Apoptosis in Bone Defect of Diabetic Rats Treated with Low Intensity Laser: Radiological and Immunohistochemical Approach

Apoptosis en Ratones Diabéticos con Defecto Oseo Tratados con Láser de Baja Intensidad: Evaluación Radiológica e Inmunohistoquímica

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SUMMARY: The aim of this study is to evaluate the efficacy of 904 nm laser diode in bone regeneration in the bone defect of diabetic rats. Six groups of 10 male Wistar rats and 2 mm bone defects drilled on the left and right tibia were used. The diabetic animals were treated with streptozotocin (40 mg/kg, i.v.). We compared the diode laser doses of treatment of bone defects: 50 w – 4 J/cm and 100 w – 4J/. The right tibia was used for immunohistochemical analysis with the apoptosis markers XIAP and Caspase-3 and the left tibia was submitted to computer tomography (CT). Caspase-3 marker showed greater amount of apoptosis in all the untreated groups compared to both laser treatments. There was no statistical significance for XIAP marker. CT scan showed improvement of bone defect area and volume in both laser treated groups, control and diabetic. Therefore, the low intensity laser therapy was effective in accelerating bone repair in both, control and diabetic groups. It was evidenced in our study that diabetes influences bone repair negatively.

KEY WORDS: Bone defect; Diabetes mellitus; Laser therapy; Regeneration; Apoptosis.

INTRODUCTION

Apoptosis is an active physiological process genetically controlled that induces cell death, but its role in the tissue repair still needs investigation (Einhorn, 1998; Dempster et al., 2005).

The bone tissue is a complex system, dynamic and constantly working between formation and bone reabsorption, changing this dynamics particularly after trauma (Dunlop & Fratzl, 2010) leading to tissue repair. Some metabolic diseases can impair this process of tissue repair, diabetes mellitus (DM) being one of the most common causes of bone repair failure. One of the mechanisms described for this failure is the decreased tissue vascularization due to the increased basement membrane thickness of the vessel wall that would directly affect the tissue metabolism (Fernandes et al., 2005). Although apoptosis might play a role in tissue repair, still very little is known about the apoptosis mechanism in the bone repair process (Imao & Nagata, 2013).

Low Light Laser Therapy (LLLT) has been applied in different tissues to further enhance and accelerate the process of regeneration and tissue repair, with positive results (Rocha Júnior et al., 2006). The effect of the LLLT in bone tissue is still poorly investigated. Moreover, the association of bone cell apoptosis with the use of low-intensity laser therapy in the regeneration of a bone defect was not yet investigated. This work aimed to study cell apoptosis in bone defects in diabetic rats, subjected or not to LLLT, comparing two different laser doses. The volume of the bone defect was also evaluated using computer tomography (CT).

MATERIAL AND METHOD

Sixty male adult Wistar rats, aged 3 months, weighing between 280 - 310 g, from the Animal Care Facility of the
University of São Paulo, Campus of Ribeirão Preto were used. The animals were housed in three to five per cage, in a carefully regulated environment maintained at 21 °C – 23 °C, 40 % - 70 % relative air humidity, and 12/12 hr light/dark cycle, receiving tap water and normal rat chow ad libitum throughout the experiment. All procedures adhered to “The ARRIVE guidelines: Animal Research: Reporting In Vivo Experiments, originally published in PLoS Biology, June 2010” and were approved by the Institutional Ethics Committee for Animal Research (CETEA - Comitê de Ética em Experimentação Animal, protocol number 160/2011). A conscious effort was done to minimize the number of animals used.

Six experimental groups were used (N=10 per group): Control Group (GC): non-diabetic animals with bone defect not submitted to laser treatment; Control Group 50 (GC50): non-diabetic animals with bone defect and treated with laser (λ = 904 nm), with fluency of 50 J/cm² for 17 seconds, providing energy 1,7 J; Control Group 100 (GC100): non-diabetic animals with bone defect and treated with laser (λ = 904 nm), with fluency 100 J/cm² for 34 seconds providing energy 3,4 J; Diabetic group (GDI): diabetic animals with bone defect not submitted to laser treatment; Diabetic Group 50 (GD50): diabetic animals with bone defect and treated with laser (λ = 904 nm), with fluency 100 J/cm² for 34 seconds providing energy 3,4 J; and Diabetic Group 100 (GD100): diabetic animals with bone defect and treated with laser (λ = 904 nm), with fluency 100 J/cm² for 34 seconds providing energy 3,4 J.

STZ (60 mg/Kg) or citrate buffer injections were performed as described previously (Fazan et al., 2009; Oliveira et al., 2013). The animals were considered diabetic when the blood glucose levels were higher than 350 mg/dl. On the STZ injected animals, the onset of diabetes occurred rapidly and was identified by polydipsia and polyuria. Non-fasting blood glucose (mg/dl) was determined with a glucose analyzer (Beckman Instruments, Inc., Brea, CA, USA) 3 days after STZ injection and immediately before the experiments, in blood droplets collected from an incision at the tip of the tail.

Ten days after the injections, all animals were submitted to a surgical procedure for creating a bone defect in the medial aspect of the proximal third of the shaft in both tibias, approximately 10 mm below the knee joint, measured with a caliper. The animals were anesthetized with a combination of Ketamine/Xylazine (80/10 mg/kg). The bone defect was made using a helical drill of 2 mm diameter coupled to a mini-drill, with a rotation of 12,000 rpm and a constant irrigation with saline. After the anesthesia, shaving and disinfecting the area to be operated, the skin was folded through an incision of 1.5 cm in length, and the periosteum was exposed and folded. The lesion was performed with the drill held vertically and at a perpendicular position relative to the longitudinal axis of the bone so as to penetrate the cortex, reaching the deep medullary canal, but without reaching the contralateral side (Fig. 1). Then, the skin was sutured with 3-0 nylon monofilament (Shalon®) at equidistant points. After surgery, the animals were reintroduced in their cages and kept with free access to water and food until the time of euthanasia (Sanada et al., 2015).

The equipment for laser radiation treatment used was a portable model HTM Compact® laser diode with the active component made of gallium and aluminum (Ga-Al-As), wave length of 904 nm and continuous emission.

Fig. 1. Surgical steps for the bone defect procedures: (A) digital trichotomy; (B) skin incision in the tibial region; (C) measurement of the site for the bone defect with a caliper; (D) drilling the bone defect with 2 mm diameter drill; (E) bone defect after drilling; (F) skin suture.
The laser was applied at a single point on the bone defect, immediately after the surgical procedure and in the second, fourth, sixth, eighth, tenth and twelfth day after the surgery. A total of seven applications in 14 days were performed. On the 14th day after surgery, the animals were killed by anesthetic overdose and the tibias were dissected and removed. The right tibia was used for conventional immunohistochemical analysis and the left tibia was frozen and submitted to the CT scan.

For protein expression analysis six animals per group were used. The immunohistochemical reactions were performed with the avidin-biotin-peroxidase (Novostain Super ABC Kit - Universal, NCL-ABCu, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) - (universal Kit mach 4 BIOCARE) method.

The animal tissue was immersed in paraffin. For immunohistochemical analysis, slides were obtained from 5 animals each being sequenced with a thickness of 5 µm. In each section used for the immunohistochemical reaction, two microscopic fields were selected in the compact bone of the tibia at 400x magnification, where higher concentration of positive or labeled cells (areas of "hot spots") for proteins related to the mechanism of apoptosis (CASPASE 3 - pro-apoptotic and XIAP - anti-apoptotic) were present. From the count of the total number of positive (nuclear staining) and negative cells in each microscopic field, the percentage of positive cells was calculated.

For radiological control and evaluation of the bone repair process, CT scans of the left tibia were performed on a CT scanner (Brilliance CT 40-slice models, and 64-slice Big Bore, PHILIPS). The images obtained on CT scans were analyzed using the software Image J® where a plug-in allowed the estimation of the volume and area of the bone defect.

A Shapiro-Wilk test was performed to test normality for all variables. When normal distribution was present, comparisons were made using analysis of variance (ANOVA) followed by Tukey post-test. On nonparametric variables, the Kruskal-Wallis test was used, followed by Dunns post-test. Analyses were performed in STATISTICA software, version 7.0 and Prism 6.0 GraphicPad. Significance level of 5 % (p ≤ 0.05) was used.

RESULTS

Nuclear positive staining was observed for CASPASE-3 protein in the diffuse layer of compact bone with several focal sites of greatest expression (Fig. 2), particularly in animal groups GC and GC50; but no significant difference was found between these two groups (Fig. 3). The expression of this protein in groups GC50, GC100, GD50 and GD100 was significantly reduced (p <0.0001, Kruskal-Wallis test) compared to GC. The expression of CASPASE 3 was larger in the GDI group compared to the GC group, with no significant difference between them. Low XIAP protein expression was observed in the compact bone layer of the tibia between the studied groups p=0, Kruskal-Wallis test. There was no significant difference between groups (Fig. 3).

In computed tomography, the volume of the bone defect and the histogram of the coefficient of the tissue in the bone defect was observed, as an indication of how much regeneration was present (Fig. 4). There was a significant difference between the non-diabetic groups treated and untreated. A decrease in the bone defect volume in treated and untreated diabetic group was also observed.
DISCUSSION

In our study, we found that the induction with the alloxan was effective in the development of experimental diabetes due to irreversible damage in beta cell, as found by Nascimento et al. (2015), who used this drug and obtained similar results, being it an excellent experimental model.

The expression of Caspase-3 in the bone defect of the tibia in diabetic rats was lower in the groups undergoing LLLT with the diode laser (l=904 nm). There was no difference between the groups treated with 50 W/cm² and 100 W/cm². The expression of XIAP in bone defects of the tibia in diabetic rats was not different in the groups undergoing LLLT with the diode laser. In computed tomography, the volume of the bone defect in the diabetic animals was higher, independently of the treatment.

The osteoclasts are multinucleated giant cells mainly responsible for bone reabsorption that quickly die due to apoptosis in the absence of trophic factors such as macrophage colony stimulating factor (M-CSF) or ligand to receptor nuclear factor kappa B (RANKL - nuclear factor kappa-B ligand). Bcl-2–interacting mediator of cell death (Bim) is a member of BH3, of the BCL-2 family, the only family of pro-apoptotic protein which regulates the mitochondrial apoptosis pathway. It is located in the mitochondria and the interaction between this protein and other BCL-2 family possibly become from oligomerization of BAX and BAK, leading to cytochrome-c release. Cytochrome-c interacts with APAF-1 and CASPASE-9 to form the apoptosome leading to apoptosis (Tanimoto et al., 2005).

Labeling of caspase-3 showed a significant difference between the normal control groups compared to those treated with low intensity laser therapy. Also, the diabetic control group increased apoptotic activation when compared to the other treated diabetic groups. Regarding the marking of XIAP, there was no statistical difference between groups (Leo et al., 2012).

The use of computed tomography helped on the evaluation of the size and behavior of the bone defect under the different experimental conditions. Garib et al. (2006) used the CT study to evaluate and monitor the behavior of implants in tibias of rabbits and found that the technique was effective for analysis. In human dentistry, cone beam tomographic evaluations are widely used to evaluate mineralized bone tissue and monitor bone remodeling since this technique allows playback of three-dimensional images of tissues with minimal distortion (Wassal Silveira, 2009).

In this work, the diabetic control group presented a larger volume of bone defect compared to the untreated control group. Thus, diabetes interfered negatively in the bone defect repair. The bone defect area results confirmed the bone volume observations.

The area compared to the treated groups, both normal and diabetic patients, and the diabetic group showed a greater area of bone defect than the normal group in our study.

LLLT is currently one of the photobiostimulation modalities most commonly used in rehabilitation. Several studies have demonstrated the positive effect of laser treatment on the bone metabolism, such as osteoblast proliferation (Einhorn), the presence of newly formed blood vessels and increase in alkaline phosphatase activity (Renno et al., 2007).
Nascimento et al. investigaron la reparación ósea en ratas diabéticas usando la LLLT. Se usaron 72 ratas divididas en 4 grupos: CTR (control no-diabético), DBT (diabético) CTRL (no-irradiado) e DBTL (irradiado diabético). Los animales fueron operados y se les dio una dosis de 50 J/cm² y 100 J/cm². Se usó un láser de diodo (904 nm). El grupo diabético requirió más terapia con láser para que recuperaran el área de la lesión. No hubo significancia estadística para el marcador XIAP. La tomografía computarizada mostró una mejoria del área y el volumen de la lesión más rápida que en el grupo control. El marcador caspasa-3 mostró una menor cantidad de apoptosis en los grupos no tratados en comparación con los tratados con láser.

Resumen: Los objetivos de este estudio fueron evaluar la eficacia del láser diodo de 904 nm en la regeneración ósea en ratas diabéticas. Se utilizaron dos grupos de 10 ratas Wistar macho y se generó un defecto óseo de 2 mm en las tibias. Se compararon las dosis de tratamiento de los defectos óseos con láser de diodo de 50 W - 4 J/cm² y 100 W - 4 J/cm². La tibia derecha fue utilizada para el análisis inmunohistoquímico con los marcadores de apoptosis XIAP y Caspasa-3. Se observó que el láser dio una mayor cantidad de apoptosis en todos los grupos no tratados en comparación con ambos tratamientos con láser. No hubo significancia estadística para el grupo XIAP. La tomografía computarizada mostró una mejoria del área y el volumen de los defectos óseos en ambos grupos tratados con láser, control y diabéticos. Por lo tanto, la terapia con láser de baja intensidad fue efectiva en la aceleración de la reparación ósea tanto en los grupos control como en los diabéticos. Se evidenció en nuestro estudio que la diabetes afecta negativamente la reparación ósea.
REFERENCES


