

Improvement of bone repair in diabetic rats subjected to 780 nm low-level laser therapy¹

Marismar Fernandes do Nascimento^I, Bernadeth Moda de Almeida^I, John Lennon Silva Cunha^{II}, Rafael Barreto Vieira Valois^{II}, Juliana Campos Pinheiro^{II}, Maria Amália Gonzaga Ribeiro^{III}, Sonia Oliveira Lima^{IV}, Ricardo Luiz Cavalcanti de Albuquerque-Júnior^V

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^IFellow PhD degree, Postgraduate Program in Industrial Biotechnology, Universidade Tiradentes (UNIT), Aracaju-SE, Brazil. Acquisition of data, histological examinations, supervision all phases of the study.

^{II}Graduate student, UNIT, Aracaju-SE, Brazil. Technical procedures, histological processing of the surgical samples.

^{III}PhD, Full Professor, Department of Dentistry, Universidade Federal de Sergipe (UFS), Aracaju-SE, Brazil. Supervision of the low-level laser therapy procedures in rodent model.

^{IV}PhD, Full Professor, Laboratory of Morphology and Experimental Pathology, Department of Medicine, UNIT, Aracaju-SE, Brazil. Technical procedures.

^VPhD, Full Professor, Laboratory of Morphology and Experimental Pathology, Department of Medicine, UNIT, Aracaju-SE, Brazil. Design of the study, histological examinations, manuscript writing, supervised all phases of the study.

ABSTRACT

PURPOSE: To investigate the effect of low-level laser therapy on bone healing in diabetic rats.

METHODS: Bone cavities (19 mm diameter) were performed in the femur of 72 alloxan-induced diabetic rats, which were assigned into four groups: CTR (non-diabetic control), DBT (diabetic) CTRL (non-diabetic irradiated) and DBTL (diabetic irradiated). Low-level laser therapy was performed every 48h for seven days. Animals were euthanized at seven, 18 and 30 days. Alkaline phosphatase serum levels and bone repair were analyzed.

RESULTS: Low-level laser therapy significantly increased alkaline phosphatase in at seven and 18 days ($p < 0.001$), and improved bone healing at seven ($p < 0.01$), 18 ($p < 0.05$) and 30 ($p < 0.01$) in diabetic animals. In addition, bone healing in irradiated diabetic group was statistically similar to control group at 30 days ($p > 0.05$).

CONCLUSION: Low-level laser therapy increased the serum levels of alkaline phosphatase and improved bone healing in alloxan-induced diabetic rats.

Key words: Laser Therapy, Low-Level. Diabetes Mellitus, Experimental. Osteogenesis. Alkaline Phosphatase. Rats.

Introduction

Diabetes mellitus (DM) is a common endocrine disorder characterized by deficiency in secretion and/or action of insulin, resulting in persistent hyperglycemia¹. DM has been associated with long-term damage, dysfunction and failure of various organs, as well as retinopathy, nephropathy, peripheral neuropathy and sexual dysfunction².

It has been reported that the DM causes a series of complications on bone metabolism, resulting in osteopenia and significant loss of bone density³. Thus, DM impacts negatively on bone repair in response to imbalance between bone formation and resorption, as well as on the dynamics of the inflammatory⁴. Furthermore, DM has also been associated with imbalance of the activation/inhibition of proteolytic enzymes, partial inhibition of the synthesis and release of angiogenic growth factors, reduced cellular proliferation rates and increased apoptosis⁵. The deleterious impact of DM on bone metabolism has motivated the search for alternative therapies to control or minimize the effects of DM on bone repair, such as new drugs and low-level laser therapy⁶.

Low-level laser therapy (LLLT) comprises a highly concentrated, non-invasive, non-ionizing radiation which, in contact with different tissues, promotes thermal, photochemical and nonlinear effects⁷. It has been reported that LLLT induces osteoblast proliferation and accelerates bone metabolism⁸. In addition, studies have suggested that LLLT can stimulate osteogenesis in fracture sites, promoting greater deposition of bone mass and accelerating fracture consolidation⁹. In addition, bone mineralization also seems to be influenced by laser irradiation once calcium accumulation can be enhanced in 46% over the controls, as well as increased serum levels of alkaline phosphatase, after LLLT irradiation of osteoblasts in culture¹⁰. Although the precise mechanism of laser photobiomodulation is not completely clear, it has been proposed that LLLT stimulates mitochondrial metabolism, culminating in an increased differentiation and proliferation of osteoblasts and subsequent higher bone matrix deposition¹¹.

Previous studies have demonstrated the beneficial effects of LLLT on bone repair in experimental models of DM^{12,13}. However, many other controversial data have been reported in the literature concerning the modulatory effect of LLLT on bone healing in diabetic animals, likely as a result of the variety of threshold parameters for energy density and intensity, including the irradiation time and the irradiation mode, used in the experiments^{14,15}.

Therefore, the purpose of this study was to assess the effect of a protocol of low-level laser therapy using diode GaAlAs laser on the bone repair in alloxan-induced diabetic rats.

Methods

All the procedures for animal research were performed in accordance with the guideline of the Brazilian Council for Animal Experimentation and followed a protocol approved by the Animal Ethics Committee of Universidade Tiradentes (approval subscription n° 020314).

Seventy-two male Wistar rats (250±25 g) were kept in plastic cages with food and water *ad libitum*, light/dark cycle of 12 hours and controlled temperature (22 ± 2°C) and humidity.

Surgical procedures and groups formation

The animals were subjected to dissociative anesthesia with intraperitoneal administration of 0.10 mL/100 g of 10% ketamine (Ketamine®, Rhobifarma Ind. Ltd. Pharmaceutical.) and 0.25 mL/100 mg of xylazine (Anasedan® Purina Agribands of Brazil Ltda.). Noncritical size bone defects were surgically created on right femur, whose depth was guided by the rupture of cortical bone. The surgical procedures were performed using spherical steel drill (n° 4) engaged in low-speed electric motor (1.500 rpm) and irrigated with saline. Subsequently, the animals were randomly assigned into four groups, as described in Table 1.

TABLE 1 - Experimental groups according to the glycemic status and irradiation protocol with low-level laser therapy.

Groups (n=18) ^(a)	Glycemic status	Energy density of low-level laser
CTR	normoglycemic (non-diabetic)	0 j/cm ² ^(b)
DBT	hyperglycemic (diabetic)	0 j/cm ² ^(b)
CTRL	normoglycemic (non-diabetic)	17,5 j/cm ²
DBLT	hyperglycemic (diabetic)	17,5 j/cm ²

^(a) Each group is divided in three subgroups (n=6) to be euthanized at 7, 18 and 30 days.

^(b) The animals were subjected to the same stress conditions of lasertherapy.

DM induction procedures

DM was induced with an intraperitoneal administration of Alloxan monohydrate (Sigma Chemical Co. St. Louis, Mo, USA), diluted in 0.01 M citrate, pH 4.5, at 150mg/ kg. After 12h, the animals received a 10% solution of glucose by gavage to prevent hypoglycemia frames. After 72h, blood samples were collected from the tail vein of animals to assess the glucose serum levels using the Accu-Chek Advantage Kit (Boehringer,

Germany). Such procedure was repeated every seven days for 30 days. Animals whose glucose serum levels were above 200 mg/dL were included in the groups of hyperglycemic rats. As glucose levels in 12 animals were found to be below this value at the monitoring times, they were excluded and replaced until the final sample was complete (18 animals/group).

Low-level laser therapy procedures

Laser irradiation was performed using a laser diode GaAlAs - 780 nm (Twin Laser®, MMOptics, Sao Paulo, Brazil) previously calibrated. The animals were subjected to transcutaneous irradiation by perpendicular contact to the bone defect in a single point, every 48 hours for 7 days. The first irradiation was performed right after the wound synthesis procedure. The irradiation parameters are shown in Table 2.

TABLE 2 - Description of low-level laser therapy parameters.

Parameters of irradiation	Protocol of low-level laser therapy
Emission	Continuous mode
Wavelength	780±10 (infrared)
Light emitter active medium	GaAlAs
Power output	70 mW
Spot size	2.3 mm
Area of the laser beam	0.04 cm ²
Power density	1,75 W/cm ²
Energy density/session	17,5 J/cm ²
Irradiation time/session	10 s
Total irradiation time	40 s
Total energy/session	0,7 J
Beam divergence	17°
perpendicular to the junction	
Tip angle	50°

Assessment of the ALK-P serum levels. Blood samples (1 mL) were collected by cardiac puncture from each animal on the day of euthanizing and stored in dry tubes for further assessment of the serum levels of alkaline phosphatase (ALK-P levels). After coagulation, the tubes were centrifuged for 10 min at 3500 rpm for serum separation. ALK-P levels were measured using an automated analyzer (Abbott, model, Architect C 8000). Previously to the blood samples collection, the animals were subjected to 12h of fasting.

Histological procedures

Six animals of each group were euthanized after seven, 18 and 30 postoperative days in a CO₂ chamber. Subsequently, the bone samples were surgically removed and fixed in 10% formalin for 48h. Thereafter, samples were subjected to decalcification (0.5 M EDTA+TRIS for 30 days) and then dehydrated, diaphanized and embedded in paraffin blocks. Histological sections (15 serial cross sections of 5 µm thick each) were obtained stained with hematoxylin-eosin (HE).

Assessment of the inflammatory reaction

Histological sections stained in haematoxylin-eosin were used for the descriptive analysis of the inflammatory reaction. The intensity of the inflammatory response was assessed in 15 serial histological sections of each animal and classified as follows: 0 (absence of inflammatory response), 1 (inflammatory cells representing less than 10% of the cell population observed within the bone healing area), 2 (inflammatory cells representing between 10% and 50% of the cell population observed within the bone healing area), and 3 (inflammatory cells representing more than 50% of the cell population observed within the bone healing area). Final scores of each group were expressed as mean ± standard deviation (SD).

Histomorphometric analysis of bone neoformation

The bone neoformation content was quantified by the same examiner in a blind study. Histological images of the bone defect were captured at x40 magnification, using an Olympus CX 31 binocular microscope (Olympus Optical do Brazil Ltda., Sao Paulo, Brazil) coupled with an NO C-7070 wide zoom camera (Olympus Optical do Brazil Ltda., Sao Paulo, Brazil). The histological sections of the whole bone defect area were digitalized using the HL Image 2005 program (Western Vision, Salt Lake City, UT, USA) and the assessment of neoformed bone procedure in 15 serial sections of bone defects per specimen were evaluated. The area of interest (under x40 magnification) was determined based on the borders of the bone defect (boundary mature compact bone) and the most superior and inferior points of the bone bridge (linear closure) of the defect. Histological images were transmitted to a microcomputer and the content of trabecular neoformed bone along the entire length of the bone defect was evaluated using the Image-J program (Version 1.32 for Windows). The percentage of bone neoformation was obtained dividing the area newly formed trabecular bone by the predetermined total area of the bone defect.

Statistical analysis

Data related to the glucose and alkaline phosphatase serum levels, as well as bone formation, were subjected to an analysis of variance (ANOVA), and statistically significant results were subjected to *post-hoc* multiple comparisons Tukey's test. Data obtained by the semiquantitative analysis of the inflammatory reaction were subjected to Kruskal-Wallis test and Dunn's multiple comparisons test. A 5% level of significance was adopted in this study ($p < 0.05$).

Results

As demonstrated in Figure 1, the previous treatment of the animals with alloxan promoted significant increase in the glucose levels compared to the control groups over the time course of the experiment ($p < 0.001$). However, the glucose levels were not influenced by the LLLT, neither in normoglycemic nor in hyperglycemic animals ($p > 0.05$). On the other hand, the ALK-P levels increased significantly in the LLLT-treated groups in seven and 18 days ($p < 0.001$), regardless the glycemic status of the animals.

Histomorphological analysis (Figure 2) revealed that, on day seven, both normoglycemic groups presented new bone formation of thin non-lamellar trabecular bone in the cavities, although they appeared to be thicker in CTRL compared to CTR. In DBT, the presence of bone formation was inconspicuous, and the cavities were still filled with loosely arranged and hypercellular granulation tissue. The LLLT-treated diabetic group (DBTL) showed more evident deposition of mineralized tissue than DBT, but it was still sparse and exhibited an irregular morphology. On day 18, bone trabecular formation was verified in all the experimental groups, but the trabeculae observed in CTRL were clearly thicker than in the others. In both irradiated and non-irradiated diabetic groups, the bone trabeculae were thin and irregularly disposed in the cavities. Although narrower intertrabecular spaces were seen in DBTL in comparison with DBT, they appeared to be larger than in CTR and CTRL. Finally, on day 30, the histological pattern of bone regeneration observed in CTR, CTRL and DBTL was similar, and represented by extensive formation of thick bone trabeculae, with narrow intertrabecular spaces. In addition, there was also full replacement of the granulation tissue by a bone marrow-like tissue in the areas adjacent to the newly formed bone trabeculae in these

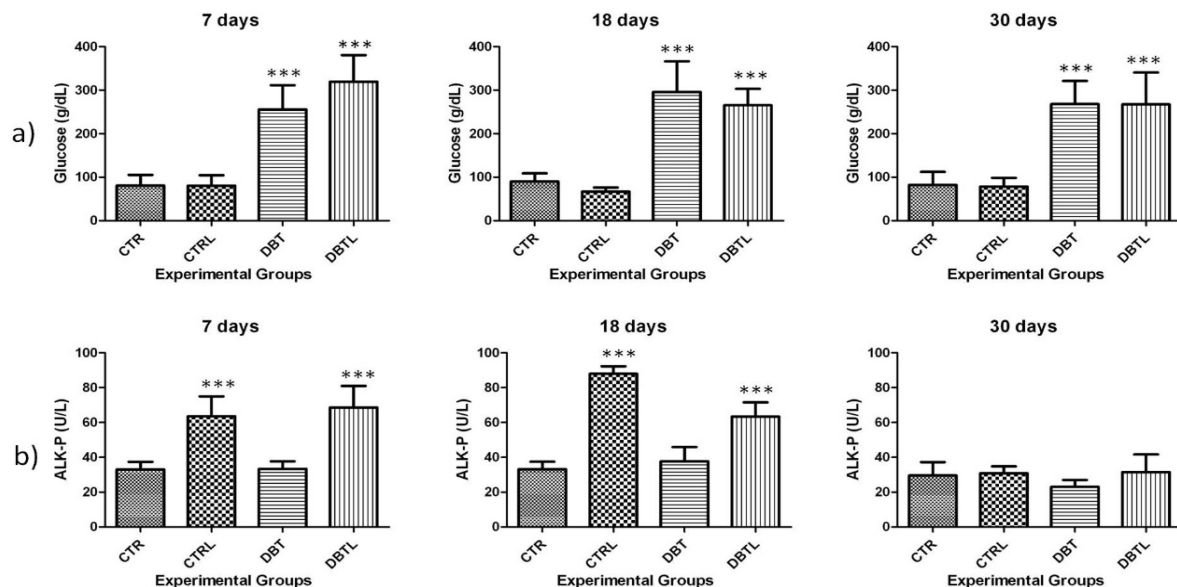


FIGURE 1 - Assessment of the serum levels of glucose and alkaline phosphatase (ALK-P) in the experimental groups throughout the experimental period. ***Significantly different from CTR ($p < 0.001$).

groups. In DBT, however, the bone trabeculae were thinner and more irregular, with still large intertrabecular spaces. Adjacent to the bone healing areas, mature residual granulation tissue was still found replacing the bone marrow.

As demonstrated in Table 3, the inflammatory reaction was significantly more intense in both diabetic (DBT and DBTL) than in non-diabetic groups (CTR and CTRL) on day 7 ($p < 0.001$). On day 18, the intensity of the inflammatory reaction observed in

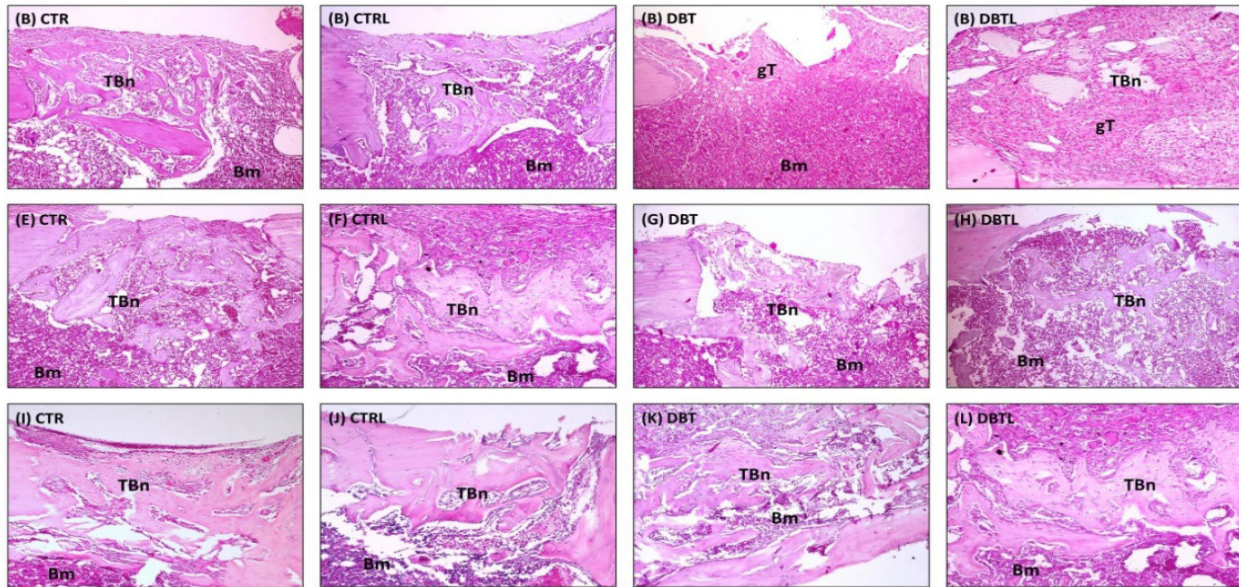


FIGURE 2 - Histological sections showing the pattern of bone healing of non-irradiated and irradiated normoglycemic (CTR and CTRL) and hyperglycemic (DBT and DBTL) groups on day 7 (A-D), 18 (E-H) and 30 (I-L) (HE, x100 magnification). TBn: trabecular neoformed bone; gT: granulation tissue; Bm: bone marrow.

the irradiated diabetic group (DBTL) was significantly decreased in comparison with the non-irradiated diabetic animals (DBT) ($p < 0.01$). Inflammatory cells were largely scanty on day 30, irrespective to the glycemic status of the animals or low-level laser therapy procedure applied, and no difference between groups was observed on day 30 ($p > 0.05$).

TABLE 3 - Semiquantitative analysis of the intensity of the inflammatory reaction observed in the bone defects of the studied groups over the time-course of the experiment.

Experimental groups	Intensity of the inflammatory reaction		
	7 days	18 days	30 days
CTR	1.25 ± 0.40^a	0.41 ± 0.50^a	0.12 ± 0.33^a
CTRL	0.79 ± 0.31^b	0.25 ± 0.44^a	0.08 ± 0.28^a
DBT	2.70 ± 0.46^c	0.98 ± 0.33^b	0.29 ± 0.46^a
DBTL	2.45 ± 0.65^c	0.50 ± 0.51^a	0.20 ± 0.41^a
p values*	0.0001	0.001	0.245

(*) Different letters in the same column represent significantly different means between groups. (Kruskal-Wallis test and Dunn's multiple comparisons test).

The bone formation area increased over the time course of the experiment in all the four groups. Significant impairment of bone healing was observed in both hyperglycemic groups (DBT and DBTL) in seven and 18 days after the surgical procedures in comparison to CTR ($p < 0.001$), but DBTL showed higher rates of bone formation than DBT in both experimental times ($p < 0.01$ and $p < 0.05$, respectively). In 30 days, the area of bone formation assessed in DBT was significantly lower than CTR ($p < 0.01$), CTRL ($P < 0.01$) and DBTL ($p < 0.01$), but no difference was found comparing the last three groups ($p > 0.05$). Only in 18 days, the area of bone formation in the irradiated normoglycemic group (CTRL) was shown to be higher than CTR ($p < 0.05$) (Figure 3).

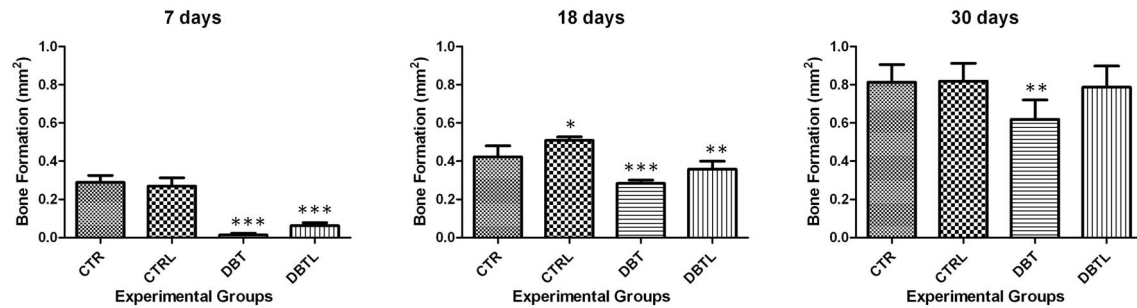


FIGURE 3 - Assessment of the area of bone formation in the experimental groups over the time-course of the study. Significantly different from CTR (** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Discussion

In this study, treatment of animals with alloxan resulted in the induction of diabetes, confirmed by the significant increase in glucose serum levels. These findings are supported by previous studies, pointing to alloxan administration as an excellent model of experimental diabetes induction¹⁶. The hyperglycemic effects of alloxan is a result of irreversible damage in β cells of the islets of Langerhans of pancreas with consequent primary insulin deficiency. This biochemical mechanism triggers a three-phase response in glucose levels during the first hours of administration, followed by the establishment of permanent diabetes in 24 subsequent hours¹⁷.

We found that the diabetic state in animals determined a significant delay in the bone formation process throughout the trial period, as previously demonstrated in other studies¹⁸. The deleterious effects of diabetes on the repair process are associated with excessive production of reactive oxygen species (ROS), decreased nitric oxide synthesis and reduction of growth factors and insulin signaling pathway proteins¹⁹. These reports indicate that intraperitoneal administration of alloxan is a suitable experimental model for the study of pathophysiological conditions in diabetic organisms.

We found that low-level laser therapy provided a significant decrease in the severity of the inflammatory response in diabetic and non-diabetic animals. Supporting our findings, similar modulatory effects of laser irradiation on inflammation over the time-course of bone healing in tibial defects of diabetic rats have been recently reported¹³. This downregulation of the inflammation is likely related to the inhibitory effect of laser energy on the release proinflammatory chemical mediators, such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 ²⁰.

Bone formation in LLLT-treated diabetic rats was significantly increased in comparison with non-irradiated ones.

Supporting our data, earlier histological finding of new bone formation at one and three weeks in the laser-treated fractures in rats has been previously demonstrated, confirming the efficacy of laser irradiation during the early stage of bone healing²¹. Moreover, the use of infrared LLLT directly to the injured tissue has demonstrated to modulate the initial inflammatory response, anticipating the resolution to normal conditions at the earlier periods of bone healing, which results in earlier bone formation and remodeling²². More recently, histological observation of increased vascularization and decreased inflammation, in addition to the increase of bone trabeculae, have also been reported as a result of low level laser irradiation after partial tibial bone osteotomy in murine model^{23,24}.

The precise mechanism underlying the LLLT-induced modulatory activity on bone healing is still under investigation. It has been suggested that at low radiation doses, intracellular chromophores, such as porphyrins and cytochromes, are able to absorb the light energy and convert it into metabolic energy, resulting in production of a transmembrane electrochemical proton gradient in the respiratory chain. Hence, LLLT is able to improve bone formation by direct stimulation of osteoblastic metabolism, with consequent increase of protein synthesis (particularly collagen) and cell levels of adenosine triphosphate²¹. LLLT also seems to increase the expression of Runx-2 gene, a transcription factor involved in osteoblast differentiation of pluripotent mesenchymal cells^{8,25}. Furthermore, LLLT promotes important changes in chemical mediators directly involved on the repair, such as reduction of cyclooxygenase-2 expression (COX-2), reducing the severity of the inflammatory response, and increase of VEGF expression, a growth factor crucial for the formation of granulation reaction²⁶. LLLT has also been demonstrated to enhance osteoblastic differentiation from stem cells in the injured area²⁷. We also found that LLLT treatment increased the meant

of bone neoformation in diabetic rats until values comparable to those observed in non-diabetic animals, whereas non-irradiated diabetic animals remained with deficient bone formation over all the time course of the experiment.

LLLT caused a significant increase in ALK-P serum levels in the first two experimental periods, regardless of the glycemic status of the animals. ALK-P is an enzyme released by osteoblasts during osteogenesis, and is associated with lysis of bloodstream-derived phosphate groups, increasing the local availability of inorganic phosphate necessary for bone mineralization²⁸. In fact, some studies showed that LLLT appears to stimulate mineralization in the process of new bone formation in surgically created bone defects, likely related to earlier and longer-term expression of osteocalcin and osteopontin, two proteins associated with extracellular matrix formation and osteoblast activity²⁴, which is consonant with our data concerning serum levels of ALK-P. The significance of the LLLT-induced improvement in bone mineralization lies in the fact that these matrix factors contribute to the growth, shape, and size of the newly formed bone matrix²⁹. The lack of significant difference between groups at day 30 suggests that, at this time, the process of bone formation/mineralization was no longer active.

In our study, the histological observation of lamellar trabecular bone in irradiated diabetic animals, but not in non-irradiated diabetic ones, support our biomechanical data and quantitative analysis of bone formation. Similar findings have also been reported and suggested that LLLT improves bone healing in diabetic hyperglycemic animals³⁰.

Conclusions

Low-level laser therapy increased the serum levels of alkaline phosphatase and improved bone healing in alloxan-induced diabetic rats.

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Correspondence:

Ricardo Luiz Cavalcanti de Albuquerque-Júnior
Laboratório de Morfologia e Patologia Experimental
Instituto de Tecnologia e Pesquisa, Universidade Tiradentes
Avenida Murilo Dantas, 300
49032-490 Aracaju – SE Brasil
Tel.: (55 79)3218-2190/ramal 2615
ricardo.patologia@uol.com.br

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