



Influence of low-level laser therapy on the healing process of autogenous bone block grafts in the jaws of systemically nicotine-modified rats: A histomorphometric study

Juliano Milanezi de Almeida^{*}, Ricardo Oliveira de Moraes,
David Jonathan Rodrigues Gusman, Paula Lazilha Faleiros, Maria José Hitomi Nagata,
Valdir Gouveia Garcia, Letícia Helena Theodoro, Alvaro Francisco Bosco

Univ. Estadual Paulista-UNESP, Division of Periodontics, Department of Surgery and Integrated Clinic, Dental School of Araçatuba, Rua José Bonifácio, n° 1193, CEP: 16015-050 Araçatuba, São Paulo, Brazil

ARTICLE INFO

Article history:

Received 14 January 2016

Received in revised form 21 October 2016

Accepted 1 December 2016

Keywords:

Autograft

Bone grafting

Nicotine

Wound healing

ABSTRACT

Objective: To analyze the influence of low-level laser therapy (LLLT) on the bone healing process of autogenous bone block grafts installed in nicotine systemically modified rats.

Methods: Seventy-two rats (Wistar) were randomly assigned into 4 groups (n = 18). SS-BG: saline application + bone graft. SS-BG/LLLT: saline application + bone graft + LLLT. NIC-BG: nicotine application + bone graft. NIC-BG/LLLT: nicotine application + bone graft + LLLT. After 30 days of application of solutions, all animals received autogenous bone block graft in the jaw, with the donation from the parietal bone's calvarial area. Treatment with LLLT was in bed-graft interface, after accommodation of the graft. The animals in each group were sacrificed at 7, 14, and 28 days after graft surgery.

Results: The histologic analyses of NIC-BG group depicted a delay of osteogenic activity in the recipient bed-graft interface and the irradiation of tissue with LLLT provided better bone healing. The histometric analysis revealed that SS-BG/LLLT and NIC-BG/LLLT groups showed increased bone formation compared to BG-SS and NIC-BG groups, after 14 days (SS-BG 24.94% ± 13.06% versus SS-BG/LLLT 27.53% ± 19.07% and NIC-BG 14.27% ± 2.22% versus NIC-BG/LLLT 24.37% ± 11.93%) and 28 days (SS-BG 50.31% ± 2.69% versus SS-BG/LLLT 58.19% ± 12.32% and NIC-BG 36.89% ± 8.40% versus NIC-BG/LLLT 45.81% ± 6.03%).

Conclusion: Nicotine harms bone formation in the bed-graft interface and LLLT action can mitigate this.

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1. Introduction

The successful design in implant dentistry is related, among other factors, to the bone area available to be restored (Depeyre, Touzet-Roumazielle, Lauwers, Raoul, & Ferri, 2016). Anatomical limitations resulting from trauma or tooth extractions result in reduced bone mass over the years, which normally limits or even prevents implant placement (Lana et al., 2012; de Lacerda et al., 2016). Given this condition, there were several options for reconstruction of the alveolar ridge, and one of them is the use

of autogenous bone (Bonfante et al., 2008; Sakkas, Schramm, Karsten, Gellrich, & Wilde, 2016). Autogenous bone block grafts are considered the gold standard in correcting bone defects due their quality and relatively high predictability of successful outcomes for presenting source of osteogenic cells and osteoinductive substances (Oliveira et al., 2015). In addition, autogenous bone block grafts lack immunogenicity and boast mechanical stability, which allows the installation of implants (Bastos et al., 2014; Restoy-Lozano et al., 2015; Schulz et al., 2016).

Some studies have evaluated the healing process of autogenous bone block grafts, including the analyze of systemic effects on the pattern of healing (Jardini, De Marco, & Lima, 2005; Luize, Bosco, Bonfante, & Almeida, 2008; Gealh et al., 2014; Moreschi et al., 2013). Hence, one of substance analyzed that may cause harmful systemic effects to the healing process of bone autografts is the nicotine (Bonfante et al., 2008).

The nicotine substance in tobacco is among the most harmful drugs to human health and causes, among other diseases,

^{*} Correspondence to: Departamento de Cirurgia e Clínica Integrada, Disciplina de Periodontia, Faculdade de Odontologia de Araçatuba, UNESP, Rua José Bonifácio, 1193, CEP: 16015-050, Araçatuba, São Paulo, Brazil.

E-mail addresses: jumilanezi@hotmail.com (J.M. de Almeida), rickfoa@hotmail.com (R.O. de Moraes), davidgusman2@gmail.com (D.J.R. Gusman), paulal.faleiros@hotmail.com (P.L. Faleiros), mjnagata@uol.com.br (M.J.H. Nagata), vg.garcia@uol.com.br (V.G. Garcia), letheodoro@foa.unesp.br (L.H. Theodoro), afbosco@hotmail.com (A.F. Bosco).

inhibition of macrophages and red blood cells, platelet aggregation leading to red cell agglutination, decreased chemotaxis, decreased phagocytosis of polymorphonuclear leukocytes, reduced antibody production, reduced viability of lymphocytes T, inhibited angiogenic mediators, peripheral vasoconstriction, ischemia, decreased tissue oxygen tension and reduced activity fibroblast and osteoblast (Cattaneo et al., 2000; Sloan, Hussain, Maqsood, Eremin, & El-Sheemy, 2010; Holt, & Keast, 1977; Ma, Zwahlen, Zheng, & Sham, 2011; Machado et al., 2010; Feitelson, Rowell, Roberts, & Fleming, 2003; Jones & Triplett, 1992; Jensen, Goodson, Hopf, & Hunt, 1991; Alpar, Leyhausen, Sapotnick, Günay, & Geurtsen, 1998; Tanur, McQuade, McPherson, Al-Hashimi, & Rivera-Hidalgo, 2000; Yuhara et al., 1999).

“Recent studies have shown that nicotine interferes with delaying the alveolar repair process after tooth extraction; interferes with repairs of bone defects treated through guided tissue regeneration; creates poor-quality bone around an implant with late loss; hinders the osseointegration of titanium implants; inhibits the expression of genes related to the bone matrix; and delays the healing process of autogenous bone block grafts (Pinto, Bosco, Okamoto, Guerra, & Piza, 2002; Saldanha et al., 2004; César-Neto et al., 2003; Berley, Yamano, & Sukotjo, 2010; Yamano et al., 2010; Bonfante et al., 2008).”

In contrast, biomodulation with low-level laser therapy (LLLT) has become common place in research and the clinic as a non-invasive method for biomodulation of osteogenesis and to reduce the healing time in bone repair (Guzzardella, Torricelli, Nicoli-Aldini, & Giardino, 2003; Ozawa, Shimizu, Kariya, & Abiko, 1998; Pogrel, Chen, & Zhang, 1997). The effects of biomodulation with LLLT could be explained by an increase of cell proliferation, or changes in the physiological activity of excitable cells (Eduardo et al., 2007; Karu, 1989). Lasers have an important benefit for the production of the fibroblast growth factor bFGF (Damante, Micheli, Miyagi, Feist, & Marques, 2009; Saygun et al., 2012) and stimulate fibroblasts, helping to produce more orderly collagen fibers, causing a higher standard of healing in injuries (Basford, 1986). This type of therapy may provide anti-inflammatory, analgesic, and biomodulation effects, increasing microcirculation of the irradiated area, promoting better repair conditions (Garcia et al., 2012). Biomodulation with LLLT increases proliferation of osteoblast cells and accelerates bone repair by stimulating the modulation of the inflammatory response by modulating cytokines (TNF- α , IL-6, IL-10, VEGF, MMP1, MMP13, IFN- γ) and chemokines (MCP-1) (Saygun et al., 2012; Pretel, Lizarelli, & Ramalho, 2007; Fukuda et al., 2013a; Pezelj-Ribarić et al., 2013; Casalechi et al., 2013; Fukuda, Tanji, Silva, Sato, & Plapler, 2013b). It also stimulates osteogenesis during the early stage of bone graft repair and has positive effects on rapid maxillary expansion and bone regeneration of the sutures, promotes better peri-implant bone healing, stability, contact implant bone and bone formation and the osseointegration of orthodontic mini-implants and dental implants (Boldrini et al., 2013; Campanha et al., 2010; Cepera et al., 2012; Gomes et al., 2015; Jakse et al., 2007; Pinto et al., 2013; Silva & Camili, 2006).

Based on these, the purpose of this study was to analyze the influence of low-level laser therapy (LLLT) on the bone healing process of autogenous bone block grafts installed in nicotine systemically modified rats.

2. Materials methods

2.1. Experimental protocol

Seventy-two 4-month-old male rats (*Rattus norvegicus* Albino, Wistar), weighing 350–400 g (UNESP, Dental School of Aracatuba, Animal Care Unit). They were kept in plastic cages

with access to food and water ad libitum, in a room with 12-h light/12-h dark cycle and temperature between 22 °C and 24 °C.

The animals were randomly assigned into 4 experimental groups (n=18) and received the following procedures: SS-BG, saline solution application, autogenous bone block graft surgery, SS-BG/LLLT, saline solution application, autogenous bone block graft surgery and application of LLLT, NIC-BG, nicotine application and autogenous bone block graft surgery; NIC-BG/LLLT, nicotine application, autogenous bone block graft surgery and application of LLLT. The applications were subcutaneous every 12 h in the dorsal region, for a period of 30 days prior to the surgical procedure. The NIC-BG and NIC-BG/LLLT groups received nicotine hemisulfate application, concentration of 5 mg/ml, administered as a dilute solution at a dose of 3 mg/kg (Okamoto, Kita, Okuda, Tanaka, & Nakashima, 1994), and SS physiological solution of sodium chloride 9%. After surgery, the applications of nicotine and saline solution continued until the euthanasia periods of 7, 14, and 28 days.

The Ethics Committee in Animal Experimentation (#2008-003196) approved the experimental protocol at Universidade Estadual Paulista – “Julio de Mesquita Filho” – Dental School of Aracatuba.

2.2. Autogenous bone block grafts

The animals were anesthetized by intramuscular injection of ketamine (0.7 mL/100 g body weight) associated with xylazine (0.3 mL/100 g body weight) and each animal received in the immediate postoperative single dose of antibiotic penicillin G benzathine (intramuscular administration of 0.2 mL/animal). The autogenous bone block graft in the jaw of the animals was based on previous studies (Bonfante et al., 2008; Jardim et al., 2005; Luize et al., 2008).

The calvaria was a donor site, and the angle of the mandible was the recipient area. After anesthesia, the skin of the donor and recipient areas was trichotomized, and a vigorous disinfection was accomplished with 0.2% chlorhexidine digluconate (Colgate-Palmolive, New York, NY).

The bone graft was harvested from the right parietal bone using a surgical trephine with an internal diameter of 3.8 mm (Prudent, Lins, São Paulo, Brazil), with a controlled speed of 800 rpm, under abundant and continuous irrigation with physiologic saline. The bone block was then drilled in its center with a 0.5-mm carbide bur (KG Sorensen, Barueri, São Paulo, Brazil) at low speed and cooled with physiological saline. The recipient area was also drilled with the same carbide bur, allowing a green polyester 5-0 suture thread (Johnson & Johnson/Ethicon, Somerville, NJ) to go through the mandible and the bone block (Fig. 1A). This procedure allowed placement and stabilization of the graft in close contact with the mandibular bone surface. The wound was sutured in layers: the muscle was sutured with 5-0 polyglactin 910 (Vicryl; Johnson & Johnson/Ethicon), and the skin in the donor and recipient areas with 4-0 black silk (Johnson & Johnson/Ethicon).

Treatment in LLLT emitting InGaAlP Thera Lase[®] (DMC Equipamentos Ltda, Sao Carlos, São Paulo, Brazil) was conducted in animals from the following groups: NIC-BG/LLLT and SS-BG/LLLT, following an agreement with a wavelength of 660 nm and power of 35 mW. Irradiation was carried out with a nozzle of 1 mm in diameter, in continuous mode, in a timely manner and in contact with the region of the bed-graft interface. The laser light was directed towards 8 equidistant points on the interface (Fig. 1B), and each spot irradiated for 3 s with a specific dose of 4 J/cm² and also a scan of 3 s skirting the area of the graft, totaling 27 s exposure time and adding a dose total of 36 J/cm². After laser irradiation, the masseter muscle was sutured with 5-0 polyglactin 910 (Vicryl, Johnson & Johnson/Ethicon) and skin with black silk wire 4-0

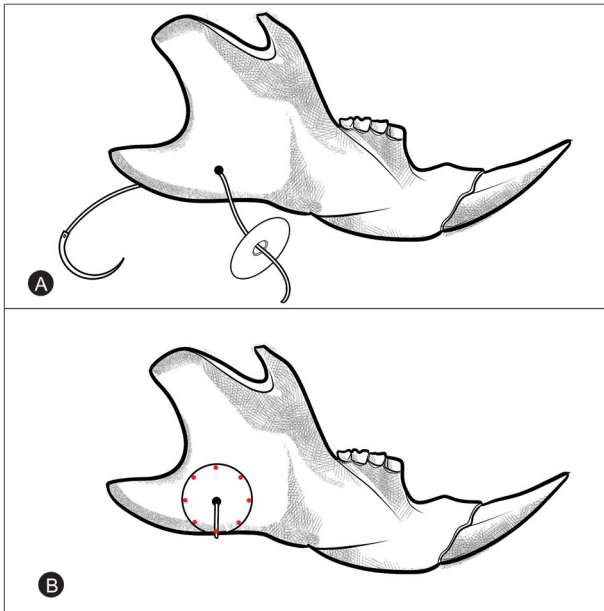


Fig. 1. Experimental surgical protocol. (A) Stabilization of the bone block graft harvested from the calvaria (drilled in its center with a carbide bur) in the recipient area of the mandible (also drilled with the same carbide bur), by a polyester 5-0 suture thread; (B) Laser irradiation points (8 equidistant points) bordering the graft area.

(Johnson & Johnson/Ethicon). In the first 48 h after surgery, the animals were kept in individual cages and were fed with minced animal food and water *ad libitum*.

2.3. Laboratory processing

After 7, 14, 28 days, animals were euthanized and the right hemimandible was removed and fixed in formalin 10% for 48 h and decalcified in EDTA solution 10%. After decalcification, the specimens were processed in paraffin in order to obtain 6- μ m thick sections, from the lower edge of the mandible in the transverse direction to the plane of juxtaposition of the bed-graft interface. Six central sections, excluding the area of drilling, were stained with Hematoxylin-Eosin (H&E).

Histologic and histometric analysis

Histologic analyses were performed by a previously calibrated examiner (ROM) who was blind to the treatments rendered. The histological characteristics of bone tissue and connective tissue in the region of bed-graft interface were described for the presence of granulation tissue, blood clot, blood vessels, fibroblasts, inflammatory cells, osteogenic activity, newly formed bone areas and fulfillment of medullar areas.

For the histometric analysis, the two most central histologic sections of each specimen block and experimental period were selected (excluding those in the center with a drilled hole), photographed with a digital camera (Olympus DP 10; Olympus Optical, Tokyo, Japan), connected to a light microscope (Olympus BX 50 F4; Olympus Optical), and then transferred to a computer. Since it was not possible to capture the full extent of the graft receptor bed to be analyzed in a single image, a digital image was created by combining three smaller images based on anatomical reference structures (such as blood vessels and trabecular bone) in each of the histological sections. Measurements were performed using the image analysis software Imagelab 2000 (Dacron Bio Informatica Ltda., Vargem Grande do Sul, SP, Brazil) by another previously calibrated examiner (JMA). The following criteria were used to standardize the histometric analysis:

- The total area of the interface was delineated corresponding to the space between the bone block graft and the receptor bed. This was considered as 100% of the area to be analyzed.
- The newly formed bone (NFB) area within the total was calculated by delineating each bone unit separately and then summing the areas.

The total area of the interface and the newly formed bone area was obtained in pixels. The data were converted to percentages (ratio of newly formed bone/interface area) and submitted to statistical analysis (Bonfante et al., 2008; Luize et al., 2008).

2.4. Statistical analysis

A kappa test was used to check the validity and reliability of the results. The data were subjected to the Shapiro-Wilk test to confirm a normal distribution, and the data were analyzed by a two-way ANOVA ($p < 0.05$) with Tukey's post hoc test for individual comparisons ($p < 0.05$). All analyses were performed using BioStat 5.0 software (Bioestat, Windows 1995; Sonopress Brazilian Industry, Manaus, AM, Brazil).

3. Results

3.1. Descriptive histologic analysis

3.1.1. 7 days

None of the groups showed newly formed bone at 7 days. SS-BG group (Fig. 2A(a)/(b)/(c)) showed mature connective tissue containing blood clot remnants (Fig. 2A(b)), inflammatory infiltrate with moderate numbers of lymphocytes and macrophages beside some fibroblasts (Fig. 2A(a)/(c)) and blood vessels (Fig. 2A(b)/(c)). SS-BG/LLLT group (Fig. 2B(a)/(b)/(c)) showed mature connective tissue containing blood clot remnants (Fig. 2B(b)), inflammatory infiltrate with a large amount of fibroblasts, macrophages and lymphocytes at the bed-graft interface (Fig. 2B(a)/(b)/(c)) and a large amount of blood vessels (Fig. 2B(a)/(b)/(c)). NIC-BG group (Fig. 2C(a)/(b)/(c)) showed immature connective tissue containing blood clot remnants (Fig. 2C(b)), inflammatory infiltrate with small quantity of fibroblasts, macrophages and lymphocytes adjacent to the bone graft interface (Fig. 2C(b)/(c)) and rare blood vessels (Fig. 2C(b)). NIC-BG/LLLT group (Fig. 2D(a)/(b)/(c)) showed mature connective tissue containing blood clot remnants (Fig. 2D(a)/(c)), inflammatory infiltrate with moderate numbers of macrophages, lymphocytes and some fibroblasts (Fig. 2D(a)/(b)/(c)) and blood vessels (Fig. 2D(b)/(c)).

3.1.2. 14 days

SS-BG group (Fig. 3A(a)/(b)/(c)) demonstrated numerous newly formed trabecular bones with osteoblasts on their edges (Fig. 3A(a)/(b)), together with a mature connective tissue rich in fibroblasts and blood vessels (Fig. 3A(c)). Most specimens of SS-BG/LLLT group (Fig. 3B(a)/(b)/(c)) presented the bed-graft interface virtually occupied by NFB tissue. In specimens with less neoformed bone tissue was observed a connective tissue quite mature and vascularized. The NIC-BG group (Fig. 3C(a)/(b)/(c)) demonstrated connective tissue with blood vessels that filled partially bed-graft interface (Fig. 3C(a)/(b)) and lower amount of NFB compared to others groups (Fig. 3C(c)). The connective tissue at the graft periphery was poorly organized with few blood vessels, discrete number of fibroblasts, macrophages and lymphocytes. The NIC-BG/LLLT group (Fig. 3D(a)/(b)/(c)) showed a significant amount of neoformation bone matrix (Fig. 3D(b)/(c)). The NFB tissue presented numerous osteocytes and osteoblasts adjacent to their edges (Fig. 3D(b)). In addition, a revascularized and connective

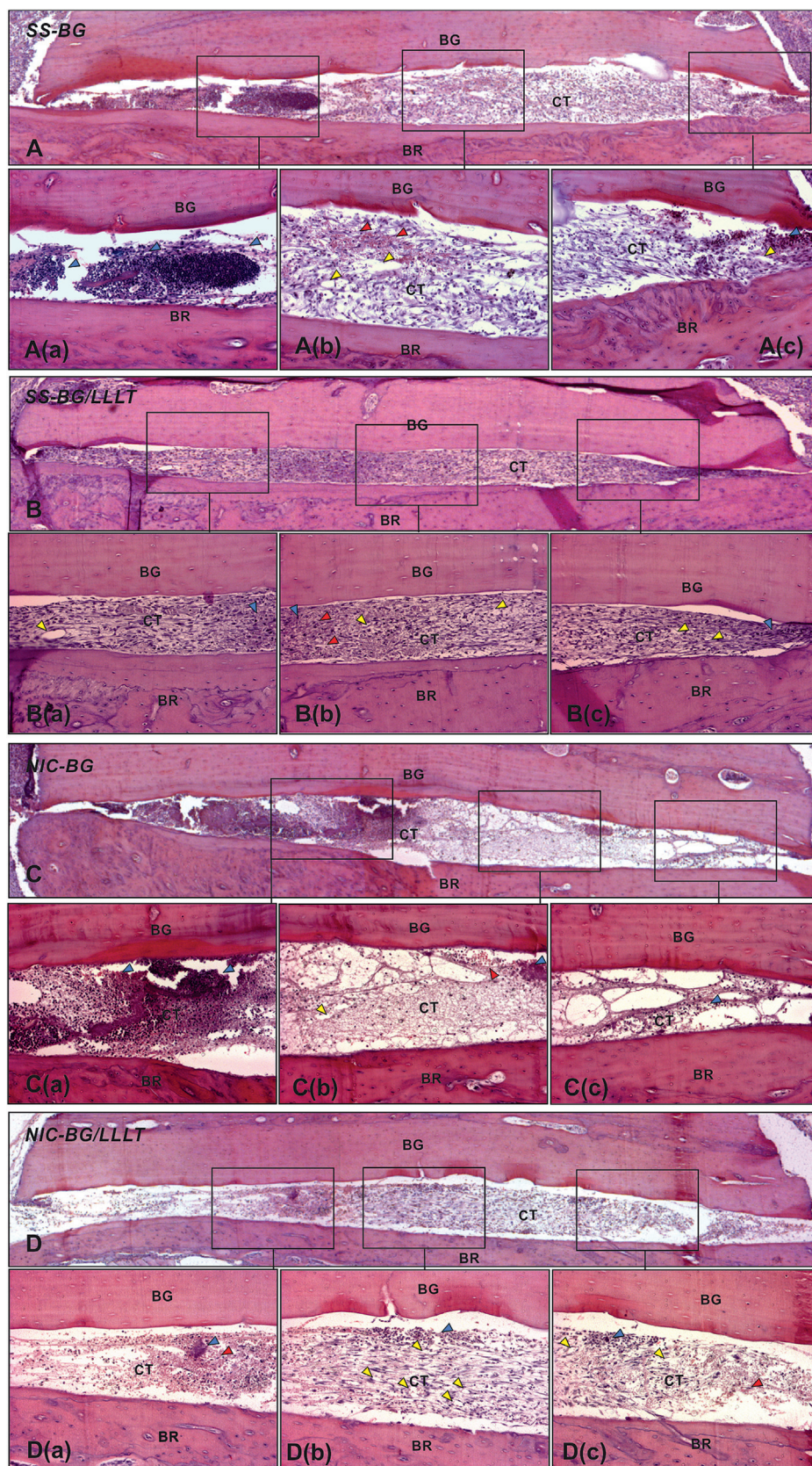


Fig. 2. Panoramic view of the bone surface of the bed recipient facing the autogenous graft and detailed histological appearance of the bed recipient-graft interface at 7 days. Photomicrographs showing the connective tissue appearance in SS-BG (A); SS-BG/LLLT (B); NIC-BG (C) and NIC-BG/LLLT (D). The connective tissue containing remnants of blood clot (red arrows), inflammatory infiltrate (blue arrows) and blood vessels (yellow arrows) are observed in all experimental groups (A(a))/(b)/(c), B(a))/(b)/(c), C(a))/(b)/(c) and D(a))/(b)/(c)). The most mature connective tissue is observed in the SS-BG/LLLT (B), while the most immature connective tissue is observed in the NIC-BG (C). (Hematoxylin and eosin staining; original magnification $\times 50$ in A, B, C and D; original magnification $\times 200$ in A(a))/(b)/(c), B(a))/(b)/(c), C(a))/(b)/(c) and D(a))/(b)/(c)). Abbreviations: BR, bed recipient; BG, bone graft; CT, conjunctive tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

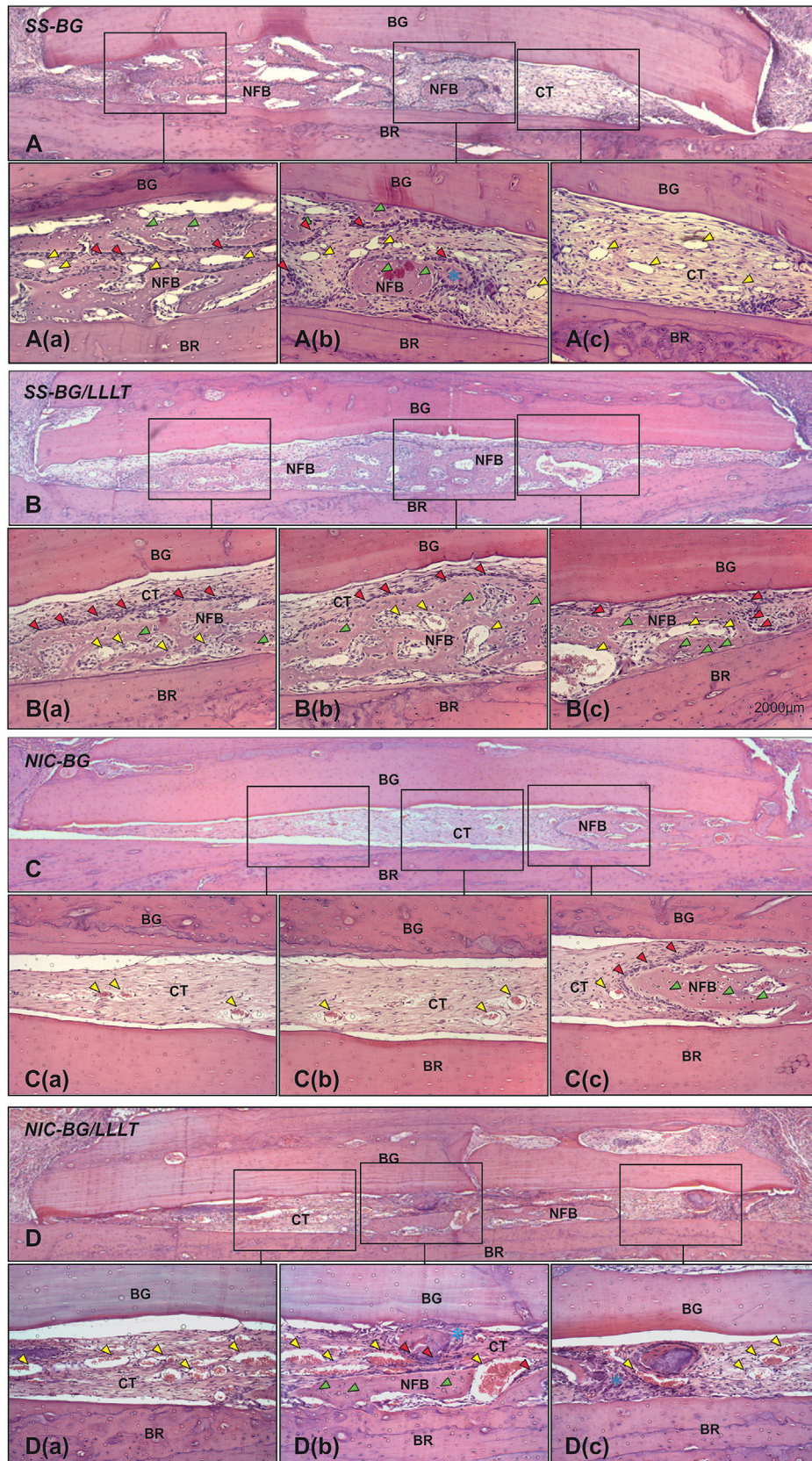


Fig. 3. Panoramic view of the bone surface of the bed recipient facing the autogenous graft and detailed histological appearance of the bed recipient-graft interface at 14 days. Photomicrographs showing the connective tissue and newly formed bone appearance in SS-BG (A), SS-BG/LLLT (B), NIC-BG (C) and NIC-BG/LLLT (D). Newly formed bone tissue containing osteocytes (green arrows) and osteoblasts adjacent to their edges (red arrows) is observed in SS-BG (A(a)/(b)), SS-BG/LLLT (B(a)/(b)/(c)), NIC-BG (C(c)), NIC-BG/LLLT (D(b)/(c)). Formation areas of osteoid matrix (asterisk) are observed in SS-BG (A(b)) and NIC-BG/LLLT (D(b)/(c)). Connective tissue containing blood vessels (yellow arrows) are observed in all experimental groups (A(a)/(b)/(c), B(a)/(b)/(c), C(a)/(b)/(c) and D(a)/(b)/(c)). Large amount of newly formed bone is observed in SS-BG/LLLT (B) and NIC-BG/LLLT (D) compared to SS-BG (A) and NIC-BG (C), respectively. (Hematoxylin and eosin staining; original magnification $\times 50$ in A, B, C and D; original magnification $\times 200$ in A(a)/(b)/(c), B(a)/(b)/(c), C(a)/(b)/(c) and D(a)/(b)/(c)). Abbreviations: BR, bed recipient; BG, bone graft; CT, conjunctive tissue; NFB, newly formed bone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tissue rich in fibroblasts and remaining granulation tissue was observed (Fig. 3D(a)/(b)/(c)).

3.1.3. 28 days

The SS-BG group (Fig. 4A(a)/(b)/(c)) presented much of the interface filled by NFB tissue integrating the graft to the receptor bed. In the same period, the SS-BG/LLLT group (Fig. 4B(a)/(b)/(c)) showed the entire length of the bed-graft interface completely occupied by NFB tissue. The NIC-BG group (Fig. 4C(a)/(b)/(c)) still had few areas of NFB interfacing by connective tissue with poor collagen fibers, few fibroblasts and inflammatory cells. Connective tissue filled most bone intertrabecular spaces Fig. 4C(a)/(b)/(c). Finally, the NIC-BG/LLLT group Fig. 4D(a)/(b)/(c) showed histological characteristics similar to the SS group. Along the entire length of the interface was a NFB tissue integrated with graft inner surface with the recipient bed.

3.2. Histometric analysis

The SS-BG group had greater bone formation compared to the NIC-BG groups. However, the group submitted to laser irradiation NIC-BG/LLLT and SS-BG/LLLT showed increased bone formation compared to the groups (NIC-BG and SS-BG), 14 days (SS-BG $24.94\% \pm 13.06\%$ versus SS-BG/LLLT $27.53\% \pm 19.07\%$ and NIC-BG $14.27\% \pm 2.22\%$ versus NIC-BG/LLLT $24.37\% \pm 11.93\%$) and 28 days (SS-BG $50.31\% \pm 2.69\%$ versus SS-BG/LLLT $58.19\% \pm 12.32\%$ and NIC-BG $36.89\% \pm 8.40\%$ versus NIC-BG/LLLT $45.81\% \pm 6.03\%$), and the results were statistically significant ($P < 0.05$). Means and standard deviations of NFB for each group are presented in Table 1.

4. Discussion

This study was conducted to analyze the influence of LLLT on the bone healing process of autogenous bone block grafts installed in nicotine systemically modified rats.

The systemic administration of nicotine to evaluate the effects of tobacco smoking on bone healing is considered a reliable method between the different in vitro (Pereira, Carvalho, Peres, Gutierrez, & Fernandes, 2008; Rothem, Rothem, Dahan, Eliakim, & Soudry, 2011; Ma et al., 2011; Kim et al., 2012) and in vivo (Bonfante et al., 2008; Ma, Zheng, Sham, & Cheung, 2010; Berley et al., 2010; Machado et al., 2010; Yamano et al., 2010) experimental models. In addition, the use of LLLT on the biostimulation of bone healing has been growing steadily, and studies have demonstrated positive results on the healing of bone tissue (Bosco et al., 2016). Considering that previous studies reported the negative effects of nicotine (Bonfante et al., 2008; Garcia et al., 2012; Machado et al., 2010), and positive effects of LLLT (Bosco et al., 2016; Garcia et al., 2014) on the bone healing process, our hypotheses were: (1) nicotine could compromise bone healing in the bed-graft interface; (2) LLLT could reverse the negative effect of nicotine on bone healing, if it occurs.

Some preclinical studies have demonstrated the effects of LLLT on bone grafts and implanted biomaterials, such as bone morphogenic proteins (BMPs), organic lyophilized bone, hydroxyapatite implants, inorganic bone and bovine bone grafts (Obradović, Kesić, & Pesevska, 2009; Bosco et al., 2016), in order to improve bone healing process in different models of study with bone augmentation procedures. Despite these findings there are only a few previous studies reporting the association of LLLT with autogenous bone (Silva & Camilli, 2006; Weber, Pinheiro, Oliveira, Oliveira, & Ramalho, 2006; Garcia et al., 2014). Among the models used have been calvarial critical size defects (Weber et al., 2006; Garcia et al., 2014; Cunha et al., 2014; Rasouli Ghahroudi et al., 2014), surgical defects created in the femur (Gerbi et al., 2008), tibia fractures (Lopes, Pacheco, Duarte, Cangussú, & Pinheiro,

2007), mandibular trauma (Rochkind et al., 2004) and titanium implants associated to biomaterial on the tibiae of rats (Lopes, Pinheiro, Sathiaiah, Duarte, & Cristinamartins, 2005). To our knowledge, this is the first study to assess the effects of LLLT in the healing process of autogenous bone block grafts fixed in the mandible, simulating a bone reconstruction model, under the systemic influence of nicotine. For this purpose, the block graft was obtained from the calvaria of the animal and fixed to the mandible following the well-established model proposed by Jardini et al. (2005) and reproduced by other authors (Bonfante et al., 2008; Luize et al., 2008) for evaluating bone healing in the graft-bed interface of different systemic-compromised models (nicotine and ovariectomy).

Although a rigid fixation model to stabilize bone block grafts has been proposed by Gealh et al. (2014), in the present study a tight suture was used for this purpose, since the fixation and force exerted by the mini-screws in the rat mandible enabled its fracture (Bonfante et al., 2008). The tight suture fixation model allowed the juxtaposition of the graft in the recipient bed and favored the process of bone formation in this interface, corroborating with the studies of Jardini et al. (2005), Bonfante et al. (2008) and Luize et al. (2008), suggesting that fixation through fair suture does not cause a risk of micro-movements.

In the present study, the animals of the NIC-BG group presented immature connective tissue, with a discrete number of fibroblasts and blood vessels adjacent to the bone graft, when compared to the SS-BG animals at 7 days. At 14 and 28 days post-operative, the NIC-BG group also presented lower bone neoformation when compared to the SS-BG animals, suggesting that nicotine compromised the bone healing process of autogenous bone block grafts fixed to the mandible. These results corroborate with the results obtained by Bonfante et al. (2008), who also observed delay in the healing process of autogenous bone block grafts due to nicotine.

It has been reported that nicotine can reduce the proliferation of macrophages, fibroblasts and red blood cells, which are important to the healing process (Berley et al., 2010). Nicotine also increases the adhesiveness of platelets, which can lead to formation of microclot and poor blood perfusion (Berley et al., 2010). In addition, in vitro studies have shown inhibition of proliferation, migration, chemotaxis and reduction in collagen types I and III in fibroblast cultures treated with nicotine (Yin, Morita, & Tsuji, 2000; Wong & Martins-Green, 2004) as well as increased levels of fibroblast collagenase, negatively affecting collagen metabolism and tissue remodeling (Yin et al., 2000). The inhibition of cellular responsiveness to collagen biosynthesis was also suggested (Yin, Morita, & Tsuji, 2003; Sørensen et al., 2010; Sørensen, 2012). Finally, nicotine is able to affect various osteogenesis-related events, such as alterations in the proliferation, differentiation and gene expression of osteoblasts (Rothem et al., 2011). All of these findings could explain the immature connective tissue and lower bone neoformation in the bed-graft interface in animals treated with nicotine in the present study and in the study of Bonfante et al. (2008).

In contrast to negative effect of nicotine, LLLT stimulates bone repair by increasing the proliferation of endothelial cells and promoting angiogenesis (Briteño-Vázquez et al., 2015; Góralczyk et al., 2015; Szymanska et al., 2013). LLLT also stimulates fibroblasts, helping to produce more orderly collagen fibers, causing a higher standard of healing in injuries (Basford, 1986; Massotti et al., 2015), corroborating with other studies demonstrating that the laser stimulates the synthesis of collagen fibers, elastic fibers and myofibroblast proliferation and reduce inflammatory response, causing wounds treated by this light to heal faster (Kana, Hutschenreiter, Haina, & Waidelich, 1981; Pugliese, Medrado, Reis, & Andrade, 2003; Hourelid & Abrahamse, 2007;

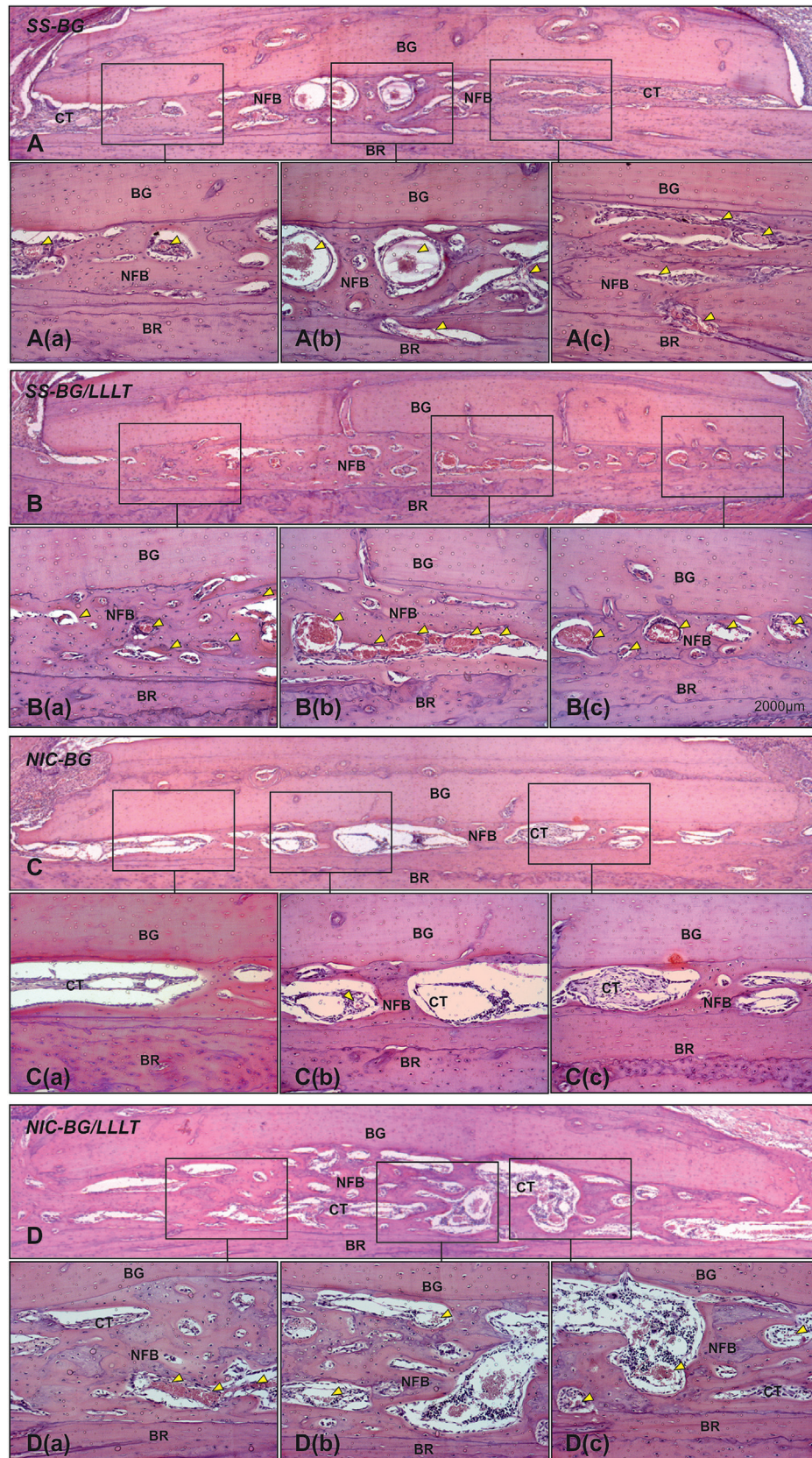


Fig. 4. Panoramic view of the bone surface of the bed recipient facing the autogenous graft and detailed histological appearance of the bed recipient-graft interface at 28 days. Photomicrographs showing the newly formed bone appearance in SS-BG (A), SS-BG/LLLT (B), NIC-BG (C) and NIC-BG/LLLT (D). Newly formed bone tissue containing osteocytes (green arrows) and osteoblasts adjacent to their edges (red arrows) is observed in SS-BG (A(a)/(b)/(c)), SS-BG/LLLT (B(a)/(b)/(c)), NIC-BG (C(a)/(b)/(c)), NIC-BG/LLLT (D(a)/(b)/(c)). Newly formed bone interfacing by connective tissue with poor collagen fibers is observed in NIC-BG (C(a)/(b)/(c)). Small areas of resorption is observed in the NIC-BG/LLLT (C(b)). Entire length of the bed-graft interface completely occupied by newly formed bone tissue is observed in SS-BG/LLLT (B). (Hematoxylin and eosin staining; original magnification $\times 50$ in A, B, C and D; original magnification $\times 200$ in A(a)/(b)/(c), B(a)/(b)/(c), C(a)/(b)/(c) and D(a)/(b)/(c). Abbreviations: BR, bed recipient; BG, bone graft; CT, conjunctive tissue; NFB, newly formed bone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Mean \pm SD amount of Newly Formed Bone (NFB) as a percentage of the total area of interface.

| | Period | |
|-------------|---------------------------------|---------------------------------|
| Groups | 14 days | 28 days |
| SS-BG | 24.94 \pm 13.06 | 50.31 \pm 2.69 [‡] |
| SS-BG/LLLT | 27.53 \pm 19.07* | 58.19 \pm 12.32 [‡] |
| NIC-BG | 14.27 \pm 2.22* | 36.89 \pm 8.40 ^{*‡} |
| NIC-BG/LLLT | 24.37 \pm 11.93 ^{#‡} | 45.81 \pm 6.03 ^{#‡‡} |

Comparison inter-groups: *Significant difference with SS-BG group (ANOVA, Tukey's test) ($p < 0.05$), # Significant difference with SS-BG/LLLT group (ANOVA, Tukey's test) ($p < 0.05$), † Significant difference with NIC-BG group (ANOVA, Tukey's test) ($p < 0.05$).

Comparison intra-groups: ‡ Significant difference with 14 days (ANOVA, Tukey's test) ($p < 0.05$).

Medrado, Pugliese, Reis, & Andrade, 2003; Mendez, Pinheiro, Pacheco, Nascimento, & Ramalho, 2004; Surinchak, Alago, Bellamy, Stuck, & Belkin, 1983; Al-Watban & Zhang, 1997). This type of therapy may provide anti-inflammatory, analgesic and biomodulation effects, increasing the microcirculation of the irradiated area and promoting better repair conditions (Garcia et al., 2012). In addition, LLLT also biomodulates the proliferation and osteoblastic differentiation (Stein, Benayahu, Maltz, & Oron, 2005; Grassi et al., 2011; Tim et al., 2014; Tim et al., 2015), bone matrix formation and mineralization, and bone maturation (Barbosa et al., 2013; Fávoro-Pípi et al., 2011; de Souza Merli et al., 2012).

In the present study, the SS-BG/LLLT group presented greater new bone formation when compared to the SS-BG group, at 14 and 28 days post-operation. Moreover, the animals of the SS-BG/LLLT group presented mature connective tissue rich in fibroblasts and blood vessels, demonstrating that LLLT can also increase bone formation and accelerate the bone healing process of autogenous bone block grafts fixed to the mandible. Although most studies have proposed the infrared laser light to stimulate bone healing because of its tissues deeper penetration, recent studies (Bosco et al., 2016; Garcia et al., 2013; Garcia et al., 2014) have demonstrated that visible laser light (660 nm wavelength) also presents biomodulatory effect when used transoperatively at the borders of calvarial surgical defect models, in contact with bone tissue, and also in a central point of the defect, only once during the surgery procedure. Based on the above mentioned studies, in the present study the autogenous bone block graft fixed to the mandible also was irradiated with visible laser light (660 nm of wavelength, 36 J/cm² of dose), only during the surgical procedure at the borders and in contact with the bone block, and also in a central point of the bone block grafted.

Many variables may affect the LLLT biostimulatory effects (such as, laser wavelength, energy, exposition time, power, and the biologic state of the cell). It was shown that the beneficial effects of LLLT are enhanced at sites with lower bone quality or involving systemically compromised models, since the susceptibility to irradiation and cell activation ability by the laser depends on the physiological state of irradiated cells (Karu, 1989). Cells with reduced redox potential, present in a diseased state such as smoking (Tinti & Soory, 2013), are more sensitive to radiation (Karu, 1989). Thus, in this study, LLLT biomodulation in a nicotine-modified rat model is proposed due to its effect being related to an increase in cell proliferation, or by changes in the physiological activity of excitable cells (Eduardo et al., 2007). This beneficial effect of laser in the healing process of autogenous bone can be related to the increased bone formation in the receptor graft interface animals NIC-BG/LLLT group compared to the animals NIC-BG group.

Extrapolating the results to clinical situations, we could consider that smoking patients or those who use nicotine would need longer periods after surgery for complete bone graft

incorporation and the use of LLLT would provide better conditions for these patients. However, further experimental studies in humans are needed to confirm the results of the present preclinical study.

Within the limits of this study, we can conclude that nicotine hinders bone formation in the bed-graft interface, and the action of LLLT can mitigate this harmful effect.

Conflict of interests

We wish to confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Funding

No sources of funding for this research.

Ethical approval

The Ethics Committee in Animal Experimentation (# 2008-003196) approved the experimental protocol at Universidade Estadual Paulista – “Julio de Mesquita Filho” – Dental School of Araçatuba.

Acknowledgment

We thank the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES) for granting a scholarship to Ricardo Oliveira de Moraes.

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