Histological analysis of the periodontal ligament and alveolar bone during dental movement in diabetic rats subjected to low-level laser therapy


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OBJECTIVE: The purpose of this research was to evaluate the histological changes of the periodontal ligament and alveolar bone during dental movement in diabetic rats subjected to low level laser therapy (LLLT).

METHODS: The movement of the upper molar was performed in 60 male Wistar rats divided into four groups (n = 15): CTR (control), DBT (diabetic), CTR/LT (irradiated control) and DBT/LT (irradiated diabetic). Diabetes was induced with alloxan (150 mg/kg, i.p.). LLLT was applied with GaAlAs laser at 780 nm (35 J/cm²). After 7, 13 and 19 days, the periodontal ligament and alveolar bone were histologically analyzed.

RESULTS: The mean of osteoblasts (p < 0.01) and blood vessels (p < 0.05) were significantly decreased in DBT compared with CTR at 7 days, whereas the mean of osteoclasts was lower at 7 (p < 0.001) and 13 days (p < 0.05). In DBT/LT, only the mean of osteoclasts was lower than in CTR (p < 0.05) at 7 days, but no difference was observed at 13 and 19 days (p > 0.05). The collagenization of the periodontal ligament was impaired in DBT, whereas DBT/LLT showed density/disposition of the collagen fibers similar to those observed in CTR.

CONCLUSIONS: LLLT improved the periodontal ligament and alveolar bone remodeling activity in diabetic rats during dental movement.

1. Introduction

Diabetes mellitus (DM) is one of the most common endocrine disorders. It is characterized by persistently raised blood glucose levels (hyperglycaemia), resulting from deficiencies in insulin secretion, insulin action, or both [1]. Chronic hyperglycaemia is associated with long-term damage, dysfunction, and failure of various organs, such as retinopathy, nephropathy, peripheral neuropathy, and autonomic neuropathy, causing gastrointestinal, genitourinary, and cardiovascular symptoms, as well as sexual dysfunction [2].

It has been reported that moderate to severe damage to the glucose metabolism directly affects the response of bone and connective tissues to injury [3,4]. Orthodontic treatment in adult diabetic patients is thus usually complicated by oral problems such as periodontal degradation and bone loss [5]. Although only a few studies have assessed the histological changes that take place in the periodontal ligament and alveolar bone during dental movement in diabetic experimental animals, they provide evidence that chronic hyperglycaemic status might impair the periodontal ligament response and bone remodeling during orthodontic treatment [6].

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Lasers emit a highly concentrated, non-invasive, non-ionizing radiation that, when in contact with different tissues, promotes thermal, photochemical and nonlinear effects [7]. Several studies have indicated that C (LLLT) modulates different biological activities, such as anti-inflammatory activity [8,9], angiogenesis [10,11] and collagen synthesis [12,13]. In particular, the acceleration of bone regeneration by laser treatment has been a focus of studies [14,15]. It has previously been demonstrated that LLLT can accelerate tooth movement, as well as alveolar bone remodeling in experimental studies [16,17] and clinical trials [18,19]. However, little is known about the effect of laser irradiation on the dynamics of dental movement in diabetic subjects. Therefore, the present study was designed to assess the histological changes taking place in the periodontal ligament and alveolar bone during dental movement in diabetic rats subjected to laser irradiation.

2. Material and methods

2.1. Ethical perspectives

The ethical principles of the COBEA (Brazilian College for Animal Experimentation) for experiments in animals were applied in this study. The institutional review board approved the study (approval n. 341208). The study was carried out at the biotherium and the Laboratory of Morphology and Structural Biology of Tiradentes University (Aracaju/SE, Brazil).

2.2. Biological assay

Sixty adult male rats (Novérgicus albinus, Wistar lineage), weighing 250 ± 30 g, were randomly assigned into four experimental groups (n = 15) (Table 1). Animals were kept in plastic cages with wood shaving bedding (replaced daily), at a controlled temperature of 22 °C, and a 12 h light/dark cycle, with water and food (diet Labina®, Purina, Sao Paulo, Brazil).

2.3. Alloxan-induced diabetes model

Diabetes status was induced by a single intraperitoneal injection of 150 mg/kg monohydrated alloxan (Sigma, St. Louis, MO, USA) dissolved in sterile 0.9% saline. After 12 h, a 10% glucose solution was offered to the animals to prevent hypoglycaemia. Blood samples were collected from the tail vein of the animals after 72 h in order to assess the plasma glucose levels via the glucose-oxidase enzymatic method, using Accu-Chek Advantage (Boehringer, Germany). Animals with glucose levels above 200 mg/dL were included in the diabetic group. The examinations were repeated every 7 days to confirm the maintenance of glucose levels. Any animals showing reversion of the signs of diabetes (glucose levels below 200 mg/dL) were excluded from this study. The animals in the nondiabetic group (CTR and CTR/LT) received an equivalent volume of citrate buffer. The orthodontic device was applied 6 weeks after diabetes was induced.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-treatment</th>
<th>Low level laser therapy (energy density)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>Citrat buffer</td>
<td>0 J/cm²</td>
</tr>
<tr>
<td>DBT</td>
<td>Alloxan (150 mg/kg)</td>
<td>35 J/cm²</td>
</tr>
<tr>
<td>CTR/LT</td>
<td>Citrat buffer</td>
<td>0 J/cm²</td>
</tr>
<tr>
<td>DBT/LT</td>
<td>Alloxan (150 mg/kg)</td>
<td>35 J/cm²</td>
</tr>
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</table>

2.4. Experimental tooth movement

At the end of 2 months, anaesthesia was induced with intraperitoneal administration of ketamine–xylazine (100 mg/kg–5 mg/kg), and an appliance exerting force to widen the space between the upper central incisors was fitted to both groups. For mesial movement of the upper left 1st molar, the wire end of a 7.0-mm length of NiTi closed-coil spring (wire size: 0.7 mm, diameter: 1/12 in., Orthometric, Marilia, SP, Brazil) was ligated with the maxillary 1st molar cleat using a 0.010-in. stainless steel ligature wire (Morelli, Sorocaba, SP, Brazil). The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a #1/4 round bar, using the same ligature wire (Fig. 1). The orthodontic force exerted by the appliance was 50 g at the start of the experiment. Tooth movement was performed for 19 days (day 0–19).

2.5. Low-level laser therapy procedures

Animals were subjected to transcutaneous irradiation using a previously calibrated semi-conductor diode laser GaAlAs (Twin Laser, MMOptics, São Paulo, Brazil) with continuous emission at 780 nm wavelength for 60 s (20 s each point). The output power was used 70 mW, with a focal spot of 0.04 cm², and a power density of 1.75 W/cm². The total energy per session was estimated as 11.6 J (0.4 J/pulse) and the energy density was 35 J/cm² distributed between three different equidistant points in the root portion. The first irradiation was performed immediately after the activation procedures, and then performed every 48 h over the course of 7 days.

2.6. Procedures for histomorphological analysis of the specimens

After 7, 13 and 19 days, animals were euthanized in a CO₂ chamber for post-mortem removal of the maxillae. Tissue specimens were fixed in buffered formaldehyde (10%, pH 7.4) for 48 h, decalcified in 5% nitric acid for 72 h, dehydrated in increasing ethyl alcohol solutions, and diaphanized in xylol for inclusion in paraffin. Subsequently, ten histological sections (5 µm thick) were obtained and stained in hematoxylin–eosin for analysis using a light microscope (Olympus CX31 optic microscope) by three trained observers.

2.7. Histomorphological analysis of the periodontal ligament and alveolar bone

The intensity of the inflammatory response was assessed in histological sections as follows: 0 (lack of inflammatory reaction); 1 (inflammatory cells representing less than 10% of the cell population observed within the wound area); 2 (inflammatory cells representing between 10% and 50% of the cell population observed within the wound area); and 3 (inflammatory cells representing more than 50% of the cell population observed within the wound area). Moreover, the inflammatory profile (IP) was classified as acute (predominance of polymorphonuclear cells) or chronic (predominance of mononuclear cells), and graded as slight/absent, moderate or severe.

2.8. Quantitative analysis of the osteoblast (OsTB)/osteoclast (OsTC) and blood vessel (BvC) count

Counting of osteoblasts, osteoclasts and blood vessels was performed using an image analysis system (Imagelab). All images were sent to a PC using an analogue video camera (PAL system), after being converted to the RGB (red–green–blue) system necessary for digitizing and processing the sections. Five histological
fields (200× magnification) of the tension and pressure areas of the periodontal ligament of the 1st molar were used to determine the mean number of osteoblasts and osteoclasts, respectively. To count the blood vessels, images of the tension and pressure areas were analyses. The images were recorded and automatically processed to find the cell density (CD) in each reference area (RA). Data were expressed as mean ± SD.

2.9. Quantitative analysis of collagenization rate (CR)

The collagen deposition rate (CR) in the periodontal ligament was determined by optical density in the image analysis system in different randomly selected fields. The system used consisted of a CCD Sony DXC-101 camera, attached to an Olympus CX31 microscope, from which the images were sent to a monitor (Trinitron Sony). By means of a digitizing system (Olympus C-7070 WIDEZOOM) the images were loaded onto a computer (Pentium 133 MHz), and processed using ImageLab software. Ten fields per case were analyzed at a magnification of 1000×. The thresholds for collagen fibers were established for each slide, after enhancing the contrast up to a point at which the fibers were easily identified as birefringent (collagen) bands. The area occupied by the fibers was determined by digital densitometric recognition, by adjusting the threshold level of measurement up to the different color densities of the collagen fibers. The area occupied by the fibers was divided by the total area of the field. The results were expressed as the percentage of the periodontal ligament area occupied by the collagen fibers.

2.10. Statistical analysis

Data obtained in the IP and HC analysis were analysed using the Kruskal–Wallis test, followed by the post hoc Dunns test. Data obtained in the OSTB, OsTC, BvC and CR counts were analysed by ANOVA followed by the post hoc Tukey’s test. Differences between groups were regarded as significant when \( p < 0.05 \).

3. Results

3.1. Descriptive analysis of histological changes in the periodontal ligament

After 7 days (Fig. 2), exuberant granulation tissue and multiple erosive alterations, consistent with howship lacunae, most of them containing osteoclasts, were seen along the alveolar wall in CTR, CTR/LT and DBT/LT, particularly in the cervical thirds of the periodontal ligament. However, intense oedematous changes in the connective tissue associated with chronic inflammatory infiltrate, and with sparse vascularization, were observed in DBT. It should be emphasized that the collagen fibers were more apparent and were well distributed in a parallel organization in the LLLT-treated groups (CTR/LT and DBT/LT). In addition, coagulative necrosis of the periodontal ligament was seen in one case of DBT, but not in the other groups.

After 13 days (Fig. 3), all groups showed a remarkable reduction in the vascular network and a substantial increase in the fibrous component. Osteoclastic activity was still present, but was lower than at 7 days. The collagen fibers had a gross and thick appearance, as well as a parallel arrangement in CTR, CTR/LT and DBT/LT. In the non-irradiated diabetic animals (DBT), however, the collagen fibers appeared to be thinner with a non-uniform distribution throughout the periodontal ligament.

After 19 days (Fig. 4), the periodontal ligament of both CTR and CTR/LT presented similar morphological features, in the form of a cell-rich and moderately vascular connective tissue, associated with thick, but delicate, parallel collagen fibers. In diabetic animals, there were parallel bundles of thin collagen fibers associated with a high content of flat spindle-shaped cells (consistent with fibroblasts), and this fibrous component was more extensive in DBT/LT than in DBT.

Data on the intensity of the inflammatory response (IR) found throughout the periodontal ligament is presented in Table 2. The IR values observed in irradiated non-diabetic animals (CTR/LT) were lower than in all other groups at 7 and 13 days (\( p < 0.05 \)). Although
at 7 days diabetic animals (DBT) showed a significantly more intense IR than the control group (CTR) \( (p < 0.05) \), irradiated diabetic rats (DBT/LT) showed no significant difference in comparison with CTR. After 13 and 19 days, leukocyte infiltration progressively decreased, and none of the groups presented significant differences regarding the intensity of the inflammatory response \( (p > 0.05) \).

### 3.2. Assessment of the mean number of osteoblasts (OsTB)

The results of the histomorphometric study of osteoblast number (OsTB) in the periodontal cortex of the alveolar wall are presented in Fig. 5. The values of OsTB were significantly lower in DBT in comparison with CTR at 7 \( (p < 0.001) \) and 13 days \( (p < 0.01) \), but not at 19 days \( (p > 0.05) \). Irradiated diabetic animals presented a significant increase in OsTB compared to non-irradiated animals at 7 and 13 days \( (p < 0.05) \), and although DBT/LT still had OsTB values lower than CTR at 7 days \( (p < 0.05) \), no significant difference was observed between these two groups at 13 days \( (p > 0.05) \). At 19 days, no significant differences in OsTB were observed between the experimental groups \( (p > 0.05) \).

### 3.3. Assessment of the mean number of osteoclasts (OsTC)

The results of the histomorphometric study of osteoclast number (OsTC) in the periodontal cortex of the alveolar wall are presented in Fig. 6. After 7 days, a significant decrease in OsTC was observed in diabetic compared with control animals \( (p < 0.001) \). Although the application of LLLT in diabetic animals (DBT/LT) promoted a slight increase in OsTC, it was not statistically significant in relation to non-irradiated animals \( (p > 0.05) \). In addition, the increase in OsTC in CTR/LT compared with CTR was not significant \( (p > 0.05) \). At 13 days, the OsTC in diabetic animals remained significantly lower than in the control group \( (p < 0.01) \), although in laser-irradiated animals (DBT/LT) this difference was not significant \( (p > 0.05) \). At 19 days, no significant differences in OsTC were observed between the experimental groups \( (p > 0.05) \).

### 3.4. Assessment of the mean number of blood vessels

The results of the histomorphometric study of blood vessel count (BVC) in the periodontal ligament on the pressure side are presented in Fig. 7. At 7 days, the blood vessel count was significantly lower in diabetic animals compared with the control group \( (p < 0.05) \). LLLT promoted a significant increase in the BVC in non-diabetic animals \( (p < 0.01) \). In addition, there was no difference between the BVC observed in laser-irradiated diabetic animals and control animals \( (p > 0.05) \). Lower numbers of blood vessels were observed at 13 and 19 days, and no significant differences were found between the experimental groups \( (p > 0.05) \).
3.5. Quantitative analysis of collagenization rate (CR)

Collagen fibers were identified due to their intense golden birefringence under polarized light (Fig. 8), and presented as fibrous structures arranged in a parallel fashion, connected on one side to the dental cement and to the alveolar bone on the other. As shown in Fig. 9, the collagenization rate (CR) was significantly lower in diabetic animals (DBT) than in controls (CTR) at 7 ($p < 0.001$), 13 ($p < 0.001$) and 19 days ($p < 0.01$). Laser irradiation induced a significant increase in CR in CTR/LT and DBT/LT in comparison with the respective controls (CTR and DBT) at 7 ($p < 0.01$ and 0.01) and 13 days ($p < 0.05$ and 0.001). In addition, the CR values observed in irradiated diabetic animals (DBT/LT) did not differ significantly from those in non-diabetic animals (CTR) throughout the experimental period ($p > 0.05$).
4. Discussion

In this study, we found substantial changes in the alveolar bone and periodontal ligament of diabetic animals in response to the application of an orthodontic force. Such changes were represented by persistence of the inflammatory response, impairment of granulation tissue formation (development of neoformed capillary vessels and deposition of type I collagen fibers), a reduction in both osteoblastic/osteoclastic differentiation and a reduced number of howship lacunae. Similar morphological and morphometric findings were recently reported by Villarino et al. [6].

The mechanisms responsible for the enhanced inflammatory response in diabetic animals have not been conclusively established. A possible association with higher levels of TNF, a product of adipose tissue, which is increased in both humans and animal models of type II diabetes, has been proposed. Thus, the enhanced production of TNF dysregulates the cytokine networks and potentiates the inflammatory response. In addition, the production of advanced glycation end products that are present at higher levels in diabetic individuals seems to enhance oxidative stress and amplify inflammatory events in tissues [20].

The impairment of angiogenesis in diabetic animals has previously been reported [21]. Although the precise cause of such impairment has not been fully clarified, it may be the result of a decrease in the amount of growth factors that are essential for wound healing, including FGF-2 and PDGF [22].

On the other hand, the existence of a bone–pancreas endocrine loop through which insulin signaling in osteoblasts ensures osteoblast differentiation and stimulates osteocalcin production, which in turn regulates insulin sensitivity and pancreatic insulin secretion, has recently been proposed [23]. Therefore, it is possible that the significant decrease in osteoblast number observed in this study might have been a result of the substantial reduction in insulin levels associated with the aloxan-induced pancreatic damage. Interestingly, the same mechanism would secondarily lead to reduced levels of calcitonin, an inhibitor of bone resorption, and should result in increased osteoclastic activity. However, it has recently been reported that osteoblasts are one the major sources of the receptor activator for nuclear factor kappa ligand (RANKL) [24], a molecule widely required for osteoclast formation from the peripheral blood-derived mononuclear precursors. Hence, the decrease in osteoblast differentiation would be ultimately responsible for reduced osteoclast formation, as observed in this study.

We found that the application of LLLT during dental movement reversed some of the deleterious effects associated with diabetic status. The major biological events modulated by laser irradiation were closely associated with a less intense inflammatory response, enhanced stromal cell proliferation, involving osteoblasts and osteoclasts, improved angiogenesis and granulation tissue formation, and better architectural reorganization of the periodontal collagen fibers.

It has previously been reported that laser irradiation causes an increase in the number of more differentiated osteoblastic cells

Table 2
Assessment of the mean scores of the histological grading of the intensity of the inflammatory response.

<table>
<thead>
<tr>
<th>Experimental period (days)</th>
<th>Experimental groups</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CTR</td>
</tr>
<tr>
<td>7</td>
<td>2.50 ± 0.5a</td>
</tr>
<tr>
<td>13</td>
<td>0.75 ± 0.3a</td>
</tr>
<tr>
<td>19</td>
<td>0.00 ± 0.0a</td>
</tr>
</tbody>
</table>

Different letters (a, b, c) in the same line represent significantly different values (p < 0.05). All values are mean ± SD.

4.3. Laser Irradiation Effects

The application of LLLT during dental movement reversed some of the deleterious effects associated with diabetic status. The major biological events modulated by laser irradiation were closely associated with a less intense inflammatory response, enhanced stromal cell proliferation, involving osteoblasts and osteoclasts, improved angiogenesis and granulation tissue formation, and better architectural reorganization of the periodontal collagen fibers.

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and bone nodule formation in vitro, by stimulating the cellular proliferation of osteoblast lineage nodule-forming cells and cellular differentiation [25]. On the other hand, increased osteoblastic differentiation might promote RANKL release by osteoblasts, resulting in improved osteoclast formation [24]. Moreover, it has also been suggested that laser irradiation might stimulate the biological events associated with osteoclast formation, such as bone marrow-derived mononuclear osteoclast precursors of (preosteoclast) fusion to mature osteoclasts [16]. As the speed of tooth movement is highly dependent on the dynamics of bone remodeling, as a result of the bone resorption and formation balance, it is possible that LLLT improved tooth movement in diabetic rats as a result of increased osteoblast/osteoclast differentiation. However, it is essential to emphasize that, despite the laser-induced histological improvement in the osteoblast and osteoclast number, the application of LLLT was not able to fully reverse the deleterious effects of the diabetic status on the differentiation of such cells, since at 7 days, both OsTC and OsTB remained lower than in CTR.

Angiogenesis and consequently granulation tissue formation is one the major biological events related to the healing process of the periodontal ligament during tooth movement. Although the formation of blood vessels was impaired in diabetic rats, LLLT promoted the functional recovery of angiogenesis, and allowed a suitable granulation tissue formation comparable to that in non-diabetic rats. The stimulatory role played by LLLT in angiogenesis and granulation tissue during wound healing has previously been reported during wound healing [26,11] and bone repair [27]. Laser light irradiation increases the production of nitric oxide (NO), which in turn can modulate the production and secretion of several cytokines, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), both of which are able to mediate endothelial proliferation and blood vessel formation, [28] which could support the histological findings regarding the periodontal vascular content observed in this study. The fact that significant results were limited to 7 days is consistent with the dynamics of wound healing, since the proliferative vascular events occur in the early stages of the connective tissue healing process [29].

Finally, improved collagenization and better spatial organization of the collagen fibers were also observed in the periodontal ligament of diabetic rats after laser irradiation. Consistent with our findings, previous reports have demonstrated that LLLT is able to improve collagen deposition and remodeling during wound healing [13,11,30], likely as a result of biomodulatory effects on fibroblast function. It has been previously demonstrated that increased expression of MMP-1 and Col-III and decreased expression of Col-I in periodontal dental ligament (PDL) of diabetic rats. After the orthodontic induction, osteoclast action was delayed, and higher Col-III/Col-I and MMP-1/TIMP-1 ratios persisted in the diabetes group compared with the normal group [31]. As observed in the current study, those data suggest that under mechanical forces, diabetes prolonged duration of degradation of PDL and remodeling of PDL and resorption of alveolar bone. More recently, molecular studies have pointed at a possible role played by LLLT on collagen (Col-I) and tissue inhibitor of metalloproteinase (TIMP-1) on the

![Fig. 6. Assessment of the mean of osteoclast number (OsTC) in the studied groups over the time course of the experiment. Columns marked with different letters represent significantly different values (ANOVA, p < 0.05).](image)
Fig. 7. Assessment of the mean number of blood vessels (BVC) in the studied groups over the time course of the experiment. Columns marked with different letters represent significantly different values (ANOVA, p < 0.05).

Fig. 8. Collagen fibers of the periodontal ligament seen under polarized light (arrows) in seven (A–D), 13 (E–H) and 19 days (I–J). Control group (CTR) is expressed in the first column (A, E, I), non-diabetic irradiated group (CTR/LT) in the second (B, F, J), diabetic group (DBT) in the third (C, G, K) and irradiated diabetic group (DBT/LT) in the fourth ones (D, H, L) (sirius red, 200× magnification; white bar – 250 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
compression and tension sides of orthodontically moved teeth. Overexpression of five MMP mRNAs was observed in both relapse and retention groups. However, TIMP-1 immunoreactivity was inhibited by LILT in both groups, whereas Col-I immunoreactivity was increased by LILT only in the retention group. These data suggest that, although LILT might act differently on the stability after orthodontic treatment according to additional retainer wearing or not, the photobiomodulation process might improve collagen remodeling [32]. Therefore, it is possible to suggest that LILT is able to stimulate collagen deposition and improve the architectural arrangement of the periodontal fibers in diabetic rats during tooth movement.

5. Conclusion

The present study suggests that LLLT leads to a substantial improvement in vascularization and collagenization on the pressure side of the periodontal ligament of diabetic rats subjected to experimental tooth movement. Notwithstanding these findings, the impairment of osteoblast and osteoclast differentiation was only partially reversed by LLLT, suggesting that diabetic patients who are not strictly monitored should not receive orthodontic treatment until their metabolic status normalizes, since the application of strong orthodontic forces has been shown to cause unwanted effects on bone remodeling.

References


