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Computerized Morphometric Assessment of the Effect of Low-Level Laser Therapy on Bone Repair: An Experimental Animal Study

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ABSTRACT

Objective: **The aim of this study was to evaluate morphometricly the amount of newly formed bone after GaAlAs laser irradiation of surgical wounds created in the femur of rats.** ***Background Data:*** **Low-level laser therapy (LLLT) has been used in several medical specialties because of its biomodulatory effects on different biological tissues. However, LLLT is still controversial because of contradictory reports. This is a direct result of the different methodologies used in these works.** ***Materials and Methods:*** **In this study, 40 Wistar rats were divided into four groups of 10 animals each: group A (12 sessions, 4.8 J/cm^2 per session, observation time of 28 days); group C (three sessions, 4.8 J/cm^2 per session, observation time of 7 days). Groups B and D acted as nonirradiated controls. The specimens were routinely processed to wax and cut at 6-μm thickness and stained with H&E. For computerized morphometry, Imagelab® software was used.** ***Results:*** **Computerized morphometry showed a significant difference between the areas of mineralized bone in groups C and D ($p = 0.017$). There was no difference between groups A and B (28 days; $p = 0.383$).** ***Conclusion:*** **It is concluded that, under this experimental condition, LLLT increased bone repair at early bone healing.**

INTRODUCTION

TISSUE HEALING is a complex process that involves local and systemic responses. The process of wound healing involves several types of cells, enzymes, growth factors, and other substances. The use of LLLT for wound healing has been shown to be effective in modulating both local and systemic response. On soft tissues, it has been shown that, depending on the wavelength, dose, and local condition, LLLT has an antiinflammatory effect, reduces pain, and accelerates cell proliferation and, consequently, the healing process.[1]

The healing of bone differs from that observed on soft tissue because of these morphological characteristics. Usually, the healing process of the bone is slower than that of soft tissues. The natural course of bone healing includes consecutive phases and differs according to the type and intensity of the trauma and also the extension of the damage to the bone.

The effects of LLLT on bone are still controversial, as previous reports show different or conflicting results. It is possible that the LLLT effect on bone regeneration depends not only on the total dose of irradiation, but also on the irradiation time and the irradiation mode.[2] Most importantly, recent study has suggested that the threshold parameter energy density and intensity are biologically independent of one another. This independence accounts for the success and the failure of LLLT achieved at low-energy density levels.[3]

Previous study on the effect of LLLT on fractures describes an increased osteoblastic activity and also increased number of blood vessels and in the amount of mineralized bone.[4] It has also been shown that liberation of prostaglandin E_2 seems to

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contribute to the process of healing of fractures.[4] Increased release of this enzyme after irradiation was described in a previous report.[5]

MATERIALS AND METHODS

Forty adult male and female Wistar rats (*Ratus norvegicus*), weighing an average of 250 g, were obtained from the Animal House of the Department of Pharmacology of the Center of Health Sciences of the Federal University of Pernambuco. The animals were kept in individual cages under natural conditions of light, temperature, and humidity, had water *ad libidum,* and were fed with standard laboratory pelted diet at the Nucleus of Experimental Surgery of the Department of Surgery of the Center of Health Sciences of the Federal University of Pernambuco, where the surgical procedures were also performed.

The animals were randomly distributed into four groups, each one with 10 animals as follows: group A (12 sessions, 4.8 J/cm^2 per session, observation time of 28 days); group C (three sessions, 4.8 J/cm^2 per session, observation time of 7 days). Groups B and D acted, respectively, as nonirradiated controls.

Under GA (Rompun® and Ketamine (Dopalen®) diluted in a relationship of one part of Dopalen® for one part of Rompun® with a dose of 0.1 mL/100 g of weight), the right leg of the animal was shaved and cleaned with a 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. After exposure the femur was divided in three portions (superior, medium, and inferior); the median portion of the bone was the place of choice for creating a mechanical bony defect with a low-speed no. 6 drill under constant refrigeration with sterile 0.9% saline solution. The defect measured approximately 1 cm^2. 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 the period.

Forty-eight hours after surgery,[6] the defects of the experimental groups A and C were irradiated in a contact mode with a continuous wave (CW) 40-mW 830-nm diode laser ($\phi \sim 1$ mm), with a total dose of 4.8 J/cm^2. The laser was applied transcutaneously, with the handpiece perpendicularly positioned on the wound.[7] Irradiation was performed three times a week, resulting in a total of 12 applications (57.6 J/cm^2) on group A and three applications (14.4 J/cm^2) on group C. The applied doses were in accordance with previous clinical studies, which varied from 1.8 to 5.4 J/cm^2.[2,8,9]

The animals were humanely sacrificed, and the specimens were surgically removed, kept in a 10% formaline solution, and routinely processed at the laboratory of Oral Pathology of the Instituto de Biociências of the PUCRS. The specimens were cut at 6 μm and routinely stained with H&E. Computerized morphometry using a specific software system of processing of the images (Imagelab®) was performed at the Biological Specimens Laboratory of the IP&D of the Universidade do Vale do Paraiba.

As the image analysis software needed similar images in terms of color for a more precise analysis, the best image of individuals of each group were selected for this analysis as follows: group A (seven images, seven animals) B (seven images, seven animals, C (six images, six animals), and D (five images, five animals); at least three animals of the experimental groups and two of their controls were used. The computerized system was calibrated to acquire a relationship of (1 pixel = 6.5 μm). The area to be measured wsa delimited (Fig. 1) and quantified by the software as described previously.[9–12] The results of the measurements were recorded and submitted to statistical analysis by Mann-Whitney test.

RESULTS

Table 1 shows the comparison between the mean number of pixels of the area measured, as shown in Figure 1 of irradiated and nonirradiated subjects. Figure 2 shows the results of the measurements' mean number of pixels of the area measured as shown in Figure 1 obtained for irradiated and nonirradiated specimens at 7 days after surgery. The Mann-Whitney test showed a significant difference between irradiated and nonirradiated groups ($p = 0.017$; Table 2) and within this experimental group ($p = 0.01$). Alternatively, the Mann-Whitney test was not significant in analysis of the mean areas of irradiated and nonirradiated defects 28 days after surgery ($p = 0.383$; Table 2). The results of the measurements can be seen on Figure 3.

DISCUSSION

Biomodulation is undoubtedly one area of controversy in the use of LLLT. Although the effect of LLLT on soft tissue has been studied by several groups, there are few works on the effect of LLLT on bone. Some previous reports do recognize that LLLT has a positive effect on bone.[2,13] These studies reflect the idea that nondifferentiated mesenchymal cells could be biomodulated positively to osteoblasts that would more rapidly change to osteocytes. This aspect is corroborated by several previous studies in which LLLT was used in fractures,[14] in bone defects,[7] in tooth extraction,[15] and after the placement of dental implants.[16] On the other hand, LLLT seems ineffective when used on normal tissues.[1,2] Biomodulating effects of LLLT observed by other researchers demand some level of tissue deficiency.[1,17]

It is known that the osteogenic potential of mesencyhmal cells depends on several genetic factors and also on systemic and local inducer factors.[18] LLLT may act as such an inducer factor. However, a report[4] suggested that LLLT would improve bone matrix production due to improved vascularization and antiinflammatory effect. These aspects would result in an increase of both the release of mediators and microvascularization, which would subsequently accelerate bone healing.

It has been observed that PGE_2 activates wound healing,[19] and increased levels of PGE_2 were observed by others.[5,20] There is evidence that PGE_2 is also produced by osteoblasts and that its effects may be therapeutic or adverse.[11]

We reported wavelength dependency of the effects of LLLT irradiation of malignant cells[21] previously and, more recently, the need of some level of deficiency for the effect of LLLT to be detected.[17]

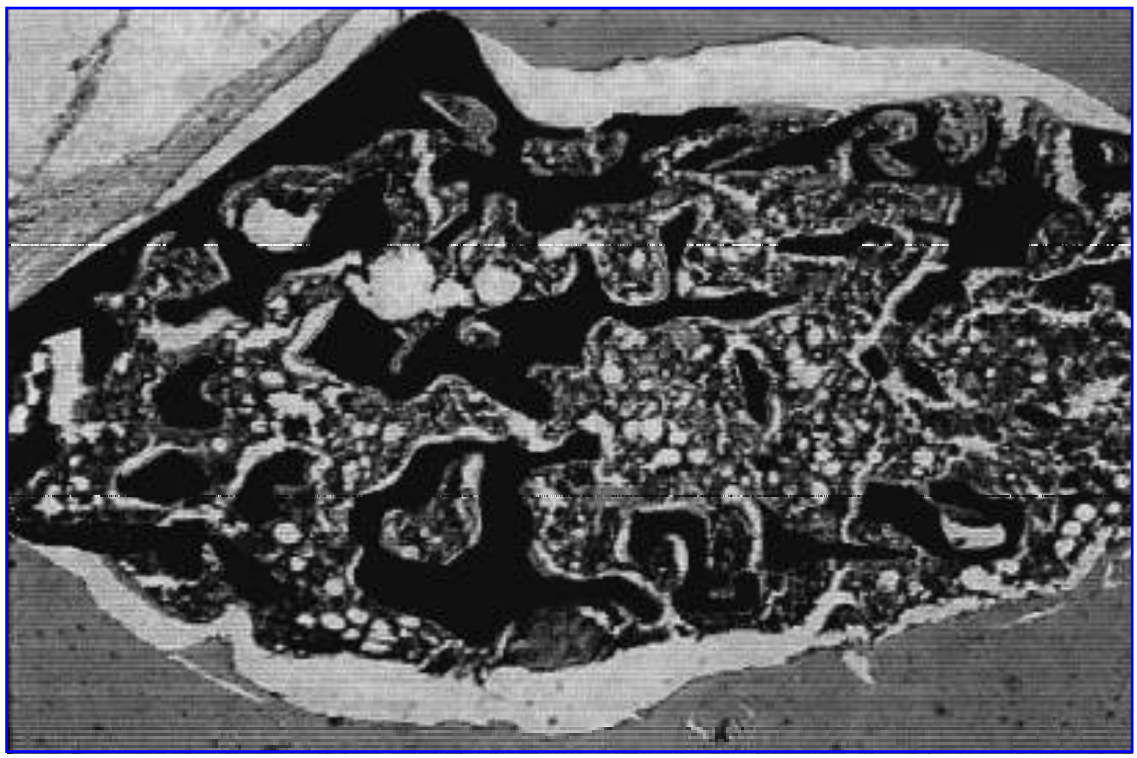

FIG. 1. Imagelab® system was used to measure the amount of newly formed bone. The area in black was measured to determine the area. H&E stain; original magnification × 40.

In the previous study, it was shown that the irradiation of bone with 830-nm laser light following the insertion of dental implants did improve both formation and quality of the neoformed bone around the implant.[16] The choice of IR laser light in this study was due to its higher penetration by the tissues, which was found to penetrate about 2 mm before significant loss occurred.[22]

In this study, in accordance with the findings of previous study[23] a low dose that a low dose ranging from 1–4 J/cm^2 was maintained. Although some studies recommended higher doses,[16,24] clinical experimentation with LLLT[8,9] has shown doses within the range of 1–5 J/cm^2 to be effective.[2,4,5,7,8,9,16,23,25,26]

Exposure time and intensity in this study were 120 sec and 40 mW, respectively, which is in accordance with suggestions that the strongest biomodulatory effects are observed at exposures timing ranging from 30 to 120 sec.[27]

TABLE 1. COMPARISON BETWEEN THE MEANS OF THE AREAS (PIXELS) ON IRRADIATED AND NONIRRADIATED BONE DEFECTS AT BOTH EXPERIMENTAL PERIODS

Group	*Time*	*Mean area (pixels)*
Irradiated	7 days	2,852,629.12
	28 days	861,794.15
Nonirradiated	7 days	1,561,740.66
	28 days	655,798.96

The controversy observed published results is due to different protocols employing varying wavelengths, association of wavelengths, modes of emission, and doses in different animal or cell models.[28,29] No method is perfect, but we tried to use a reproducible method of measurement in the present investigation. Tissue morphology and the shape and distribution of the trabeculae may differ in the samples, which would lead to imprecise interpretation of the results found in this study. However, we used serial cuts to prevent greater variation in the reading, Approximately the same serial cut of each specimen was used for the computerized analysis.

The computerized analysis showed itself effective in measuring the area of new-formed bone and confirmed the findings

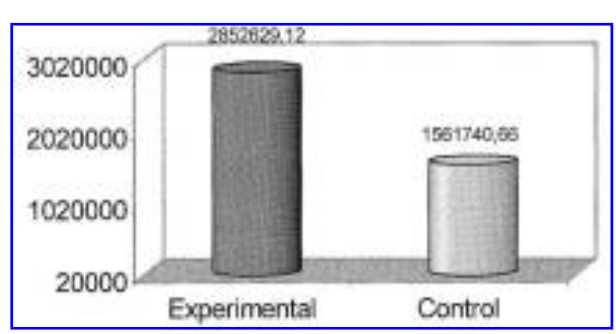

FIG. 2. Comparison of the mean area (pixels) of newly formed mineralized bone between the experimental group and control in the period of observation of 7 days.

TABLE 2. COMPARISON OF THE MEAN AREAS OF NEWLY FORMED MINERALIZED BONE MEASURED (PIXELS) ON IRRADIATED AND NONIRRADIATED SAMPLES AT DAYS 7 AND 28 AFTER SURGERY

Group	*Mean (area in pixels)*	*SD*	*Variation coefficient*	*p*
Irradiated (7 days)	2,852,629.12	745,985.83	26.15%	0.01
Nonirradiated (7 days)	1,561,740.66	248,036.22	15.88%	
Irradiated (28 days)	861,794.15	470,949.95	54.61%	0.383
Nonirradiated (28 days)	655,798.96	298,272.25	45.50%	

of a previous report[24] that also found increased bone proliferation following LLLT using a similar software and immunohistochemistry. This was not aligned with other groups, which did not find a positive effect of LLLT in healing bone[30,31] and others. It is important that some previous reports, which found no biomodulating effects of LLLT, presented some problem in the method used,[32] did consider the systemic effect of LLLT[4,7] and used contralateral procedures as controls.

The findings of this investigation are very close to a study which found intense activity and high numbers of osteoblasts 5–6 days after the procedure was performed on bone defects using the same model as that in on this study.

Previous work using GaAlAs (790-nm) laser with a similar dose as that used in this investigation demonstrated a 10% increase in the amount of mineralized bone at 7 days on irradiated animals.[33] Another study[34] verified progress on bone consolidation with increased formation of trabecular bone and in the number of osteoblasts after the use of HeNe (632.8-nm) laser. The experimental period was 7 days and a total dose of 94.7 J/cm^2 was used for the treatment, higher than our 4.8 J/cm^2. These may indicate a more positive effect of 830-nm laser light in comparison to lasers emitting on 632.8 or 790 nm. This is probably due to a higher penetration of laser light with higher wavelength (Infrared) on the tissues.

CONCLUSION

The results of this study suggest that, under the experimental conditions of the present investigation, the use of LLLT at 830 nm improve bone healing at early stages.

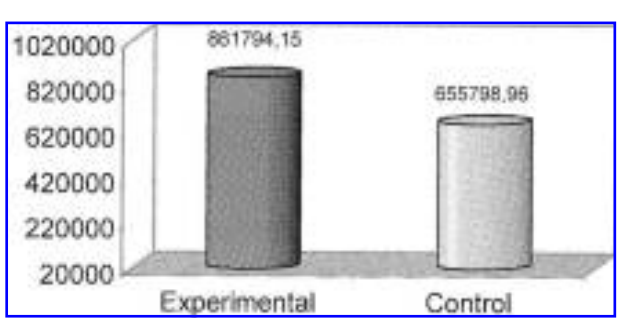

FIG. 3. Comparison between the experimental group and control in the period of observation of 28 days.

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