a promising bone graft.

Key words: bioglass • collagen • magnesium • scaffolds • LLLT • bone repair
ites has been emerging as a promising alternative. Collagen (Col) is a common and efficient approach for bone tissue engineering applications. Many authors have evidenced that BG/Col composites are able of increasing mineralization and cell proliferation using in vitro studies. Vargas et al. (2013) demonstrated that BG/Col films are very attractive matrices for tissue engineering and regenerative medicine, especially due to its capacity to induce an earlier angiogenic response.

Therefore, an ideal bone graft would present similar composition and mechanical properties than bone tissue (which could be obtained by the association of BG/Mg and Col). In a recent study of our group, Gabbai-Armelin et al. (2017) successfully introduced Mg into BG/Col and demonstrated that the composites presented increased mechanical properties and were biocompatible and non-cytotoxic, being a promising intervention to be used for bone tissue engineering.

Although the biological osteogenic potential of Mg-enriched BG/Col composites, in specific situations, such as loss of a great amount of bone tissue or fractures related to diseases, the effects of biomaterial based grafts may be not sufficient to stimulate bone tissue. In this context, combining biomaterial matrices and post-operative therapeutic approaches (such as low level laser therapy) may be necessary to properly stimulate bone tissue.

Low level laser therapy (LLLT) is an electromagnetic energy that has been widely used to accelerate tissue repair and to modulate inflammatory processes. Due to these stimulatory effects, LLLT has been used to treat injuries in a series of tissues including bone. Many authors demonstrated that LLLT is able of up-regulating the synthesis of genes and proteins related to bone cell proliferation and differentiation, modulating the inflammatory process, stimulating angiogenesis and increasing newly formed bone deposition during the process of fracture healing.

Despite the evidenced potential of the above cited therapeutic resources, there is still limited understanding of the simultaneous use of Col-enriched BG/Mg composites with LLLT irradiation on the process of bone healing. The combination of intra with extra-operative technologies may constitute an optimized treatment for improving bone regeneration. In this context, the objective of this study is to investigate the effects of intra- and extra-operatively bone regenerative therapeutic strategies, based on the association of Col-enriched BG/Mg composites with LLLT. For this purpose, an in vivo study was conducted, comparing the bone regenerative capacity in an experimental model of tibial bone defects in rats. Histology, histomorphometry, immunohistochemical analysis and mechanical test were performed after 15 and 45 days of implantation.

### Materials and methods

#### Materials

For the obtainment of BG, mineral silica 98.0 wt% powder was purified by attack with hot hydrochloric acid (Merck, P.A.) followed by filtration (fast paper filtration Whatman 40) and held 30 washings with boiling distilled water for elimination of impurities (R2O3) and analyzed to ensure 99.5 wt% purity. Additionally, the following reagent analytical-grade was used: sodium hydroxide, calcium oxide and sodium phosphate. The chemicals were weighed and mixed for 30 min in a polyethylene bottle. Premixed batches were melted in an alumina crucible at a temperature of 1500°C (Lindberg Blue vertical super kanthal furnace - USA). The melting time was fixed as 2 h. Samples were quenched in deionized water and milled to powder grain (particle size: 125 – 250 µm).

A commercially available Mg powder (particle size of 74 to 105 µm) was purchased from Alfa Aesar (purity: 99.6%; Massachusetts, USA). Also, a commercially available collagen type I from bovine tendon was obtained from United States Biological (particle size: < 500 µm; US Biological Life Sciences, Massachusetts, USA).

Furthermore, for sample manufacturing, Poly (methyl methacrylate) (PMMA, particle size: 15 µm) and methyl methacrylate (MMA, purity: 99.09%) (VPI Dental Products Pirassununga, São Paulo, Brazil) and carboxymethyl cellulose (CMC) (Sigma Aldrich (Missouri, USA) were used. Polymer and monomer were utilized exclusively to aggregate all the tested materials, i.e., BG, Col and Mg. PMMA is well stablished to be biocompatible and inert.

#### Preparation of BG/Mg and BG/Col/Mg composites

For BG/Col/Mg composite manufacturing, all the materials (i.e., PMMA, MMA, BG, Col, Mg, CMC and distilled H2O) were added at different ratios according to the respective group (Table 1). The resulting amounts (wt%),

| Groups       | PMMA (wt%) | MMA (wt%) | Bioglass (wt%) | Collagen (wt%) | Magnesium (wt%) | CMC (wt%) | Water (wt%) |
|--------------|------------|-----------|----------------|----------------|----------------|-----------|-------------|
| BG/Mg        | 13.28      | 26.57     | 22.97          | 0.00           | 2.94           | 2.42      | 31.83       |
| BG/Col/Mg    | 13.80      | 27.59     | 15.43          | 4.56           | 3.05           | 2.51      | 33.05       |
considering only the bioactive (i.e., BG, Col and Mg), were obtained considering the mass (g) of each one of these components used in the formulations (Table 2). CMC was the porogenic agent and the amount utilized for this component in each formulation is toward inducing porosity of 60% (29-31). All the materials, in powder form, were weighed and mixed in a silicone container using a spatula. Afterwards, water was added and the blend was mixed again. Finally, the MMA monomer was added and mixed in order to start the crosslink. Then, the mixture was rapidly transferred to a silicon mold of 3 mm diameter x 1 mm height. After, the molds were wrapped and submitted to a pressure air chamber at 0.6 MPa for 30 min. Subsequently, the unsealed molds were vacuum dried (10⁻³ Torr) for 15 min and the composites were dried at room temperature.

Low level lasertherapy (LLLT)

A Ga-Al-As laser equipment (Photon lase III, DMC Equipment, São Carlos, SP, Brazil) was used in this study. The LLLT parameters used in this protocol treatment are shown in Table 3 (22). The irradiation was performed at one point, above the area of the created defect, by the punctual contact technique. LLLT treatment was performed for 3 times per week, in non-consecutive days (32-35).

Experimental design

Eighty male Wistar rats (12 weeks, weighing 300–350 g) were utilized in this study. They were maintained under controlled temperature (24 ± 2 ºC), light–dark periods of 12 h, with water and commercial diet ad libitum. Animal handling and surgical procedures were conducted in accordance with protocols approved by Animal Care Committee guidelines of the Federal University of São Paulo (CEUA8007051116). Animals were distributed into 4 groups: (i) bioglass/magnesium (BG/Mg); (ii) bioglass/collagen/magnesium (BG/Col/Mg); (iii) bioglass/magnesium + Laser (BG/Mg + LLLT) and (iv) bioglass/collagen/magnesium + Laser (BG/Col/Mg + LLLT). All experimental groups were divided into two different sub-groups (n = 10) and euthanized in 2 periods (15 and 45 days after implantation). As described below, a non-critical size bone defect was performed on both tibiae.

Surgical procedures

Surgery was performed under sterile conditions and general anesthesia induced by intraperitoneal injection of 8 mg/kg xylazine (Anasedan; Sespo Industry and Trade Ltda, Jacareí, SP, Brazil) and 80 mg/kg ketamine (Dopalen; Sespo Industry and Trade Ltda.), associated with 1 mg/kg acepromazine (Dopalen; Sespo Industry and Trade Ltda, Jacareí, SP, Brazil) and 0.05 mg/kg fentanyl (Dopalen; Sespo Industry and Trade Ltda, Jacareí, SP, Brazil). Then, bilateral noncritical size bone defects (3 mm diameter) were created using a motorized drill (Beltec®, Araraquara, SP, Brazil) and under copious irrigation with saline solution at the upper third of the tibia (10 mm distal of the knee joint). The composites were placed in the created defect, according to a randomization scheme. Thereafter, the wound was closed with resorbable Vicryl® 5-0 (Johnson&Johnson, St.Stevens-Woluwe, Belgium) after which the skin was closed by staples (Agraven®; InstruVet BV, Cuijk, The Netherlands). Post-surgery, all animals received, 2 mg/kg meloxicam (Maxicam, Ourofino, Osasco, SP, Brazil) and cephalothin 60 mg/kg (Keflin, Neutro®, Ely Lilly, SP, Brasil), for 5 consecutive days. All animals were monitored daily. After 15 and 45 days of implantation, rats were euthanized by CO₂ asphyxia and the specimens were harvested, fixed, and analyzed. All the data obtained were subjected to statistical analysis by the Student’s t-test and a significant difference was considered at p < 0.05.

Table 2: Amounts of BG, Col and Mg in the BG/Mg and BG/Col/Mg-based composites, considering only the wt% of these components

| Groups     | Bioglass (wt%) | Collagen (wt%) | Magnesium (wt%) |
|------------|----------------|----------------|-----------------|
| BG/Mg      | 89             | 0              | 11              |
| BG/Col/Mg  | 67             | 20             | 13              |

Table 3: Laser parameters

| Parameters | In vivo |
|------------|---------|
| Wavelength | 808 nm (infrared) |
| Laser frequency | Continuous output |
| Optical output | 30 mW |
| Spot size | 0.028 cm² |
| Power density | 1.07 W/cm² |
| Dose | 100 J/cm² |
| Energy | 2.8 J |
| Time per point | 94 s |
| Application mode | Stationary in skin/contact mode |

Table 4: Means and standard deviation for the mechanical test of the tibiae

| Groups     | Maximal load (kN) |
|------------|-------------------|
| 15 days    |                   |
| BG/Mg      | 0.074 ± 0.015     |
| BG/Col/Mg  | 0.087 ± 0.020     |
| BG/Mg + LLLT | 0.079 ± 0.011 |
| BG/Col/Mg + LLLT | 0.069 ± 0.010 |
| 45 days    |                   |
| BG/Mg      | 0.091 ± 0.023     |
| BG/Col/Mg  | 0.086 ± 0.012     |
| BG/Mg + LLLT | 0.131 ± 0.018*   |
| BG/Col/Mg + LLLT | 0.084 ± 0.010*   |

* BG/Mg + LLLT compared to BG/Mg, BG/Col/Mg and BG/Col/Mg + LLLT (p = 0.0007, 0.0022 and 0.0017 respectively).
were harvested.

**Histopathological analysis**

In the histopathological and immunohistochemistry analysis, the right tibiae were removed, fixed in 10% buffer formalin (Merck, Darmstadt, Germany) for 24 h, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany) and embedded in paraffin blocks. Thin sections (5 μm) were prepared using a microtome (Leica Microsystems SP 1600, Nussloch, Germany). 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) 33, 36, 37. The area of the bone defect was qualitatively evaluated considering inflammatory process, granulation tissue, newly formed bone and material degradation. The analysis was performed in a blinded way (PRGA and HMC).

The histomorphometric analysis was performed by using a Zeiss microscope (Carl Zeiss Vision GmbH, Germany) and the semiautomatic image-analyzing computer program Osteoméasure (Osteometrics, Inc., Atlanta, Georgia). Percent bone volume (BV/TV) was acquired according to the percentage of area within the region of interest (ROI) occupied by bone (magnification of 40x).

**Immunohistochemistry**

The immunostaining of runt-related transcription factor-2 (runx-2) and activator of nuclear factor kappa-B ligand (Rank-L) was evaluated as described earlier 36, 37, utilizing the streptavidin–biotin-peroxidase method. The immunostaining of runx-2 and Rank-L was assessed qualitatively (presence and location of the immunomarkers) and quantitatively using a light microscopy (Leica Microsystems AG, Wetzlar, Germany) in accordance with a previously described scoring scale from 1 to 4 (1 = absent, 2 = weak, 3 = moderate, and 4 = intense) 36. The analysis was performed in a blinded way (PRGA and HMC).

**Mechanical test**

Biomechanical properties of the left tibia were determined by a three-point bending test with a 1 kN load (3340 Series Single Column Systems, Instron, Norwood, MA, USA). 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 posterior-anterior direction at the exact site of the bone defect. A 5 N pre-load was applied to avoid specimen sliding. Finally, the bending force was applied at a constant deformation rate of 0.5 cm/min until fracture occurred. From the load-deformation curve, the maximum load at failure (N) was obtained.

**Statistical analysis**

Data were analyzed and displayed in tables and graphs, and the values expressed as mean and standard deviation. The distribution of variables was tested using the Shapiro-Wilk normality test. ANOVA with post hoc Tukey was used for parametric data and Kruskal-Wallis test with post hoc Dunn for nonparametric data. The level of significance was set at 5 % (p ≤ 0.05). All statistical analyses were performed using GraphPad Prism version 7.0.

**Results**

**Histopathological analysis of tibial implants**

**Fifteen days**

The qualitative histological evaluation of BG/Mg and BG/Col/Mg implants indicated an initial degradation of the samples, accompanied by bone neoformation, mainly, in the periphery of the defect (Figures 1A and 1B). Moreover, the defect line still could be noticed, and granulation and medullary tissues were noticed in some regions of the defect site (Figures 1A and 1B).

The histological assessment for BG/Mg + LLLT e BG/Col/Mg + LLLT, 15 days post-implantation, showed a similar pattern compared to the one found for groups without LLLT. It could be observed an initial degradation of the material and some areas of neoformed bone in the periphery of the defect (Figures 1C and 1D). The defect line also could be noticed, separating the healthy cortical from the region of induced defect. Particles of the material were found in different parts of the defect, however less evidently compared to the groups without LLLT. It is worth mentioning that a more evident and organized granulation tissue was also found for the LLLT-associated groups compared to the non-associated ones (Figures 1C and 1D).

**Forty-five days**

The histological findings for BG/Mg and BG/Col/Mg, 45 days after implantation, revealed a progressive biomaterial degradation and newly formed bone replacing the area previously occupied by the material (Figures 1E e 1F).

Several samples showed a more tenuous defect line compared to the previous period. Biomaterial particles could be observed, mostly in the center of the defect, among trabecular bone. Moreover, granulation and medullary tissues were also found among fragments of the biomaterial (Figures 1E e 1F).

In a similar way, the histological analysis for BG/Mg and BG/Col/Mg associated to LLLT, 45 days post-surgery, showed material degradation and newly formed bone, mainly in the borders of the defect (Figures 1G e 1H). The bone tissue showed to be more mature, resembling healthy cortical. The biomaterial still could be observed, mainly, in the core of the defect. Additionally, a more organized granulation tissue could be found compared to the previous period (Figures 1G e 1H).
Figure 1: Representative histological sections of BG/Mg 15 days (A); BG/Col/Mg 15 days (B); BG/Mg + LLLT 15 days (C); BG/Col/Mg + LLLT 15 days (D); BG/Mg 45 days (E); BG/Col/Mg 45 days (F); BG/Mg + LLLT 45 days (G); BG/Col/Mg + LLLT 45 days (H).

* Material, B Bone, D Defect line, GT Granulation Tissue, MT Medullary Tissue. The line separates the experimental period of 15 days from the 45 days. Magnification of 100x. Bars: 20 µm.
Histomorphometrical analysis

The data of the quantitative histomorphometrical analysis are presented in Figure 2. After 15 days, no statistical difference were observed among groups ($p > 0.05$). At day 45 post-implantation, the groups presented increased values for newly formed bone compared to the previous period, with values between ~23 and 32 % of the defect area. Statistical difference was found when comparing BG/Col/Mg + LLLT to BG/Col/Mg after 45 days of surgery ($p = 0.0499$).

Immunohistochemistry

**Qualitative analysis**

The immunolabeling for runx-2 was detected for all groups, 15 days post-implantation, mainly in the borders of the newly formed bone and granulation tissue (Figure 3A-3D). The immunoexpression was also found in the medullary tissue and osteoblastic cells. After 45 days, runx-2 labeling was still detected throughout the defect, more evidently in the neoformed bone among the particles of the biocomposite and in the remaining medullary tissue (Figures 3E-3H).

Similarly, 15 and 45 days after implantation, Rank-L immunolabeling was detected for BG/Mg and BG/Col/Mg associated or not with LLLT (Figure 4). Rank-L factor was more evident in the newly formed bone among the particles of the material and in specimens whose medullary and granulation tissues were still present.

**Quantitative analysis**

The results for runx-2 and Rank-L quantitative analysis are depicted in Figures 5 and 6 respectively. All groups presented immunostaining values from 2.5 to 3.2 which are indicative of moderate immunostaining. However, no statistical difference was found among any group at both time points ($p > 0.05$; Figures 5 and 6).

Mechanical test

The mechanical test indicated statistically differences in the maximal load comparing BG/Mg + LLLT (0.131 kN) to BG/Mg, BG/Col/Mg and BG/Col/Mg + LTTT (values ranging between 0.084 and 0.091 kN) after 45 days, with a significant increase in BG/Mg + LLLT group ($p < 0.05$). No other difference was observed among groups ($p > 0.05$).

Discussion

The present study investigated if the introduction of Col and post-operative treatments (LLLT) would improve the osteogenic potential of BG/Mg composites in an *in vivo* study. The results showed that Col could be successfully introduced into BG/Mg, but it did not offer any extra stimulatory effect to the composites in means of bone formation and runx-2 and rank-L immunostaining. Interestingly, the association of BG/Mg/Col and LLLT constituted an optimized treatment for accelerating material degradation and increasing newly formed bone deposition (especially 6 weeks after the surgical procedure). However, LLLT irradiation also did not present any effect on immunostaining markers evaluated. The mechanical test showed an increased maximal load for BG/Mg + LLLT compared to all other groups 45 days post-implantation.

The wavelength of 808 nm, utilized in this work, is well-known to penetrate deeper into tissues. This fact makes this choice suitable to be used for bone tissue stimulation. Also, previous works have been demonstrating the positive effects of this wavelength, including the application time of 94 s, to improve bone fracture healing and stimulate bone metabolism.

**Figure 2:** Histomorphometrical data for the composites associated or not with LLLT.

* BG/Col/Mg + LLLT compared to BG/Col/Mg at day 45 ($p = 0.0499$).
Figure 3: Representative histological sections for runx-2 of BG/Mg 15 days (A); BG/Col/Mg 15 days (B); BG/Mg + LLLT 15 days (C); BG/Col/Mg + LLLT 15 days (D); BG/Mg 45 days (E); BG/Col/Mg 45 days (F); BG/Mg + LLLT 45 days (G); BG/Col/Mg + LLLT 45 days (H).

* Material, B Bone, D Defect line, GT Granulation Tissue, MT Medullary Tissue, Ob Osteoblast. The line separates the experimental period of 15 days from the 45 days. Magnification of 200x. Bars: 20 µm.
**Figure 4:** Representative histological sections for Rank-L of BG/Mg 15 days (A); BG/Col/Mg 15 days (B); BG/Mg + LLLT 15 days (C); BG/Col/Mg + LLLT 15 days (D); BG/Mg 45 days (E); BG/Col/Mg 45 days (F); BG/Mg + LLLT 45 days (G); BG/Col/Mg + LLLT 45 days (H).

* Material, B Bone, D Defect line, GT Granulation Tissue, MT Medullary Tissue, Oc Osteocyte. The line separates the experimental period of 15 days from the 45 days. Magnification of 200x. Bars: 20 µm.
An accelerated material degradation and higher bone formation were found in the irradiated groups. It is well known that resorption of the bone substitute material (e.g. biodegradation of the material) is required in order to allow newly bone tissue ingrowth. In this context, the results of the present study indicate that the degradation rate of the material stimulated by laser irradiation influences the deposition of the tissue and formation of bone. The stimulatory effects of LLLT on bone growth are widely known, being able of increasing bone cell proliferation and accelerating newly formed bone deposition. As a result, the positive histological findings, showed by laser treated animals, may be related to the attraction of osteoprogenitor cells and osteoblasts, increasing the rate of bone formation and bone ingrowth into the defect area. Interestingly, the extent of new bone formation in the presence of the Col enriched BG/Mg composites was not higher than the other samples. These were unexpected findings and do not corroborate those of Nijsure et al. (2017), demonstrating that BG/Col/copper scaffolds constituted a suitable environment for osteoblast growth and attachment, being a promising alternative for bone tissue engineering purposes.

Concerning the immunohistochemistry analysis, it is noteworthy that similar findings of runx-2 immunostaining was observed for all experimental groups. Runx-2 immunofactor is mainly expressed in osteoblasts and it is required for the differentiation of mesenchymal progenitors toward osteoblast cell lineage. It is well known that runx-2 is fundamental for upregulation of other osteoblastic markers, like osteocalcin, osteopontin and alkaline phosphatase, influencing osteoblast cell differentiation and consequently, bone formation and deposition. Many authors observed an increase of runx-2 immunostaining in the presence of BG and/or LLLT. Fernandes et al. (2013) demonstrated an increased runx-2

Figure 5: Runx-2 quantitative analysis for BG/Mg and BG/Col/Mg composites associated or not with LLLT after 15 and 45 days post-implantation ($p > 0.05$).

Figure 6: Rank-L quantitative analysis for BG/Mg and BG/Col/Mg composites associated or not with LLLT after 15 and 45 days post-implantation ($p > 0.05$).
immunostaining after LLLT irradiation compared to control group in an experimental model of bone defects in rats. Magri et al. (2017) observed a runx-2 increase in BG-treated animals in a tibial bone defect model (37). Although the lack of difference among groups, it is possible to suggest that all the treatments were able of stimulate runx-2 immunostaining, as can be observed in both qualitative and quantitative evaluations (which indicate a moderate immunostaining), positively affecting osteoblast activity.

Additionally, the resorption and remodeling of bone tissue by osteoclasts is also necessary for a successful bone healing process. In this context, rank-L is known as a key factor for differentiation and activation of osteoclasts (45-47). No difference for rank-L immunostaining was found among groups. Pinto et al. (2012) found an increased rank-L immunostaining in rats with a tibial induced bone defect and treated with BG and LLLT (48). The same statements made for Runx-2 immunostaining can be applied for rank-L mainly based on the moderate expression of the immunomarkers. In this context, increased expression of rank-L may indicate an increased presence of osteoclasts in an attempt of degrading the material.

Appropriated mechanical strength is a requirement for scaffolds with bone tissue engineering purposes, being able of supporting bone ingrowth (48, 49). Mechanical tests demonstrated an increased maximal load for BG/Mg + LLLT compared to other groups 45 days post-surgery. The introduction of Mg into bone grafts is highly desirable based mainly on its biocompatibility and, especially, on its mechanical properties, making it very attractive as orthopedic implants. Also, many authors have demonstrated that LLLT, alone or associated to biomaterials, is able of improving the mechanical properties of tibial bone callus in healthy and osteoporotic rats (50, 51). Fangel et al. (2014), in an experimental study, demonstrated that tibial bone defects treated with Biosilicate (with similar composition to BG) associated to LLLT presented a superior mechanical callus strength (51). Interestingly, the groups treated with Col enriched composites had no difference in the mechanical properties. It is known that, although Col has many biological applications, it presents low mechanical properties, which limit its application in hard-tissue scaffolds (52), corroborating the findings of the present study.

As previously showed by the results, the LLLT stimulus had an influence over material degradation, accompanied by bone formation and enhanced mechanical properties. Nevertheless, the LLLT, using the present parameters and conditions, could not over stimulate the studied immunohistochemical bone markers, and further investigations, utilizing different parameters, should be performed to elucidate this issue.

The results of this study confirmed one of our hypotheses that the association of LLLT and Col to BG/Mg would constitute a therapeutical intervention, with improved biological properties. It has been attributed to LLLT a stimulatory effect on neovascularization by the increased secretion of angiogenic factors (53) which, together with the osteopromotive properties of BG, might further influence bone formation when using BG/Mg-supplemented Col. Thus, the higher amount of newly formed bone may explain the increased bone callus strength of these animals. However, to further understand the effects of BG/Mg/Col composites associated to LLLT, information on the long-term performance of the material and under compromised conditions (such as osteoporosis) remains to be provided. Also, other experiments, including a negative control should be performed.

**Conclusion**

The present investigation lead to us to conclude that the Col enriched BG/Mg composites irradiated with LLLT presented superior biological and mechanical properties, demonstrating to be a promising bone graft for tissue engineering applications. Further long-term studies should be carried out to provide additional information concerning the late stages of material degradation and bone regeneration.

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Conflict of interest
No benefit of any kind will be received either directly or indirectly by the authors.