Lasers Med Sci (2003) 18: 89–94
DOI 10.1007/s10103-003-0260-z

ORIGINAL ARTICLE

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Effect of low-power GaAlAs laser (660 nm) on bone structure and cell activity: an experimental animal study

Received: 15 January 2003 / Accepted: 22 January 2003

Abstract Low-level laser therapy (LLLT) is increasingly being used in the regeneration of soft tissue. In the regeneration of hard tissue, it has already been shown that the biomodulation effect of lasers repairs bones more quickly. We studied the activity in bone cells after LLLT close to the site of the bone injury. The femurs of 48 rats were perforated (24 in the irradiated group and 24 in the control group) and the irradiated group was treated with a GaAlAs laser of 660 nm, 10J/cm^2 of radiant exposure on the 2nd, 4th, 6th and 8th days after surgery (DAS). We carried out histomorphometry analysis of the bone. We found that activity was higher in the irradiated group than in the control group: (a) bone volume at 5 DAS ($p = 0.035$); (b) osteoblast surface at 15 DAS ($p = 0.0002$); (c) mineral apposition rate at 15 and 25 DAS ($p = 0.0008$ and 0.006); (d) osteoclast surface at 5 DAS and 25 DAS ($p = 0.049$ and $p = 0.0028$); and (e) eroded surface ($p = 0.0032$). We concluded that LLLT increases the activity in bone cells (resorption and formation) around the site of the repair without changing the bone structure.

Keywords Bone · Histomorphometry · Low-level laser therapy · Rats

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Introduction

The stimulation of cellular activity in bone tissue post injury has been studied in recent decades. Several of these studies were performed using grafts of autogenous bone [1], bone morphogenetic protein [2–4], insulin growth factor-I [5, 6], ultrasound [7–9], electromagnetic waves [10], and recently with low-level laser therapy (LLLT) [11–13]. LLLT in soft tissues has been used clinically in many countries to speed up the healing of wounds and to control pain [14–18]. Several groups have studied the effect of LLLT on soft tissue, but few have studied its effect on bone. Using LLLT on bone has been shown to be effective in modulating inflammation [19] and accelerating cell proliferation [20] and the bone healing process [21–27]. On the other hand, LLLT seems to be ineffective when it is used on normal tissue [23]. Conclusions about the effects of LLLT on bone are still controversial, as reports have shown different or conflicting results [28, 29] or have used qualitative methods of analysis [21, 30].

In this study we quantitatively evaluated the effects of GaAlAs laser (660 nm) on the activity of rat bone cells during the inflammatory period in vivo close to the site of the bone injury. Using the experimental model in vivo we studied the cell activity and structural behavior of the bone tissue post LLLT, and carried out a histomorphometric analysis [31, 32].

Materials and methods

The experiment was performed using 48 male *Rattus norvergicus albinus* (Wistar lineage) whose body weights ranged from 250 to 300 g. These were randomly divided into irradiated ($n = 24$) and control ($n = 24$) groups. The animals were kept under constant conditions of temperature (20 ± 1 °C) and light (12 h light/dark cycle) with ad libitum access to food and water.

Surgical procedure

The animals were anaesthetised with Zoletil (Virbac) administered by intramuscular injection at a dose of 50 mg/kg (1 ml/kg) of

weight. The right legs of the animals were shaved and cleansed with 2% alcoholic iodine solution. Access to the femur was obtained by means of a longitudinal incision 2.0 cm long on the skin and subcutaneous tissue, and a small bone window was opened without cutting muscle tissue. After exposure, the femur distal epiphysis was perforated with a Zeckrya (Maillefer) surgical bone drill 1 mm diameter × 2 mm in depth, coupled to a micromotor (25 000 rpm, 405n Dentec) under constant refrigeration with sterile 0.9% saline solution (Fig. 1). The wound was sutured using nylon (4.0). The animals were kept under daily observation throughout the experimental period. No clinical evidence of complications was observed during that period [13].

On the same day of surgery and 24 h before sacrifice, the mineral apposition rate was marked with the administration of Terramycin (Pfizer) by intramuscular injection at a dose of 20 mg/kg (1 ml/kg) [32].

Laser irradiation

We used a GaAlAs laser of 660 nm wavelength from Dermolaser (Solaris, Brazil), with a spot area of 0.08 cm^2, a power output of 0.005 W and an energy of 0.8 J. We irradiated on the 2nd, 4th, 6th and 8th days after surgery (DAS), with l0 J/cm^2 of radiant exposure and 0.0625 W/cm^2 of irradiance. We irradiated one point, perpendicular to the surface of the sutured skin. The animals were anaesthetised with Zoletil (Virbac) administered by intramuscular injection at a dose of 15 mg/kg (0.3 ml/kg). The control animals received the same procedure as the irradiated animals but with the laser shut off.

Sample collection and analytical procedure

The animals were sacrificed on the 5th, 15th and 25th DAS. After dissection of the femur, the distal segment was fixed in 70% ethanol, dehydrated, embedded in methylmethacrylate, and sectioned longitudinally using a Policut S microtome (Reichert-Jung, Heidelberg, Germany). We obtained 5 and 10 μm sections from the centre of each specimen. The 5 μm section was stained with 0.1% toluidine blue at pH 6.4 and at least two non-consecutive sections were examined for each sample. The static indices of the bone structure were: (a) the bone volume as a percentage of the tissue volume (BV/TV, %); (b) trabecular thickness (Tb.Th, μm); (c) trabecular separation (Tb.Sp, μm); and (d) trabecular number (Tb.N, /mm). The static indices of bone formation were: (a) the osteoid volume as a percentage of the bone volume (OV/BV, %); (b) the osteoid surface as a percentage of the bone surface (OS/BS, %); (c) the osteoblast surface as a percentage of the bone surface (Ob.S/BS, %); and (d) the osteoid thickness (O.Th, μm). The static indices of bone resorption were: (a) the osteoclast surface as a percentage of the bone surface (Oc.S/BS, %); and (b) the eroded surface as a percentage of the bone surface (ES/BS, %). These indices were measured at a standardised site below the growth plate in the secondary spongiosa using a semiautomatic method (Osteometrics Inc., Atlanta, GA).

We obtained dynamic data using a Zeiss integrating eyepiece II or a calibrated eyepiece. The dynamic bone formation index was the mineral apposition rate (MAR, μm/day), obtained from unstained 10 μm sections examined by fluorescent light microscopy (Nikon, Tokyo, Japan).

All histomorphometric indices were reported according to the standard nomenclature recommended by the American Society of Bone and Mineral Research [32]. All data of the animals were obtained by double-blind measurements.

Statistical analyses

We used Prism 2.0 software to carry out the statistical analyses. We also used Student's *t*-test and one-way analysis of variance (ANOVA), complemented by the Bonferroni test. The significance was set at $p < 0.05$.

Ethical aspects

The animals were cared for in accordance with national guidelines for the humane treatment of laboratory animals. The study was approved by the Committee of Ethics in Research (CER) at the Vale of Paraiba University, São José dos Campos (SP), Brazil, under protocol number 026/2000/CER.

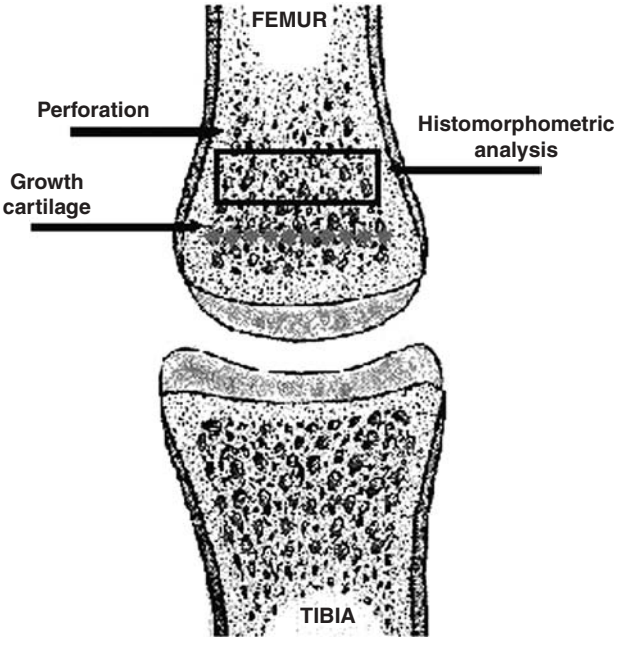

Fig 1 Schematic representation of the perforation areas and bone histomorphometry

Results

Indices of bone structure (Table 1 and Fig. 2)

Bone structure analysis. The bone volume, trabecular thickness, trabecular separation and trabecular number of both groups (irradiated and control) behaved in a similar way. The curves tended to coincide at 25 DAS. However, bone volume in the irradiated animals at 5 DAS was significantly higher statistically ($p = 0.035$) than in the control group (Fig. 2).

Indices of static and dynamic bone formation (Table 2, Figs. 3, 4 and 5)

For static bone formation we analysed osteoid volume, osteoid surface, osteoblast surface and osteoid thickness. At 15 DAS the osteoblast surface in the irradiated animals was significantly higher statistically ($p = 0.0002$) than in the control group (Fig. 3). At 25 DAS, the osteoid volume in the treated animals was significantly higher statistically ($p = 0.047$) than in the control group (Fig. 4). At between

Table 1 Analysis of histomorphometry of the structural indices of the control and irradiated groups

DAS	Tb.Th (μm) *CTR vs. IRD*	Tb.Sp (μm) *CTR vs. IRD*	Tb.N (/mm) *CTR vs. IRD*
5	48.6 + 2.6 vs. 54.5 + 1.7	112.1 + 6.6 vs. 99.4 ± 4.9	6.3 ± 0.3 vs. 6.6 ± 0.2
15	48.9 ± 1.0 vs. 51.6 ± 2.3	101.9 ± 8.0 vs. 83.2 ± 4.8	6.8 ± 0.4 vs. 7.5 ± 0.3[a]
25	65.3 ± 2.9[c, d] vs. 61.3 ± 2.5[b]	104.2 ± 6.4 vs. 102.9 ± 4.3[b]	6.0 ± 0.2 vs. 6.1 ± 0.2[d]

Tb.Th (μm), trabecular thickness; **Tb.Sp (μm)**, trabecular separation; **Tb.N (/mm)**, trabecular number. Level of statistical significance in the comparison of the control (CTR) and irradiated (IRD) groups and experimental times, $p < 0.05$: [a] (5 vs. 15 DAS), [b] (5 vs. 25 DAS), $p < 0.01$: [c] (5 vs. 25 DAS), [d] (15 vs. 25 DAS). Data are expressed as means ± SEM

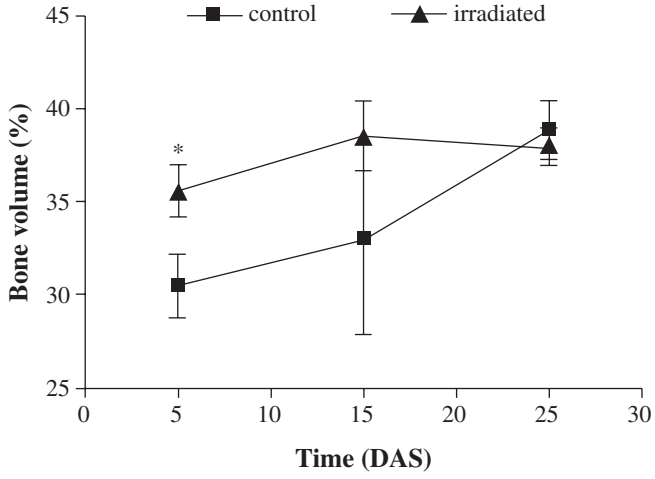

Fig 2 Bone volume (BV/TV, %): mineralised and non-mineralised trabecular bone tissue in relation to tissue volume against DAS. Analysis of bone injury in the control and irradiated groups. Symbols represent mean ± SEM.*5 DAS $p = 0.035$

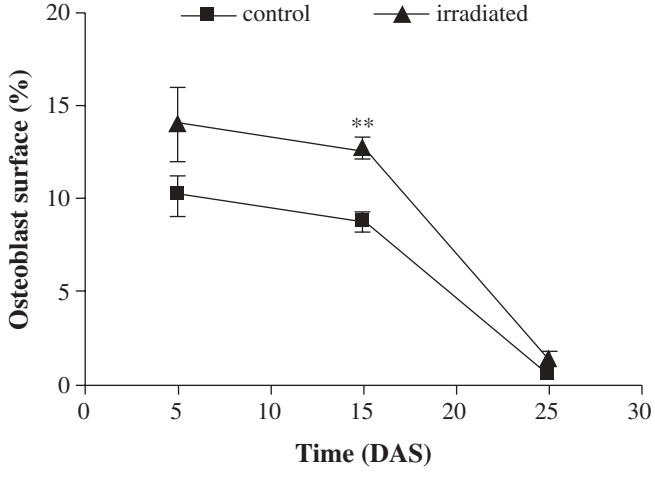

Fig. 3 Osteoblast surface (Ob.S/BS, %): osteoblast cell surface as a percentage of bone surface against DAS. Analysis of bone injury in the control and irradiated groups. Symbols represent mean ± SEM.** 15 DAS $p = 0.0002$

5 and 25 DAS the osteoid thickness had decreased significantly in the control group ($p < 0.01$), but did not reach statistical significance in the irradiated group during the time of the experiment (Table 2).

Between 15 and 25 DAS the mineral apposition rate (dynamic index) decreased significantly ($p < 0.01$) in

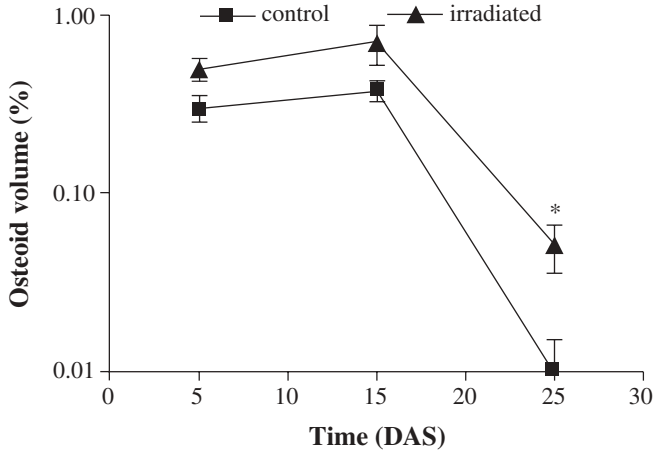

Fig. 4 Osteoid volume (OV/BV, %): bone tissue not mineralised in relation to bone volume against DAS. Analysis of bone injury in the control and irradiated groups. Symbols represent mean ± SEM. * 25 DAS $p = 0.047$

both groups. Moreover, in the irradiated group the rate was higher on 15 DAS ($p = 0.0008$) and 25 DAS ($p = 0.0061$) than in the control group (Fig. 5).

Indices of bone resorption (Figs. 6 and 7)

On 5 DAS and 25 DAS the osteoclast surface was significantly higher ($p = 0.049$ and $p = 0.0028$, respectively) in the irradiated group than in the control group (Fig. 6). The eroded surface (Fig. 7) was also higher ($p = 0.0032$) in the irradiated group than in the control group at 25 DAS.

Discussion

It is difficult to compare the results of studies involving LLLT in the literature because of the wide variety of methods used [25, 30, 33]. Few studies have used quantitative methods to analyse LLLT on bone tissue [27]. Mester [34] and Sarti et al. [35] reported that LLLT had systemic effects, which may explain the negative results of other authors who used the control and irradiated sites in the same animal [28].

LLLT in vitro studies referred to DNA synthesis improved alkaline phosphatase levels and protein synthesis

Table 2 Analysis of histomorphometry of bone formation indices of the control and irradiated groups

DAS	OS/BS (%) *CTR vs. IRD*		O.Th (μm) *CTR vs. IRD*
5	16.7 ± 2.1	vs. 25.1 ± 3.8	1.0 ± 0.1 vs. 1.2 ± 0.1
15	15.2 ± 1.8	vs. 19.4 ± 1.4	1.2 ± 0.1 vs. 1.6 ± 0.4
25	1.1 ± 0.4[a, b]	vs. 2.8 ± 0.8[a, b]	0.7 ± 0.1[a] vs. 1.1 ± 0.2

OS/BS (%), osteoid surface; **O.Th (μm)**, osteoid thickness. Level of statistical significance in the comparison of the control (CTR) and irradiated (IRD) groups and experimental times: $p < 0.01$. [a] (5 vs. 25 DAS), [b] (15 vs. 25 DAS). Data are expressed as means ± SEM

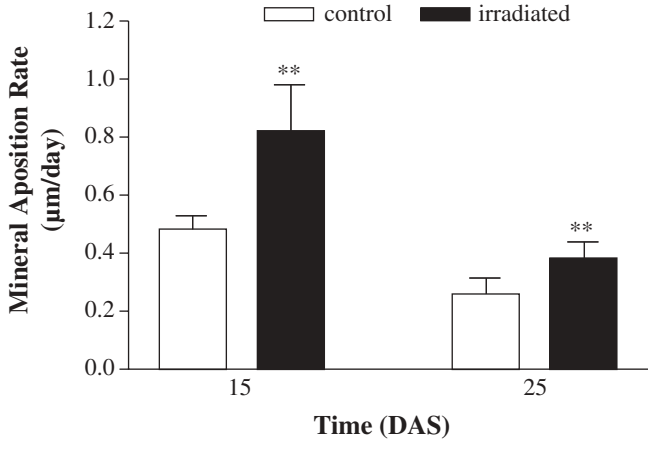

Fig. 5 Mineral apposition rate (MAR, μm/day): distance between two demarcations for tetracycline divided by the time interval between the two demarcations, expressed in micrometers/day, against DAS. Analysis of bone injury in the control and irradiated groups. Bars represent mean ± SEM. ** 15 DAS $p = 0.0008$ and ** 25 DAS $p = 0.0061$

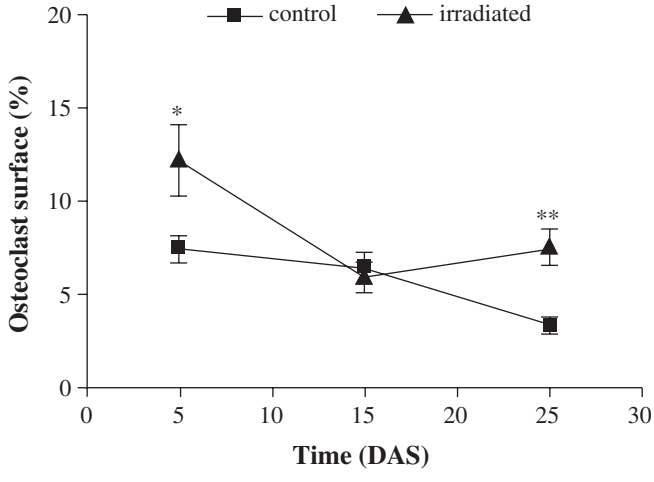

Fig. 6 Osteoclast surface (Oc.S/BS, %): osteoclast cells surface as a percentage of bone surface against DAS. Analysis of bone injury in the control and irradiated groups. Symbols represent mean ± SEM. * 5 DAS , $p = 0.049$ and ** 25 DAS $p = 0.0028$

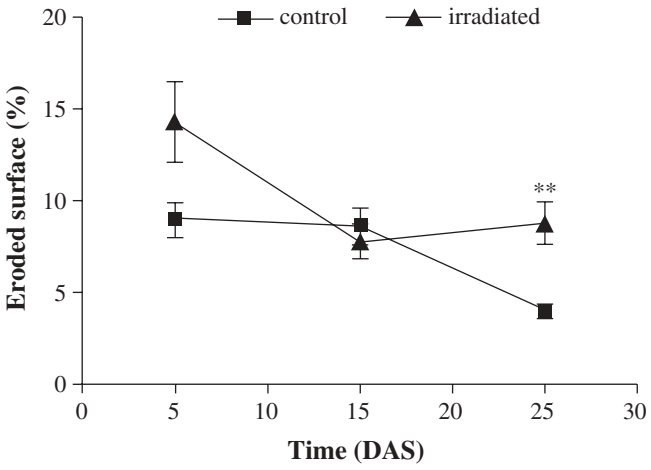

Fig. 7 Eroded surface (ES/BS, %): trabecular surface with gaps of eroded bone (gaps of Howship) with the presence or otherwise of osteoclasts as a percentage of bone surface against DAS. Analysis of bone injury in the control and irradiated groups. Symbols represent mean ± SEM. ** 25 DAS $p = 0.0032$

[36]. Osawa et al. [37] analysed the number of mitoses and the alkaline phosphatase levels in osteoblasts and observed similar stimulatory results with irradiated culture cells. In in vitro studies, Baruska et al. [33] and Yaakobi et al. [29] evaluated osteoblast activity through the levels of alkaline phosphatase, and found elevated levels after irradiating animals with HeNe laser. Using the same technique, Yaakobi et al. [29] analysed the calcium accumulation rate. This was 50% higher in the irradiated group than in the control group. Sathaiah et al. [11] used an HeNe laser and analysed the bone using Raman spectroscopy. They found that after 15 DAS the concentration of calcium hidroxyapatite increased. They reported that this showed increased osteoblast activity and, therefore, improvements in the organic matrix formation and mineralisation [38]. Our results corroborate those of Sathaiah et al. and Freitas et al. [26] because both the osteoblast surface (Fig. 3) and the mineralisation apposition rate (Fig. 5) were greater in our irradiated animals than in our controls. This high osteoblast activity at 15 DAS was directly related to that at 25 DAS (Fig. 4), which was significantly higher in the irradiated animals than in the control group.

The osteoclast cell behaviour we found has not previously been reported in the literature. We found that the osteoclast surface increased significantly at 5 DAS (Fig. 6) in animals treated with LLLT. We assume that two mechanisms were involved. First, this laser wavelength directly influenced the osteoclast. Osteoclast are multinuclear cells, with many mitochondria of high activity [39]. According to Karu et al. [40], the rmtochondrial cytochromes absorb the photon energy in the visible part of the electromagnetic spectrum, and this absorption increases ATP synthesis and improves the potential activity of the cells. Second, osteoclast activity may influence posterior osteoblast activity [38, 41], and vice versa. On the other hand,

Wiegand-Steubing et al. [42] showed that LLLT increases the concentration of tumour necrosis factor (TNF) in culture cells. Schenk [39] and Gerstenfeld et al. [43] also reported that TNF contributes positively to bone cell activity post injury. Zaidi et al. [44] reported that both osteoblasts and osteoclasts have hormonal interaction. Hentunen et al. [45] reported that the bone matrix liberates a protein that stimulates osteoclast formation, which is light-dose dependent. In agreement with this, our increased osteoclasts at 5 DAS and increased osteoblasts at 15 DAS explain the high osteoclast surface and the eroded surface at 25 DAS. They also explain the higher resorption levels in the irradiated animals. The greater development of the resorption index at 25 DAS is directly proportional to the stimulated indices of formation in the intermediate stage (15 DAS).

Our results with LLLT show that activity in the bone cells around the injury site, especially osteoclast activity, increases. However, there were no alterations in the bone architecture with normal structural indices. In conclusion, LLLT, used in the inflammatory period of the process of bone repair, increases normal cell activity (resorption and formation).

Acknowledgement The authors are grateful to Martin J. C. Van Gemert of the Academic Medical Center in Amsterdam, The Netherlands, for commenting on the manuscript.

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