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Bone regeneration by low-level laser therapy and low-intensity pulsed ultrasound therapy in the rabbit calvarium

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ABSTRACT

Objective: We evaluated the efficacy of low-level laser therapy (LLLT) and low-intensity pulsed ultrasound (LIPUS), alone and in combination, in triggering new bone formation.
Study design: Sixteen New Zealand white rabbits were given two calvarial defects by using a 6-mm trephine bur, then divided into four treatment groups: control, LLLT, LIPUS, and LLLT + LIPUS. The LLLT and LIPUS groups were treated three times a week for two weeks. The LLLT + LIPUS group received each treatment on the same day, 12 h apart, three days a week for two weeks. The animals were sacrificed after three weeks.
Results: LLLT and LIPUS, alone and in combination, enhanced new bone formation in comparison to the untreated controls after three weeks ($P < 0.05$); the combined therapy did not produce an additive effect.
Conclusions: Our results demonstrate the efficacy of LLLT or LIPUS in triggering bone regeneration. Therapeutic dose and duration requires further study.

1. Introduction

Maxillofacial bone defects can be caused by trauma, tooth extraction, surgery, congenital anomalies, and pathologies (Giannoudis, Dinopoulos, & Tsiridis, 2005). Regeneration of bone defects is a long healing process requiring recruitment and differentiation of new bone cells in maxillofacial surgery (Sena, Leven, Mazhar, Sumner, & Virdi, 2005). Various graft materials are used to repair bone defects, although they are prone to failure. Several therapies have been explored for their ability to enhance bone regeneration such as low-level laser therapy (LLLT), ozone therapy, platelet therapy, and low-intensity pulsed ultrasound (LIPUS) (Kazancioglu, Ezirganli, & Aydin, 2013; Bronoosh et al., 2015; Acar et al., 2015).

Laser therapies are nondestructive and induce photobiological responses (da Silva & Camilli, 2006; Nascimento et al., 2010; Barbosa et al., 2013 Pires Oliveira, de Oliveira, Zangaro, & Soares, 2008; Stein et al., 2008). When laser light is absorbed by tissue, several biochemical reactions are occurred. In vitro studies have shown that application of LLLT increases mitochondrial activity, DNA/RNA synthesis in osteoblasts, cell viability and alkaline phosphatase (ALP) (Pires Oliveira et al., 2008; Stein et al., 2008). Also in vivo studies have shown that LLLT enhances bone regeneration by inducing osteoblast activity and vascularization (Kazancioglu et al., 2013; da Silva & Camilli, 2006; Nascimento et al., 2010; Barbosa et al., 2013). Thus LLLT is considered as an effective procedure on bone healing (Kazancioglu et al., 2013; da Silva & Camilli, 2006).

Ultrasound therapies have a long history and are proven to improve fracture healing process (Williams, 1983; Maintz, 1950; Azuma et al., 2001; Gebauer, Lin, Beam, Vieira, & Parsons, 2002; Heckman, Ryaby, McCabe, Frey, & Kilcoyne, 2002). Although the mechanism of LIPUS on bone regeneration is not fully understood, it has been shown that LIPUS has positive effect on the cellular reactions involved in ALP activity and osteogenic differentiation (Azuma et al., 2001; Angle, Sena, Sumner, & Virdi, 2011). LIPUS has been approved by FDA as accelerates bone fracture healing (Azuma et al., 2001; Angle et al., 2011). It also enhances bone healing in cases with bone defects and distraction osteogenesis in animal models via increasing angiogenesis (Azuma et al., 2001; Hasuike et al., 2011; Chan et al., 2006).

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Several studies have investigated the effect of LLLT on bone defect healing (Kazancioglu et al., 2013; Nascimento et al., 2010; Barbosa et al., 2013). LIPUS has largely been studied only in the context of bone fracture and distraction osteogenesis (Azuma et al., 2001; Heckman et al., 1994; Hasuike et al., 2011; Chan et al., 2006), however, studies on its effect on bone defect healing are rare (Hasuike et al., 2011). To the best of our knowledge, the effect of LLLT combined with LIPUS on bone defect healing has not been addressed. Our objective was to investigate the possible effects of LLLT on bone defect healing when used alone and in combination with LIPUS.

2. Materials and methods

2.1. Ethics and animals

The experimental procedures and study protocol were approved by the Animal Ethics Committee of Inonu University (Approval No. 2014/a-43). The study was performed according to the principles of the Basel Declaration.

Sixteen New Zealand white rabbits, aged 7 months and weighting 3.2–3.5 kg, were used for the study. The animals were obtained from the Animal Breeding and Research Center of Inonu University. Experimental procedures were performed at the same center. The rabbits were housed in individual cages at 1 atm pressure, 25 °C, and a 12-h light/dark cycle. All animals were given free access to standard laboratory pellet chow and water.

2.2. Surgical procedure and study design

All surgical procedures were performed under sterile conditions. Animals were anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. The dorsal part of the skull was shaved with a razor and disinfected with povidone iodine. A longitudinal incision of approximately 3–4 cm was made in the skull along the sagittal suture from the occipital region to the nasal bone. First, a cutaneous flap was raised and the periosteum was shifted to expose the calvarial bone. Two bicortical calvarial defects (6-mm diameter) were prepared with a trephine burr on the right and left side of the parietal bone without damaging the dura in each animal (Fig. 1). Sterile saline was used to cool the trephine burr. The periosteum and scalp were sutured with 4.0 vicrly. The scalp was sterilized with povidone iodine. After surgical procedures were completed, animals were expected wake up under veterinary supervision and they were placed into individual cages. First 24 h animals did not receive any treatment.

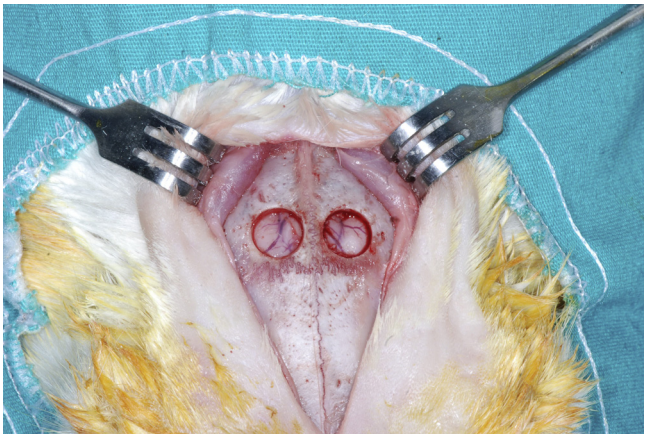

Fig. 1. Two 6-mm calvarial defects.

Sixteen rabbits were randomly divided into four groups of four animals.

- Control Group (Group A): The animals did not receive treatment after surgery.
- Laser Group (Group B): 24 h after surgery, the animals received LLLT (CHEESE Dental Laser System, DEN4A) three times a week (every other day) for two weeks (six sessions). A gallium–aluminum–arsenic (GaAlAs) diode laser was applied at a continuous wavelength of 810 nm, a power output of 0.1 W, and 120 s. A dose of 4 J/cm^2 was applied to the defect per session.
- LIPUS Group (Group C): 24 h after surgery, LIPUS (200-ms application of a 1.5 MHz wave, repeating at 1.0 kHz and delivering 30 mW/cm^2) was applied within 20 min three times a week (every other day) for two weeks (six sessions).
- Combined Group (Group D): In this group, animals received LLLT and LIPUS on the same day with a 12-h interval. Each treatment was performed three times a week (same with group B and group C) for two weeks (six sessions for each one).

All animals received 200 mg/mL pentobarbital sodium (Pentothal; Abbott, North Chicago, IL) at the end of the third week for sacrifice. All surgical and sacrifice procedures were performed by same surgeon (A.H.A).

2.3. Micro-CT analysis

After the animals were sacrified, tissue samples were obtained and immediately fixed in 10% formaldehyde for 5 days. Then the samples were rinsed with saline solution (0.9%) to remove the formaldehyde. The micro-CT system (SkyScan1172) was set at 100 kV and 100 μA with the aid of a 0.5-mm Al + Cu filter. The image resolution was set at 13.68532-μm pixels. Scaned data were transformed into images with NRecon v.1.6.3 software (Bruker-micro CT), and CTAn v.1.12 software (Bruker-micro CT) was used for data analysis. Three-dimensional images were created by CTVol v.2.2.1 software (Bruker-micro CT). Because our defect size was 6 mm, the region of interest (ROI) was adjusted to a diameter of 6 mm (Fig. 2) (Acar et al., 2015). The lower and upper limit of the defect was identified. The total volume of the study area and new bone volume were expressed as a percentage.

2.4. Histology and histomorphometry

Samples were placed in 10% formic acid for decalcification after micro-CT analysis. Decalcification was performed for 12 days, at which time the defects were coronally divided into two blocks and processed for routine histology. Tissue samples were embedded in paraffin blocks and 6-μm serial sections were cut on a microtome. Hematoxylin-eosin was used for staining and samples were examined using a Leica DFC280 light microscope; images were captured with Leica Q Win Plus. Total tissue area, newly formed bone, and connective tissue area were measured. New bone formation was expressed as a percentage.

2.5. Statistical evaluation

Results were analyzed in SPSS (Statistical Package for Social Sciences) for Windows 15.0. To identify differences in parameters, we used the Kruskal–Wallis test followed by the Mann–Whitney *U*-test to identify differences between groups. Results were considered significant at $P < 0.05$.

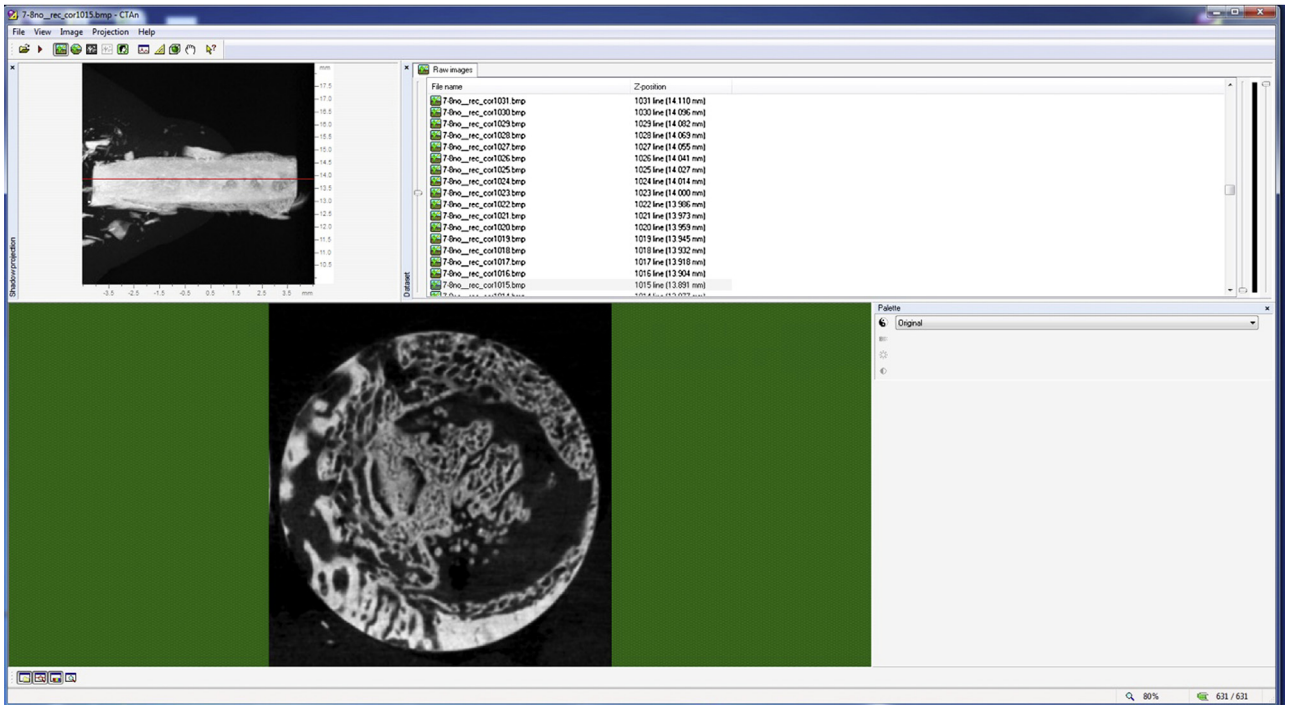

Fig. 2. Upper and lower borders of the defect area were calculated in CTAn software.

3. Results

3.1. Histological findings

Complete recovery did not occur in all groups by the end of the study. In all groups, fibrous connective tissue nearly filled the area between the surgical bone margins and new bone and osteoblast activity were observed in the defect area. Acute inflammatory cells and particles of dura matter were not observed.

3.1.1. Control group

The defect area was filled with fibrous connective tissue with a small calcified bone island at the middle of the defect (Fig. 3). Limited osteoblast activity was detected around the calcified bone islands compare to experimental groups (Fig. 4).

3.1.2. Experimental groups

All experimental groups yielded similar results. The defect areas were filled with fibrous connective tissue, but less than compare to control group. The experimental groups also showed more calcified bone islands than the control group (Fig. 3). Significant osteoblast activities were detected near the calcified bone islands in experimental groups (Fig. 4).

3.2. Histomorphometry

Histomorphometry revealed a significantly higher percentage of new bone formation in the experimental groups than in the control group ($P < 0.05$), however there was no significant difference between experimental groups ($P > 0.05$) (Table 1).

3.3. Micro-CT Analysis

New bone volume in each group is shown in Table 2. Micro-CT and histomorphometry analysis yielded similar results. The percentage of new bone formation was significantly higher in the experimental groups than the control group ($P < 0.05$), however there was no significant difference between experimental groups ($P > 0.05$). The micro-CT images of 6-mm defects are shown in Fig. 5.

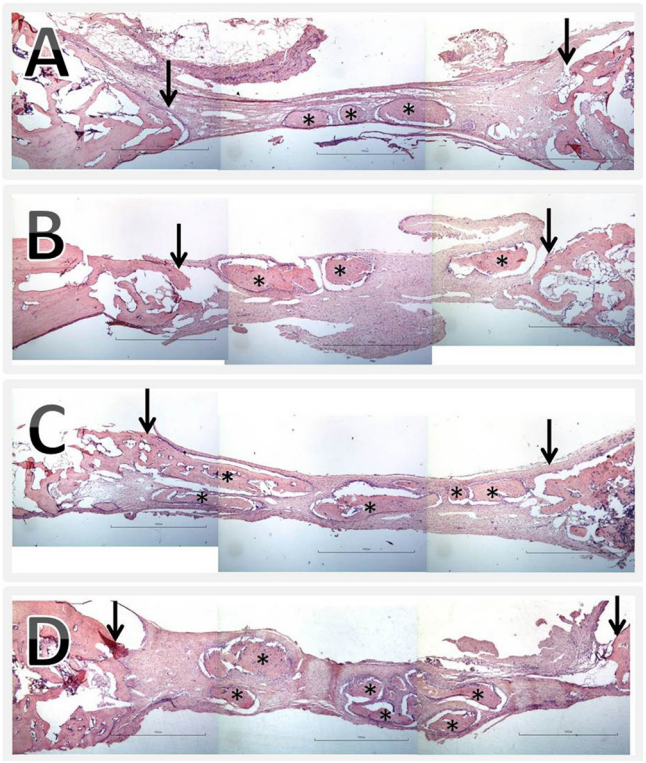

Fig. 3. Surgical bone margins (arrows) and new bone island (*); hematoxylin and eosin staining; (A) control group; (B) LLLT group; (C) LIPUS group; (D) LLLT + LIPUS group; bar: 1000 μm.

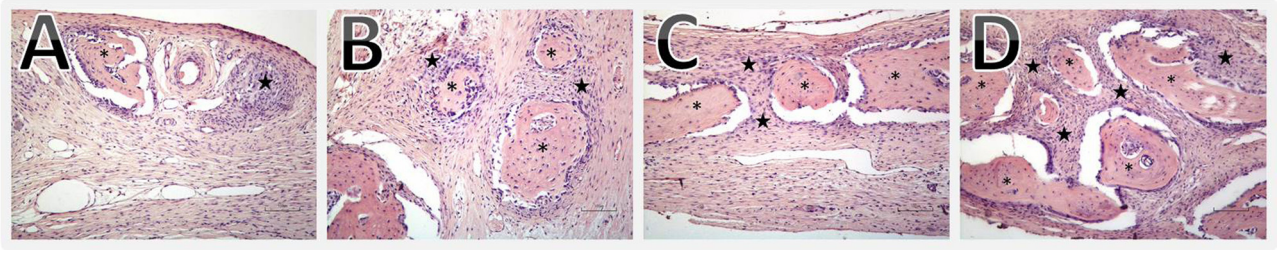

Fig. 4. New bone island (*) and osteoblast activity (stars); hematoxylin and eosin staining; (A) Control group. (B) LLLT group. (C) LIPUS group. (D) LLLT + LIPUS group; bar: 100 μm.

Table 1
Histomorphometric evaluation of new bone.

New bone percentage	Week 3 Ave ± SS
Group A	10.94 ± 1.44%
Group B	13.43 ± 2.27%
Group C	14.44 ± 1.49%
Group D	15.69 ± 1.34%
[a]p	0.05*
A–B	0.026*
A–C	0.002*
A–D	0.001*
B–C	0.2
B–D	0.053
C–D	0.165

[a] Mann–Whitney *U*-test.
* $P < 0.05$.

Table 2
Micro-CT evaluation of new bone.

New bone percentage	Week 3 Ave ± SS
Group A	13.31 ± 2.17%
Group B	17.82 ± 3.67%
Group C	19.12 ± 4.44%
Group D	19.34 ± 4.88%
[a]p	0.05*
A–B	0.005*
A–C	0.015*
A–D	0.015*
B–C	0.382
B–D	0.574
C–D	0.959

[a] Mann–Whitney *U*-test.
* $P < 0.05$.

4. Discussion

In this study we target to investigate the effects of LLLT and LIPUS on bone healing. Both methods improved new bone formation in the early stage of healing (three weeks after surgery), but did not produce an additive effect when used in combination.

In this study, we created two calvarial defects on each rabbit to increase the number of defects without increasing the number of animals (Nyan et al., 2009). There is an ongoing debate about the size of bone defects in rabbit calvarial bone. It is important to ensure the bone defect in the control group does not heal spontaneously during the study. Previous studies have shown that 6-mm diameter defect in rabbit calvarium is sufficient for evaluating new bone formation within a short timeframe, so we used this in our study (Acar et al., 2015). Indeed, the defect in our control group was not completely healed by the end of the third week.

Histomorphometry is considered the gold standard for evaluating bone healing, as it facilitates in situ analysis of bone cells and their activities (Acar et al., 2015; Iwaniec, Wronski, & Turner, 2008). In addition, micro-CT is considered a useful and reliable method for evaluating bone healing. Maréchal et al. (2005) reported that micro-CT is as effective as histomorphometry, yielding concordant results. Indeed, micro-CT and histomorphometry yielded similar results in our analysis of new bone formation.

Bone healing is a complex and lengthy process of inflammation, bone formation, and bone remodeling; however, medical applications such as laser and ultrasound may accelerate the healing process. Laser applications have been used in medical treatments since the 1960s. Despite their long history of medical use, the mechanism by which LLLT enhances tissue healing is not fully understood. When laser light is absorbed by tissue, mitochondrial activity increases, as does local blood circulation, ATP synthesis, cellular activity, collagen synthesis, and the release vascular endothelial growth factor (VEGF) (Kazancioglu et al., 2013; Pires Oliveira et al., 2008; Stein et al., 2008; Fávaro-Pípi et al., 2010; Matsumoto, Ferino, Monteleone, & Ribeiro, 2009). The positive effects of LLLT on bone healing have been demonstrated in several in vitro and in vivo studies (Kazancioglu et al., 2013; da Silva & Camilli, 2006; Nascimento et al., 2010; Barbosa et al., 2013; Pires Oliveira et al., 2008; Stein et al., 2008). LLLT is used to enhance bone healing and to accelerate the regeneration of fractures and osteointegration of orthopedic implants (da Silva & Camilli, 2006).

It is important to ensure that the dose and duration of laser application are within the therapeutic range. The optimal LLLT protocol has not been defined yet, but dose range between 0.001 and 16 J/cm^2 have been accepted as a therapeutic window (Kazancioglu et al., 2013; Matsumoto et al., 2009). In this study, we applied the GaAlAs diode laser for 120 s at a dose of 4 J/cm^2 per session based on a previous study of rat calvarial bone defects (Kazancioglu et al., 2013). Based on our results, 4 J/cm^2 per session seems to be effective for stimulate bone regeneration.

Ultrasound may increase protein and collagen synthesis in human fibroblasts (Webster, Harvey, Dyson, & Pond, 1980). Wang et al. (2004) reported that ultrasound increases expression of vascular endothelial growth factor (VEGF) in human osteoblasts. In addition, several in vivo studies have shown that LIPUS accelerates healing in bone fracture, distraction osteogenesis, and sutural bone. Toy, Oztürk, Altindiş, Kozacioğlu, and Toy (2014) reported that LIPUS enhances transforming growth factor beta (TGF-β), VEGF, and osteocalcin expression in rat premaxillary sutures after expansion. LIPUS affects the inflammation, bone formation, and bone remodeling stages of bone healing (Azuma et al., 2001).

Several studies have investigated the effect of LLLT and LIPUS on bone healing, but few have explored their effect on new bone formation. Fávaro-Pípi et al. (2010) compared the effects of LLLT and LIPUS on new bone formation in rat tibial defects. They reported a positive effect for LLLT but 12 sessions of LIPUS had no effect on new bone formation 25 days post-surgery. In contrast,

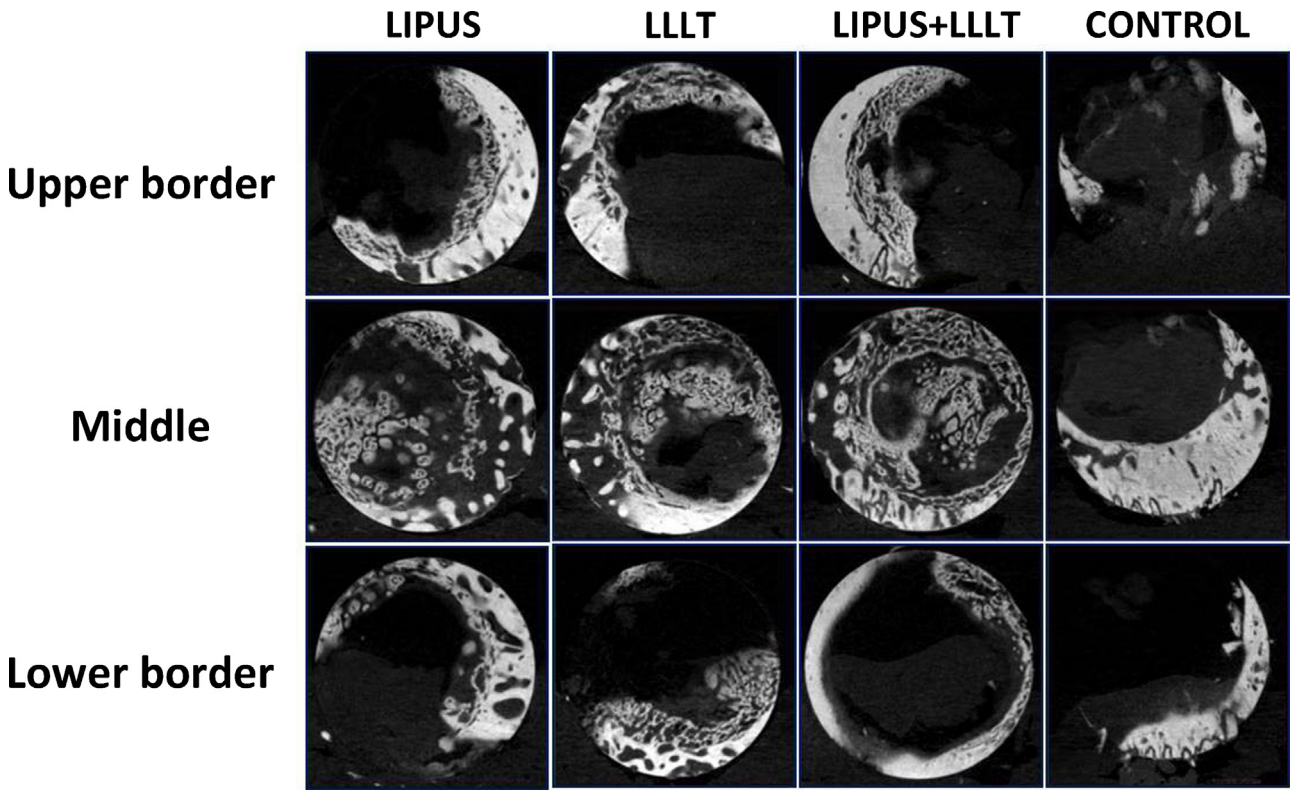

Fig. 5. Micro-CT images of new bone formation.

Lirani-Galvão, Jorgetti, and da Silva (2006) found that 12 sessions of LLLT and LIPUS enhanced bone repair in rat tibial defects after 20 days. Fındık and Baykul (2014) reported that 15 days of daily 20-min session of 30 mW/cm^2 LIPUS treatment accelerates bone healing. In addition, Hasuike et al. (2011) demonstrated that daily application of LIPUS increases bone regeneration on rat calvarial defects at 21 and 28 days after surgery. In our study, six LIPUS sessions over two weeks had a positive effect on new bone formation at three weeks post-surgery. The variation in results may be explained by differences in LIPUS application or in differences between animal and defect models.

To the best of our knowledge, the combined effect of LLLT and LIPUS on bone defects has not been explored. LLLT and LIPUS have similar effects on bone tissue; both accelerating osteogenesis and bone healing. Matsumoto et al. (2009) stated that LLLT improves bone healing in rat tibia by upregulating cyclooxygenase-2 (COX-2) expression in bone cells. LIPUS may also up-regulate COX-2 (Tang et al., 2006), which is a key regulator of prostaglandin synthesis. Simon, Manigrasso, and O'Connor (2002) reported that COX-2 improves bone fracture healing and enhances endochondral ossification. Several groups have demonstrated that LLLT and LIPUS stimulate alkaline phosphatase (ALP), which is a marker of osteoblast activity (Angle et al., 2011; Matsumoto et al., 2009; Coombe et al., 2001). In a study of rat femur defects, it was reported that application of daily ultrasound enhanced ALP values after 18 days (Fındık & Baykul, 2014). In both cases, the stimulatory effect of therapy was observed in the early stage of bone healing (Angle et al., 2011; Fındık & Baykul, 2014).

Inflammation is the first response to bone injury. A hematoma forms and lymphocytes, endothelial cells, and fibroblasts migrate to the injury site. Several studies have shown that laser and ultrasound stimulate proliferation of fibroblasts and endothelial cells. Laser and ultrasound also stimulate VEGF and may thus enhance angiogenesis in during bone healing (Pires Oliveira et al., 2008; Stein et al., 2008; Gebauer et al., 2002; Angle et al., 2011; Coombe et al., 2001; Doan, Reher, Meghji, Harris, 1999; Matić, Lazetić, Poljacki, Duran, & Ivkov-Simić, 2003).

Our results showed that LIPUS and LLLT enhance new bone formation, but provide no additive effect when applied in combination. First, this may be related to the duration and dose of the LLLT and LIPUS applications or to the mechanism by which these treatments enhance bone formation. Second, we think there is a limit to the biological response to stimuli, such that additional stimulus may produce no more effect. Third, combined applications may be tend to inhibit of each other positive effect on bone tissue.

In conclusion, LLLT and LIPUS enhanced new bone formation in the third week of our experiment. The optimal dose and duration of LLLT and LIPUS remain unknown, pending future investigation.

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