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Faculdade de Odontologia de Araraquara

LUIZ GUILHERME MARTINS MAIA

Avaliação histológica da movimentação ortodôntica em ratos diabéticos ou osteopênicos sob ação da fotobiomodulação a laser

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Orientador: Prof. Dr. Luiz Gonzaga Gandini Jr

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Maia LGM. Avaliação da movimentação ortodôntica em ratos diabéticos ou osteopênicos sob ação da fotobiomodulação a laser. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

RESUMO

O objetivo deste estudo foi avaliar o efeito da terapia com laser de baixa intensidade (LBI) sobre as alterações histológicas do ligamento periodontal, osso alveolar e complexo dentina-polpa de dentes submetidos à movimentação ortodôntica em ratos diabéticos e osteopênicos. Para tanto, induzimos o diabetes e osteopenia em ratos Wistar, por meio da administração de aloxana e por ovariectomia, respectivamente. Animais normoglicêmicos e ratas não ovariectomizadas funcionaram como controles. Posteriormente, o primeiro molar superior direito foi submetido à tracionamento mesial. A LBI foi realizada a 780 nm. Após o sacrifício em 7, 13 e 19 dias, os tecidos foram removidos, processados e analisados histologicamente. Em animais osteopênicos e diabéticos, a LBI reduziu significativamente a intensidade da resposta inflamatória, modulou a diferenciação osteoclástica/osteoblástica e aumentou a vascularização do ligamento periodontal, além de aumentar a organização arquitetural das fibras periodontais. Adicionalmente, a LBI também atenuou a inflamação e incrementou a vascularização em polpas dentais de dentes tracionados em ratos diabéticos, embora tenhamos observado um aumento importante na colagenização pulpar. Concluímos que ambos os distúrbios metabólicos promoveram alterações morfológicas importantes nos tecidos pulpar e periodontais durante a movimentação ortodôntica e que o laser de baixa intensidade é capaz de reverter parcial, mas não totalmente, as modificações histológicas periodontais e do complexo dentina-polpa.

Palavras-chave: Movimentação dentária, doenças ósseas metabólicas, diabetes mellitus

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ABSTRACT

The purpose of the current study was to assess the effect of the low level laser therapy (LLLT) on the histological changes of the periodontal ligament, alveolar bone, and dentin-pulp complex of teeth subjected to orthodontic movement in diabetic and osteopenic rats. Therefore, we induced diabetes and osteopenia in Wistar rats, by the administration of aloxan and by ovariectomy, respectively. Normoglycemic and non-ovariectomized animals were regarded as controls. Subsequently, the upper right first molar was submitted to mesial traction. The LLLT was carried out at 780 nm. After the sacrifice of the rats in 7, 13 and 19 days, the tissues were removed, processed and analyzed in light microscope. In both osteopenic and diabetic animals, the LLLT significantly reduced the intensity of the inflammatory response, modulated the osteoblastic/osteoclastic differentiation, and increased the blood vessels content of the periodontal ligament, as well as provided better architectural arrangement of the periodontal collagen fibers. In addition, the LLLT also attenuated the inflammation and improved vascularization in dental pulps of pulled teeth of diabetic rats, although we also observed a significant increment in the pulp collagenization. We concluded that both metabolic disturbances promoted morphological changes in the pulp and periodontal tissues during dental movement, as well as that LLLT is able to partially, but not completely, reverse the histopathological findings seen in the periodontal tissues and pulp-dentin complex.

Key Words: Tooth movement, bone diseases, diabetes mellitus

SUMÁRIO

INTRODUÇÃO EXPANDIDA

O movimento ortodôntico é um processo biológico que envolve reabsorção óssea pelos osteoclastos no lado de pressão e neoformação óssea pelos osteoblastos no lado de tensão, em resposta a estímulo mecânico celular consequente de força de compressão e tração[32] . O dente move-se através do osso carregando as estruturas adjacentes preservando os componentes do complexo dentina-polpa, como se houvesse migração da cavidade onde o mesmo está localizado[37,22,42].

A movimentação dentária ortodôntica promove uma série de reações biológicas[15,23] que vão ocorrer por intermédio de mediadores químicos. Os principais mediadores químicos locais são constituídos pelas interleucinas, fator de crescimento transformador β e prostaglandinas, enquanto os sistêmicos são representados pela calcitonina, hormônios da paratireóide, hormônios sexuais e vitamina D[39]. Estes são sintetizados e segregados pelas células locais e estimulam o processo de reabsorção óssea.

Durante o movimento ortodôntico, as células do ligamento periodontal são comprimidas e o fluido extracelular do periodonto é extravasado para os espaços medulares. Na zona de pressão, o tecido de sustentação fibroso é reconstituído através da substituição quase completa das fibras velhas por novos elementos fibrosos[17,36]. Nesta situação ocorre estase, isquemia, diminuição gradual de capilares, presença de trombos, completa obliteração de vasos sangüíneos e degeneração vascular também são descritas no lado de pressão do ligamento periodontal durante a movimentação ortodôntica[19,33]. Por outro lado, Lew[19] e Tang[38], afirmam que distensão e dilatação dos vasos sangüíneos são relatadas no lado de tração do ligamento periodontal.

Estudos efetuados em modelos experimentais *in vivo* também têm demonstrado que a movimentação ortodôntica parece afetar a homeostase do complexo dentina-polpa dos dentes tracionados, refletindo em alterações morfológicas ou funcionais destes tecidos[1,34,35,43], embora outros autores defendam a hipótese de que tais alterações pulpares estariam relacionadas a forças ortodônticas que excedem a capacidade responsiva da polpa dentária[22,37,42].

Alguns Distúrbios metabólicos como Diabetes *Mellitus* (DM), e a osteoporose, podem acometer pacientes em tratamento ortodôntico e influenciar a velocidade de reabsorção e

neoformação óssea, tempo de tratamento, quantidade de crescimento, cicatrização, entre outros.

Por um lado, o Diabetes Mellitus (DM), é caracterizada pelo aumento persistente dos níveis glicêmicos como conseqüência da deficiência na secreção de insulina[7]. O DM tipo 1 resulta da destruição das células β, das ilhotas de Langerhans do pâncreas e conduz a uma acentuada deficiência de insulina, o que acarreta uma dependência na administração da mesma[41]. Segundo a Organização Mundial da Saúde (OMS)[27], 346 milhões de pessoas no mundo têm Diabetes. Em 2004, houve uma estimativa de 3,4 milhões de pessoas mortas devido ao alto nível de açúcar no sangue, sendo mais de 80% em países de baixo a médio nível. A OMS estima que as mortes por diabetes duplicará entre 2005 e 2025 e a Associação Americana de Diabetes (ADA)[3] afirma ainda que nos Estados Unidos 20,8% de pessoas sofrem de todos os tipos de Diabetes, sendo que 6,2 milhões não possuem conhecimento sobre essa doença. Tais dados demonstram a importância de iniciativas científicas no cuidado de pacientes portadores desta doença.

As manifestações clínicas bucais mais freqüentes no indivíduo com Diabetes *Mellitus* incluem aftas, cáries, líquen plano, candidíase, xerostomia e disfunções salivares, além da doença periodontal, considerada a sexta complicação mais freqüente do Diabetes[7,21,40]. Correa et al.[11] (2007) definiram a doença periodontal como um processo inflamatório causada por alterações do microbiota. Uma das primeiras manifestações clínicas causadas pela periodontite é a perda de inserção do periodonto de sustentação, havendo a proliferação de microrganismos patogênicos associada a alterações vasculares na gengiva, propiciando uma maior freqüência da doença periodontal[20].

Hiperglicemia crônica está associada a danos em longo prazo e disfunção de vários órgãos e tecidos[3], tendo sido demonstrado que danos de moderada a grave intensidade no metabolismo da glicose são capazes de afetar diretamente a resposta do tecido ósseo e conjuntivo fibroso à lesão[7].

Nesse sentido, estudos tem demonstrado que o estado hiperglicêmico crônico pode prejudicar a resposta vascular e inflamatória e remodelação óssea durante o tratamento ortodôntico, afetando particularmente a resposta inflamatória e a dinâmica da reabsorção e neoformação dos tecidos periodontais[24,41].

Estudos efetuados por Real et al.[31] (2009), relataram que as alterações da resposta inflamatória/reparativa associadas ao DM parecem resultar de complicações como diminuição de células polimorfonucleares e da função leucocitária, metabolismo anormal do colágeno e maior tempo para cicatrização das feridas, além das alterações no metabolismo de proteínas.

Madeiro et al.[20] afirmaram ainda que o difícil controle da cicatrização tecidual no diabético decorre da presença de hiperglicemia, microangiopatias, acidez metabólica, fagocitose ineficaz pelos polimorfonucleares e macrófagos[20]. Além disso, o DM altera a síntese, a maturação e o *turnover* do colágeno, um dos constituintes do tecido periodontal, o que acarreta a deficiência no reparo do mesmo em resposta a um processo inflamatório, seja bacteriano ou não[4].

O envolvimento das estruturas periodontais no DM confere a esta enfermidade um especial interesse por parte dos ortodontistas, pois são estes os sítios trabalhados durante a movimentação dentária ortodôntica. Além disso, não foram encontrados estudos sobre as alterações do complexo dentina-polpa de dentes movimentados ortodonticamente em modelos experimentais diabéticos.

A osteoporose é uma doença metabólica / hormonal caracterizada por diminuição da massa óssea com a ausência de alterações da mineralização dos ossos. Considerada como a mais comum patologia óssea metabólica, que surge como conseqüência de mudanças no turnover nível do tecido ósseo em que a quota de reabsorção óssea supera a formação de novo osso[30]. Osteoporose pós-menopausa é causada por uma redução acentuada nos níveis de estrogénio que leva a um aumento da taxa de remodelação óssea [9,25].

Estudos têm apontado para uma possível influência exercida pela osteoporose no desenvolvimento e progressão de lesões ósseas em estruturas craniofaciais e oral, mas o papel preciso desempenhado por tal doença na perda de anexos periodontais, dentes e altura do rebordo não tem sido claramente elucidado[18].

O modelo animal mais utilizado nas pesquisas experimentais com organismos osteopênicos está representado pela realização de ovariectomia em ratas. Este procedimento promove diminuição substancial na produção de estrogênio, que consequentemente altera a produção de proteínas osteo-indutivas, tais como a osteogenina e proteína morfogenética do osso[9,25], e iniciam-se os fenômenos característicos da menopausa. Com a ovariectomia realizada ocorre a condição de osteopenia determinada pelos níveis séricos de cálcio e fosfatase alcalina (ALK-P) nos animais experimentais[2,10,26,28,29].

Distúrbios no metabolismo da neoformação óssea em organismo com osteoporose pós-menopausa, são capazes de afetar a dinâmica do movimento dentário e, desta forma, acelerar a fase de correção ortodôntica durante a terapia[4,6,8]. Adicionalmente, outros estudos relatam que apesar deste movimento acontecer mais rápido, observa-se que há uma diminuição de osteoblastos, o que poderia acarretar um aumento na fase de contenção, assim como um aumento das taxas de recidiva[27,41].

O laser de baixa intensidade (LBI) é uma forma de energia altamente concentrada, não-invasiva, e não-ionizante que, quando em contato com tecidos diferentes, promover efeitos fotoquímicos e não lineares no tecido tratado. Vários estudos têm indicado que a laserterapia modula diferentes atividades biológicas, tais como a magnitude da resposta inflamatória, proliferação celular, diferenciação miofibroblástica e angiogênese. Além disso, tem-se utilizado a LBI para melhorar as propriedades biomecânicas do osso durante a regeneração óssea de fraturas, em modelos animais[12,13,14].

Relatos anteriores comprovam que o LBI pode ser útil para acelerar a dinâmica da movimentação dentária durante o tratamento ortodôntico, tanto em modelos experimentais[5,16] quanto em modelos de ensaios clínicos[12,13,14]. Propriedade biológica é provavelmente um resultado do estímulo da remodelação óssea, biomodulação da magnitude da resposta inflamatória e da produção aumentada de osteoclastos diferenciados.

Apesar das claras repercussões dos efeitos da DM e da osteopenia sobre os tecidos dentários e suas estruturas periodontais durante a movimentação ortodôntica, verifica-se a necessidade de trabalhos com ênfase na ação do LBI sobre a dinâmica destas alterações do periodonto e complexo dentina-polpa, visto que estes ainda são bastante escassos. Diante do exposto, constitui proposição do presente trabalho realizar um estudo experimental analisando o efeito da laserterapia de baixa intensidade sobre as alterações pulpo-periodontais induzidas pela movimentação dentária em ratos diabéticos e osteopênicos.

Neste estudo verificou-se que a terapia com laser de baixa potência é capaz de reverter as alterações deletérias histopatológicas observadas no ligamento periodontal comprometido por organismo diabéticos e osteopênicos acelerando o processo de remodelação do ligamento periodontal e osso alveolar durante a movimentação dentária induzida, porém essa propriedade na reversão da reação desmoplásica do tecido conjuntivo pulpar não foi significante.

Histomorphological changes induced by low level laser therapy during dental movement in ovariectomized rats*

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Abstract

The movement of the tooth is a process involving the resorption of alveolar bone and bone formation in response to stimuli compression and traction. The metabolic and hormonal changes in bone turnover, such as postmenopausal osteoporosis, are able to affect the dynamics of tooth movement. This study investigated the histopathological changes of the periodontal ligament of rats with estrogen deficiency induced by low level laser therapy (LLLT) during tooth movement. We found the treatment with LLLT is able to reversing the deleterious histopathological changes observed in the periodontal ligament of ovariectomized during experimentally induced tooth movement. Furthermore, based on the results of this study, it is possible that the LLLT can accelerate the remodeling of the periodontal ligament and alveolar bone during orthodontic tooth movement.

Key words: Tooth Movement, Bone Remodeling

Introduction

Osteoporosis is a metabolic/hormonal disease characterized by reduction of bone mass with no alterations of the mineralization in the bones. Considered as the most common bone metabolic pathology, it appears as a consequence of changes in the turn-over level of the bone tissue in which the quota of bone re-absorption overtakes the new bone formation.[1] Postmenopausal osteoporosis is caused by a sharp decrease in estrogen levels that leads to an increased rate of bone remodeling.[2] Studies have pointed at a possible influence exerted by osteoporosis on the development and progression of bone lesions in craniofacial and oral structures, but the precise role played by such disease in the loss of periodontal attachments, teeth and height of the residual ridge has not been clearly elucidated.[3]

Tooth movement is a complex physiopathological process which involves the resorption and neoformation of the alveolar bone in response to compressive and distractive stimuli, respectively.[4] Studies have demonstrated that metabolic and hormonal changes in the bone turnover, such as postmenopausal osteoporosis, are able to affect the dynamics of the tooth movement and accelerate the correction phase of orthodontic therapy.[5,6,7] However, other studies have previously reported that despite tooth movement is facilitated in rats with estrogen deficiency-derived osteoporosis, there is a decrease in the counts of osteoblasts, cells responsible for the new bone formation in regions of both tension and pressure of the periodontal ligament, which could lead to a longer-term maintenance phase, as well as increased relapse rates.[8,9]

Low level laser treatment (LLLT) is a highly concentrated, non-invasive, non-ionising radiation and contactless laser treatment performed with a low-energy output that, when in contact with different tissues, promote photochemical and nonlinear effects in the treated tissue. [10] Several studies have indicated that LLLT modulates different biological activities, such as anti-inflammatory action,[11,12] cell proliferation,[13] myofibroblastic differentiation[14] and angiogenesis.[15] In addition, LLLT has proved to enhance biomechanical properties of bone during fracture healing in animal models.[6,17]

There are some previous reports providing evidence that LLLT can be useful to accelerate the dynamics of tooth movement during orthodontic therapy, both in experimental models[8,18] and clinical trials models.[19,20,21] Such biological property is likely a result of bone

turnover stimulation, biomodulation of the magnitude of the inflammatory response and augmented production of differentiated osteoclastos.[22]

Therefore, the purpose of this study was to investigate the histopathological changes of the periodontal ligament of estrogen-deficient rats submitted to low level laser therapy during tooth movement.

Material and methods

Ethical Perspectives. 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 nº 341208). The study was carried out at the biotherium and the Laboratory of Morphology and Structural Biology of Tiradentes University (Aracaju/SE, Brazil).

Biological Assay. Sixty adult non-pregnant female rats (*Novergiculs 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 daily replaced wood shavings bedding, under controlled temperature at 22˚C, and 12 h light/darkness scale, with water and food (diet Labina®, Purina, Sao Paulo, Brazil).

Rat osteoporosis model. General anesthesia was induced by intraperitoneal injection of 50 mg kg -1 ketamine hydrochloride (Ketalar, Eczacıbaşı, Lüleburgaz, Turkey) and 5 mg kg -1 xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey). Bilateral ovariectomy was performed and the excised tissues were histopathologically verified as ovarian. The success of ovariectomy was confirmed by observing marked atrophy of the uterine horns. Ovariectomy was followed by a resting period of 2 months to allow the full effect of estrogen deficiency.[23]

Serum Analysis. The test was carried out using diagnostic reagent kit (Span diagnostic Ltd. Surat, India) for the In vitro determination of calcium (Ca). The calcium present in the serum was precipitated with naphtyl hydroxamic acid (calcium reagent). The precipitated was then dissolved in EDTA reagent and calcium from this solution was complexed with color reagent to give a colored complex that was measured colorimetrically. In vitro determination of Alkaline Phosphatase (ALK-P) was carried out by using diagnostic reagent kit (Span diagnostic Ltd). ALK-P from serum converts phenyl phosphate to inorganic phosphate and phenol at PH 10. Phenol so formed in alkaline medium with 4-aminoantipyrine in presence of

oxidizing agent potassiumferricyanide and forms an orange colored complex, which was measured by colorimetric method using an autoanalyzer (450 nm wavelength).[24]

Experimental Tooth Movement. At the end of 2 months, anesthesia was induced by the same method, 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 inch, Orthometric. Marilia, SP, Brazil) was ligated with the maxillary 1st molar cleat by a 0.010-inch 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, by using the same ligature wire. The orthodontic force exerted by the appliance was 50N in the beginning of the experiment. Tooth movement was performed for 19 days (day 0 ~ 19).

Low Level Laser Therapy Procedures. Animals were submitted 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 60s (20S each point). The output power used was 70mW, with a focal spot of 0.04 cm^2, and power density of 1W/cm2. The total energy per session was estimated in 4,2 J and the energy density was 35 J/cm^2 distributed in three different equidistant points in the root portion. The first irradiation was performed immediately after the burning procedures, and then performed every 48 h over the course of 7 days.

Procedures for the histomorphological analysis of the specimens. After seven, 13 and 19 days, animals were euthanized in a CO_2 chamber for post-mortem removal of the maxillas. 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, 10 histological sections (5µm thick) were obtained and stained in hematoxylin-eosin for analysis using a light microscope (Olympus CX31 optic microscope) by three trained observers.

Assessment of the inflammatory profile (IP). 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 profile inflammatory (IP)

was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as slighter/absent, moderate or severe.

Assessment of hyalinization content (HCt). The intensity of the hyalinization content was assessed in histological sections as follows: - (lack of hyalinization); 1 (hyalinized areas correspond to less than 150 μm); 2 (hyalinized areas representing between 150 and 400 μm); and 3 (hyalinized areas correspond to more than 400 μm).

Quantitative analysis of the osteoblasts (ObT)/osteoclastss (OcT) and blood vessels (BV) count. Counting of osteoblasts, osteoclastos and blood vessels were performed with an image analysis system (Imagelab). All the images were sent to the PC using an analog video camera (PAL system), after being converted to the RGB (red-green-blue) system necessary for digitizing and processing the sections. Five histological fields (200x magnification) of the tension and pressure areas of the periodontal ligament of 1st molar were used to determine the mean of osteoblasts and osteoclastos, respectively. For the assessment of the blood vessels count, images of the tension and pressure areas were analyzed. The images were recorded and automatically processed to find the cell density (CD) in each reference area (RA). Data were expressed as mean ± SEM.

Descriptive analysis of the collagen fibers of the periodontal ligament. Histological sections stained in picrosirius and analyzed under polarized light were used to the descriptive analysis of the collagen deposition. Collagen fibers were analyzed according to their birefringence pattern (greenish/yellow-greenish or orange, orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long), and disposition (reticularly or parallel arranged or interlaced).

Statistical Analysis. Data obtained in the IP and HC analysis were analyzed using the Kruskal-Wallis test, followed by *post-hoc* Dunn´s test. Data obtained in the ObT, OcT and Bv count analyses were analyzed by Anova followed by *post-hoc* Tukey‟s test. Differences among the groups were regarded as significant when $p<0.05$.

Results

Serum Analysis

As shown in Table 2, after 30 and 60 days from the surgical management, a significant increase in the serum levels of ALK-P ($P<0.001$). Ca levels were also increased in 60 days

($p<0.01$). However, no significant difference was observed in the serum levels markers of CTR neither in 30 nor in 60 days ($p>0.05$).

Histomorphological analysis

As demonstrated in Table 3, the peak of the inflammatory response was seen in seven days, progressively decreasing until 19 days. Although there was no significant difference among the groups, irrespective the ovariectomy or laser therapy, over the time course of the experiment ($p>0.05$), the pattern of the morphological changes in the periodontal ligament and alveolar bone was found to be distinct.

In seven days, both non-irradiated groups (CTR and OVR) presented intense infiltrate of lymphocytes and plasma cells were observed in the upper areas of the periodontal ligament in the pressure side, particularly near the gingival tissues. In the middle and apical thirds of the periodontal ligament, an immature granulation tissue, composed of misshapen blood vessels and macrophage/lymphocyte-rich infiltrate, was observed. Extensive resorptive changes were observed throughtout the alveolar bone in both non-irradiated, but it was clearly more expressive in OVR. Application of LLLT accelerated the reorganization of the granulation tissue into a more collagenized connective tissue, and reduced the extent of the resorptive changes. No remarkable difference was noted between L/CTR and L/OVR regarding the inflammatory response and granulation tissue. In the tension side, the inflammatory response ranged from mild to moderate in non-irradiated groups, whereas in the irradiated ones it was mild (Fig. 1).

In 13 days, the inflammatory infiltrate observed in the pressure sides was mild and restricted to the cervical areas of the periodontal ligament in CTR and OVR, but was absent in both irradiated groups. The surface of the alveolar bone showed irregularities resulted from resorption activity, but the presence of osteoclasts was more evident in OVR than in the other groups (CTR, L/CTR and L/OVR). No remarkable inflammatory, vascular or resorptive changes were noted in the tension sides of all the experimental groups (Fig. 2).

In 19 days, there was complete regeneration of the periodontal ligament. However, the surface of the alveolar bone of the pressure side in OVR still presented various “jagged-like” areas in the alveolar bone surface conferring an irregular appearance to the bone. In addition, such irregular appearance was no longer observed in the ovariectomized rats treated with low level laser irradiation (Fig. 3).

Quantitative Analysis of the Osteoclasts Count (OcT)

As demonstrated in Fig.4, the peak of osteoclastic activity was in seven days, decreasing significantly after 13 and 19 days in the mesial pressure sides of both CTR and

OVR. In seven and 13 days, the OcT was significantly increased in OVR compared to CTR ($p<0.001$ and 0.01, respectively), whereas no significant difference was seen in the other experimental times ($p>0.05$).

As demonstrated in Fig. 5 shows the mean count of osteoblasts (ObT) in the tension sides of the mesial roots of the experimental groups. The peak of osteoblastic activity was seven days, decreasing in 13 days and remaining relatively stable until 19 days. In seven days, both irradiated groups (L/CTR and L/OVR) presented increased ObT when compared to CTR ($p<0.05$ and 0.01m respectively) and OVR ($p<0.01$ and 0.001, respectively). However, there was no difference between this CTR and OVR or between the irradiated groups ($p>0.05$).

As described in Fig. 6, the peak of Blood Vessels Count (BvC) was also in seven days, and the amount of newly-formed vessels was slightly higher in the tension side than in the pressure side, although no significant difference was observed ($p>0.05$). At this experimental time, laser irradiation significantly improved the BvC in the pressure sides of CTR and OVR ($p<0.01$). In the tension side, although both irradiated groups presented high rates of BvC, only L/OVR showed significant increase in relation to its non-irradiated counterpart ($p<0.05$). No difference among the groups was observed in 13 and 19 days ($p>0.05$).

Hyalinization (hyaline degeneration) of the periodontal ligament was characterized by intense eosinophilic change of the connective tissue, accompanied of loss of histologic architectural details (Fig. 7). Hyalinization was seen in seven and 13 days in the pressure sides of both experimental groups, but it was more expressive in the first experimental period. In seven days, HCt was significantly increased in OVR in relation to CTR ($p<0.01$), whereas in l/CTR it was found to be decreased ($p<0.05$). No significant difference was observed between L/CTR and L/OVR ($p>0.05$). In 13 days, despite HCt was less expressive, it was still greater in OVR than in CTR ($p<0.01$). Both irradiated groups showed rates of hyalinization comparable to CTR ($p>0.05$) (Fig. 8).

There was no difference in the pattern of collagenization of the periodontal ligament between ovariectomized and sham groups, which presented, in general, orange birefringence consistent with type-I collagen. However, the LLLT promoted relevant changes in the rearrangement of the collagen fibers over the time course of the experiment.

In seven days (Fig. 9), the non-irradiated animals showed disruption of the morphological features of the periodontal ligament. The collagen fibers were found to be thin and delicate, intensely interlaced, and permeated by newly formed blood vessels throughout the extent of the periodontal ligament in the pressure sides. In the tension ones, the fibers were outstretched and distended. Both irradiated groups, on the other hand, showed

morphological signs of rearrangement of the horizontal fibers of the periodontal ligament, but it was more expressive in the pressure sides.

In 13 days (Fig. 10), both non-irradiated groups presented greater density of the collagen fibers in the periodontal ligament, particularly in the tension sides. However, the disposition of such fibers tended to be parallel to each other in the tension side, but disorganized in the pressure one. Besides, their appearance was still thin and delicate. In the irradiated groups, the fibers appeared gross, thick and clearly parallel to each other in the tension side, but still poorly organized in the pressure one.

In 19 days (Fig. 10), the periodontal ligament was completely recovered, and showed thick and well-compacted collagen fibers with parallel disposition in both tension and pressure sides, regardless of the laser therapy.

Discussion

The most common type of osteoporosis is the bone loss associated with menopause-induced ovarian hormone deficiency. The precise mechanism underlying bone loss secondary to ovary and hormone deficiency remains uncertain and several hypotheses have been linked to this condition, such as the augment plasma calcium levels as a result of increased bone resorption.[25] The most commonly used animal model for osteoporosis studies are the ovariectomized rats. The ovariectomized rat exhibits most of the characteristics of human postmenopausal osteoporosis, including biochemical changes of serum markers of bone metabolism.[26,27]

In attempt to verify whether the osteopenic condition, we determined the serum levels of Ca and ALK-P in the experimental animals. The biochemical markers and biomechanical results were similar to the earlier studies in ovariectomized rats.[24,27,28,29,30] These data are suggestive of intense bone metabolism and are consistent with bone loss caused by ovariectomy. In addition, the absence of significant difference between the non-operated rats and those subjected to sham surgery is a strong indicative that the biochemical changes are, in fact, promoted by ovariectomy.

There have been previous biomechanical and histological studies reporting that estrogen deficiency affects the dynamics of bone neoformation.[33,32] It has been previously proposed that osteogenesis decreases in ovariectomized rats because estrogen deficiency alters the production of osteo-inductive proteins, such as osteogenin and bone morphogenetic

protein, and disrupts bone matrix formation.[33] In this study, we found that the most expressive morphological changes observed in the pressure sides of the ovariectomized rats were associated to increased bone resorption. Supporting our findings, other studies have also demonstrated that the ovariectomy-induced estrogen deficiency affects the orthodontic therapy in experimental rodent model.[6,8,34] On the other hand, the fact that no significant difference in the osteoblasts and blood vessels count was noted in the alveolar bone of ovariectomized and non-ovariectomized animals reinforces the hypothesis that estrogen deficiency leads to increased resorption but does not influence neither the osteoblastic proliferation nor the granulation tissue formation.

In fact, the increased formation of osteoclasts seems to indicate that estrogen deficiency apparently is able to promote more rapid bone resorption and, consequently, facilitate the tooth movement.[8] However, it is possible to speculate whether a too fast dental movement would be harmful for the periodontal ligament. Hyalinization is considered an undesirable side-effect of toothe movement, and it is represented by focal areas of sterile necrosis of connective tissue in response to the disruption of the blood supply in the pressure side of the periodontal ligament movement.[35] It has been suggested that long-term persistence of hyalinization can be interpreted as a morphological sign of periodontal injury during induced tooth movement.[36] Thus, as in this study ovariectomized rats showed more expressive hyalinization zones, even in the latter stages of the experiment, it strongly suggests the occurrence of severe injury in the periodontal fibers.

In this study, the laser irradiation promoted no significant difference in the osteoclasts number in non-ovariectomized rats. Controversially, Kawasaki and Shimizu[18] reported an up-regulatory effect of LLLT on the osteoclasts formation. However, such increase is these resorptive cells occurred in two days of movement, but, as described in the current study, no difference was observed in seven days. These data are suggestive that the stimulatory effect of LLLT on osteoclasts formation is supposed to occur only on earlier phases of tooth movement, likely in response to the greater recruitment of macrophages into the injured site.[37] As a matter of fact, the laser-induced augmented resorption in the first steps, but not in the latter ones, of bone repair has been previously described site.[12] Besides, LLLT was found to stimulate the expression of metalloproteinase-9, the main MMP involved in the osteoclastic activity,[38] which seems to indicate that the faster dental movement associated to LLLT might result not only of early increased osteoclastic differentiation but also of greater resorptive activity of these cells.

On the other hand, the application of low level laser therapy (LLLT) on the ovariectomized rats reduced significantly the osteoclasts count in seven and 13 days. As LLLT accelerates the chronification of the inflammatory response,[15] it also promotes faster elimination of phlogiston constituents, such as necrotic material, possibly due to a stimulated macrophage activity.[39] This mechanism would reduce the migration of macrophages, as well as osteoclasts-precursor mononuclear cells to the periodontal ligament. Moreover, it has been previously reported that LLLT downregulates the RANKL:OPG mRNA ratio in osteoblasts,[40] suggesting that an overstated osteoclasts formation might be inhibited by laser irradiation. In addition, Saad et al,[41] have also demonstrated that LLLT was effective in promoting significant reduction of the osteoclasts number during bone repair in ovariectomized rats, thus supporting our findings. As in this study, the number of osteoclasts in irradiated ovariectomized and control rats was statistically similar, it is possible to suggest that LLLT was able to avoid excessive bone resorption in the pressure side of the periodontal ligament during tooth movement in estrogen-deficient ovariectomized rats.

A significant laser-induced increase of the osteoblasts was also verified in the initial times of this study, regardless the ovariectomy surgery. The stimulatory effects of LLLT on osteoblasts proliferation during bone healing have been recently described in estrogen deficient ovariectomized rats.[42] In addition, Da Silva et al,[43] reported that LLLT increase cell levels of ALK-P, Runx2, osteocalcin, type I collagen, and bone sialoprotein mRNA, suggesting that not only the proliferation but also the functional activity of osteoblasts is improved after laser irradiation. Therefore, it is possible to speculate that, after laser irradiation, bone formation is likely increased in the tension side of the periodontal ligament during the initial stages of experimental tooth movement. Moreover, once this improvement seems to be limited to seven days, and also considering the lack of overeactive bone and/or cementum formation, it is suggested that LLLT does not induce any long-term damaging changes to the periodontal ligament, such as hypercementosis or ankylosis.

The increase in blood vessels count induced by LLLT in the initial stages of this experiment was already expected. Laser-induced slight increase of the number capillary vessels during tooth movement has also been described by Ibrahim et al.[4] Such increase is probably related to an improvement in the granulation tissue after the pressure-associated injury of the periodontal ligament. Supporting this theory, other investigations have described the improvement of the blood vessels network after LLLT in experimental wound healing.[15,45] Besides, because no significant difference was observed between irradiated ovariectomized

and non-ovariectomized rats, it is possible to suppose that estrogen deficiency appears to present no relation with the dynamics of granulation tissue formation.

Low level laser therapy also reduced significantly the occurrence of hyalinization in ovariectomized rats. These beneficial effects might have resulted of the possible improvement of macrophage activity,[39] which would rapidly phagocyte the aseptic necrotic connective tissue (hyalinized tissue). In addition, it is also possible that the improved blood supply promoted by LLLT has allowed greater migration of monocyte-derived macrophages into the injured ligament, as well as provided higher rates of oxygenation, thus facilitating the replacement of the damaged/hyaline tissue for a newly formed collagen. Therefore, as long as long-term persistence of hyalinization has been widely related to impairment of tooth movement, it is suggested that LLLT might have provided a better quality for this movement.

In this study, the rearrangement pattern of the collagen fibers of the periodontal ligament was analyzed under polarized light. We found a clear predominance of type-I collagen instead of type III, suggesting that the collagenization process was already installed. The fact that there were disorganized fibers in the pressure side in seven days, but parallel in 13 and 19 days, seem to indicate that the remodeling process of the collagen is a late event in the periodontal healing after tooth movement. As no difference was observed in the collagenization pattern between ovariectomized and sham groups is suggestive that, as expected, the osteopenic status seems to exert no influence on the collagen synthesis and remodeling. However, LLLT substantially accelerated the rearrangement of the periodontal ligament fibers. Supporting our findings, the beneficial effect of LLLT on the collagen synthesis and remodeling was already reported in previous investigations performed in dermal tissues.[15,45]

In conclusion, we found that low level laser therapy is able to reverse the deleterious histopathological changes observed in the periodontal ligament of ovariectomized rats during experimentally-induced tooth movement. Moreover, on the basis of the findings of the present study, it is possible that low-energy laser irradiation may accelerate the remodeling process of the periodontal ligament and alveolar bone during orthodontic tooth movement.

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Tables and figures legends

Groups	Surgical procedures	Low Level Laser Therapy (Total Dose)
CTR	Sham Surgery*	0 j/cm^2
OVR	Ovariectomy	20 j/cm^2
L/CTR	Sham Surgery*	0 j/cm^2
L/OVR	Ovariectomy	20 j/cm^2

Table 1. Distribution of the animals in the experimental groups according to treatment

* Sham surgery (also placebo surgery) is a faked operative intervention that omits the step of ovaries removal.

Analytical Times	Serum Levels	
	Ca ± SD (MG/dL)	**ALK-P**
Non-operated rats	57.35 ± 7.48	8.1 ± 2.30
CTR - 30 days after sham surgery	69.4 ± 8.26	11.5 ± 3.41
OVR - 30 after ovariectomy	114.5 ± 16.2***	9.0 ± 4.11
CTR - 60 days after sham surgery	67.39 ± 7.68	7.66 ± 4.94
OVR - 60 after ovariectomy	116 ± 6.10***	15.2 ± 0.14**

Table 2. Determination of the serum levels of Alkaline Phosphatase and Calcium before and after the surgical management of the animals.

SD – Standard Deviation. ALK-P: Alkaline Phosphatase. Ca: Calcium

* Significantly different from non-operated rats $p<0.01$.

** Significantly different from non-operated rats $p>0.001$

	Pressure Side				Tension Side			
	CTR	**OVR**	**L/CTR**	**L/OVR**	**CTR**	**OVR**	**L/CTR**	**L/OVR**
	2	2	2	2	2	1	1	1
	2	2	2	2	2	1	1	1
7 days	2	2	2	2	1	2	1	1
	2	2	2	2	2	2	1	1
	2	2	2	2	1	1	1	1
	0	1	1	0	0	0	0	1
	1	1	1	1	1	1	0	0
13 days	1	1	0	1	1	1	0	1
	1	1	1	1	0	0	1	0
	1	1	0	0	1	1	1	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
19 days	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0

Table 3. Assessment of the inflammatory infiltrate in the studied groups in both pressure and tension sides of the periodontal ligamentum of the first molar mesial root.

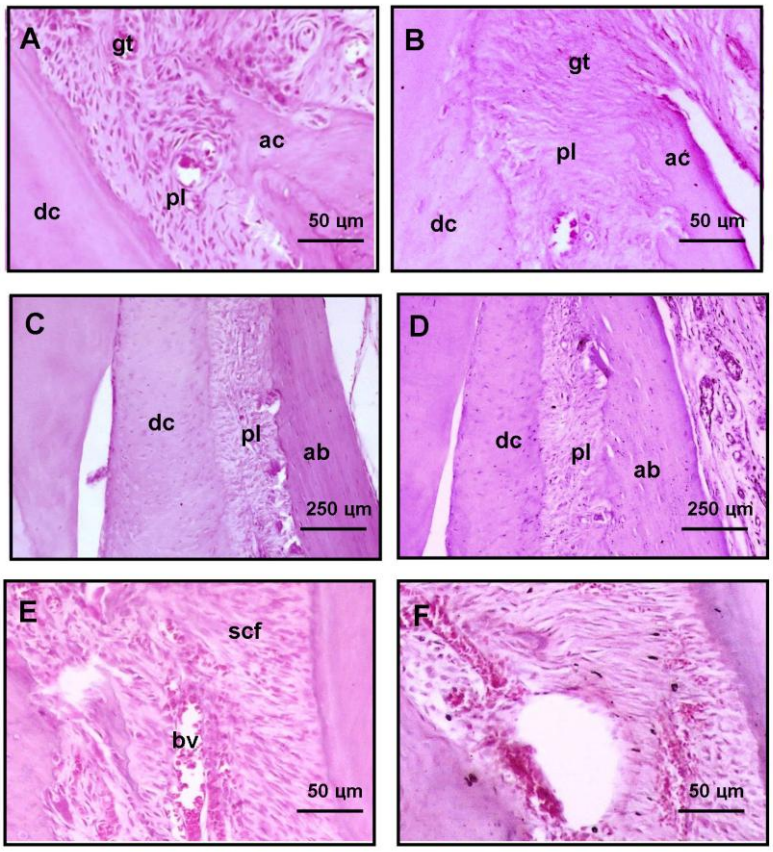

Fig. 1 – Histological sections of the seven days samples stained in HE. Pressure sides of the tooth roots showing (A) upper third of the periodontal ligament (pl) with intense inflammatory infiltrate particularly in the interface with the gingival tissue in OVR but (B) mild inflammation in L/OVR. (C) Middle third of the periodontal ligament presenting highly vascular granulation tissue in OVR and (D) more collagenized ligament in L/OVR. Tension side of the tooth roots showing (E) moderate inflammation in OVR, but (F) mild in l/OVR. Note that the collagen fibers are stretched and the blood vessels are hyperemic. **Legend**: dc (dental cementum); gt (gingival tissues); ac (alveolar crest); pl)periodontal ligament); ab (alveolar bone); scf (stretched collagen fibers); bv (congested blood vessels).

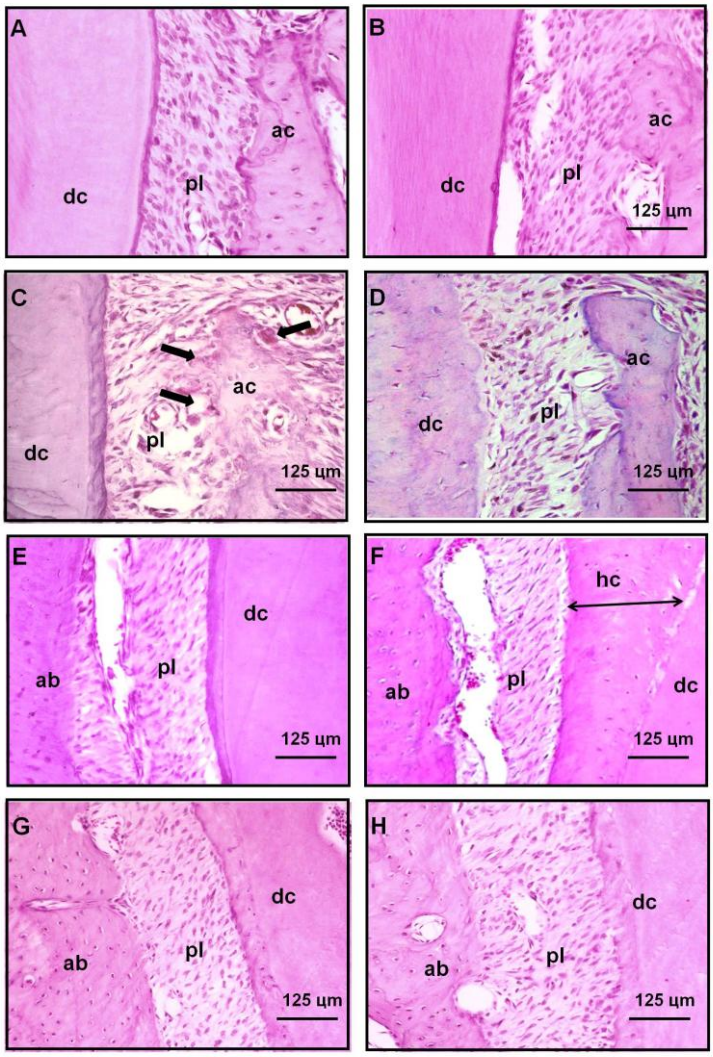

Fig. 2 – Histological sections of the 13 days samples stained in HE showing the cervical third of the periodontal ligament in the pressure sides. (A) CTR, (B) L/CTR, (C) OVR (note the expressive osteoclastic actibity highlighted by the arrows) and (D) L/OVR; Tension side of the tooth roots showing distended fibers and lack of inflammatory infiltrate. (E) CTR, (F) L/CTR (note the marked hypercementosis), (G) OVR and L/OVR. **Legend**: dc (dental cementum); ac (alveolar crest); pl (periodontal ligament); ab (alveolar bone); hc (hypercementosis).

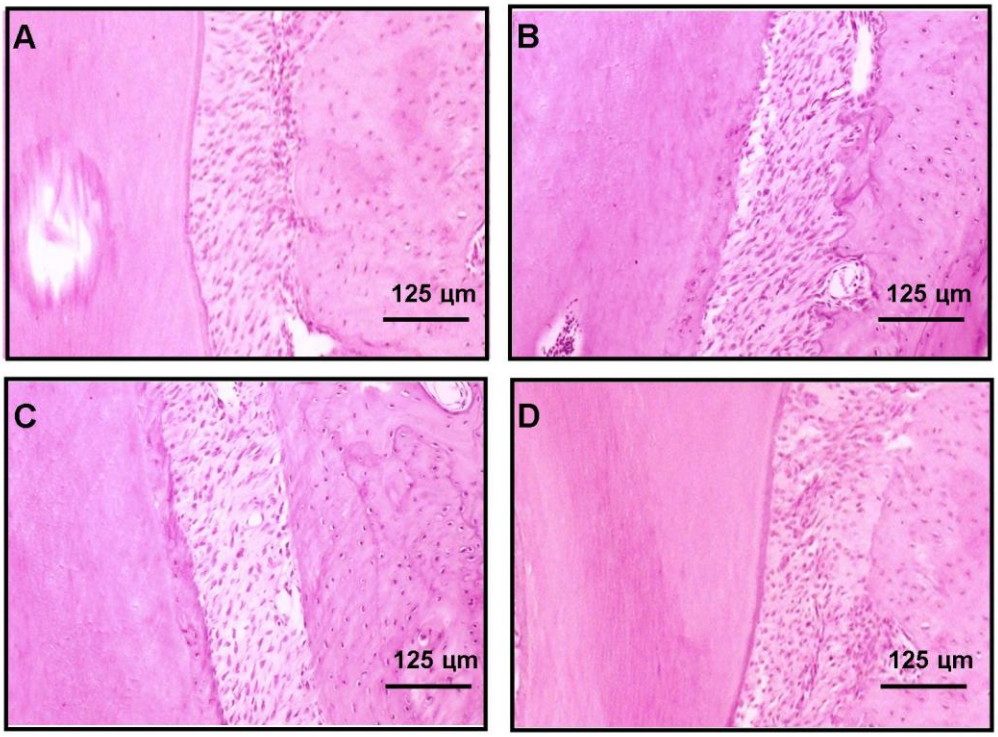

Fig. 3 – Histological sections of the 19 days samples stained in HE. Pressure sides of the tooth roots showing recovery of the typical morphological appearance in (A) CTR, (B) OVR, (C) L/CTR and (D) L/OVR. Note the presence of irregularities in the alveolar bone surface in OVR.

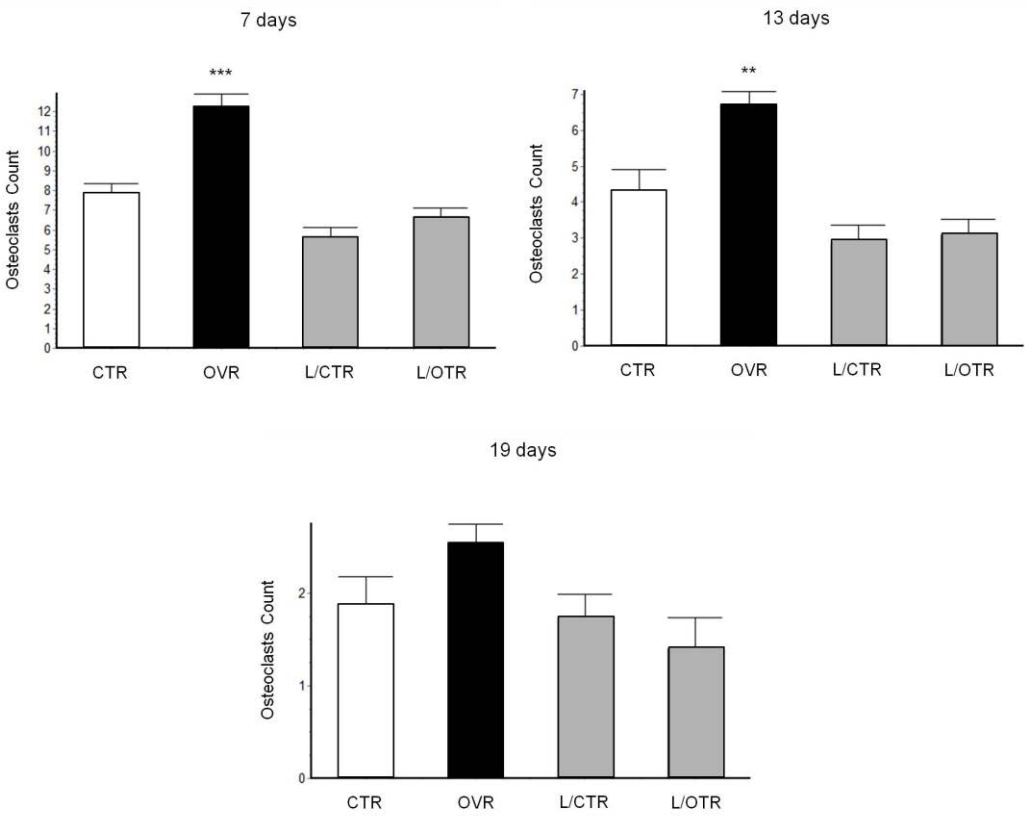

Fig. 4 – Osteoclasts Count (OcT) in the pressure sides of the periodontal ligament in the experimental groups over the time course of the study (mean ± SEM). *** Significantly different from CTR ($p<0.001$). ** Significantly different from CTR ($p<0.01$).

Quantitative Analysis of the Osteoblasts Count (ObT)

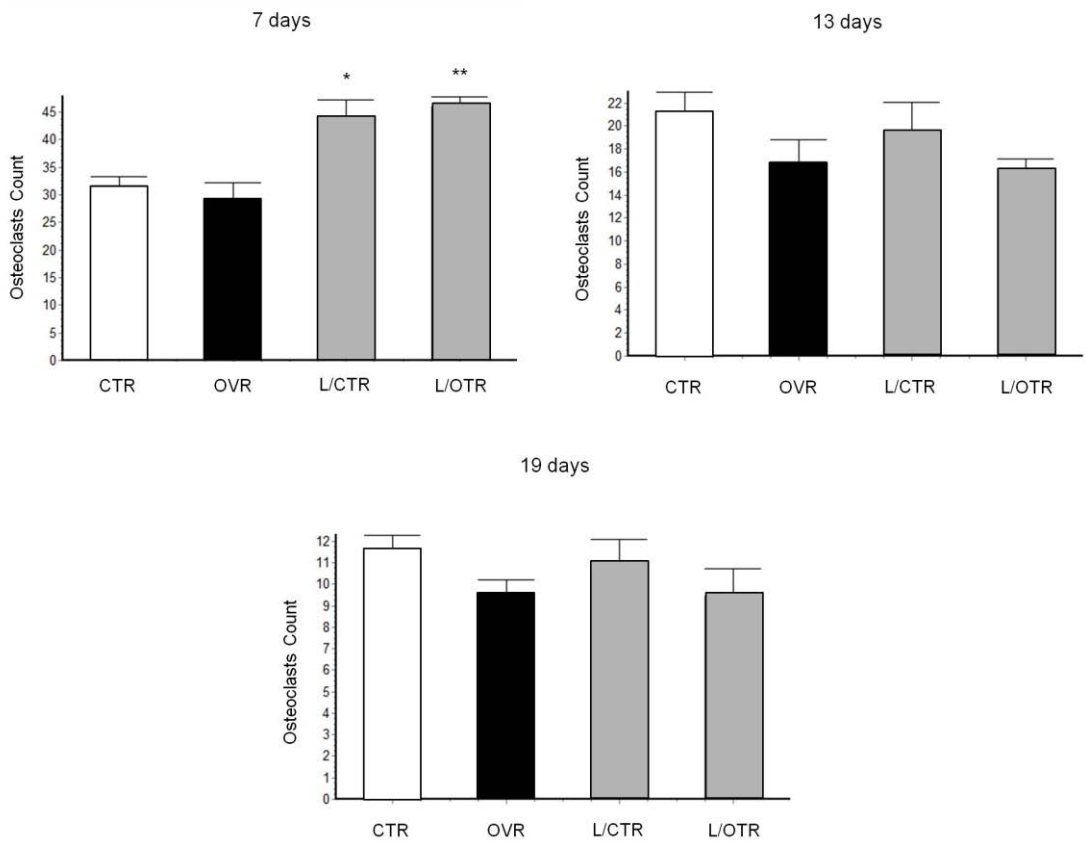

Fig. 5 –Osteoblasts Count (ObT) in the pressure sides of the periodontal ligament in the experimental groups over the time course of the study (mean ± SEM).

Quantitative Analysis of Blood Vessels Count (BvC)

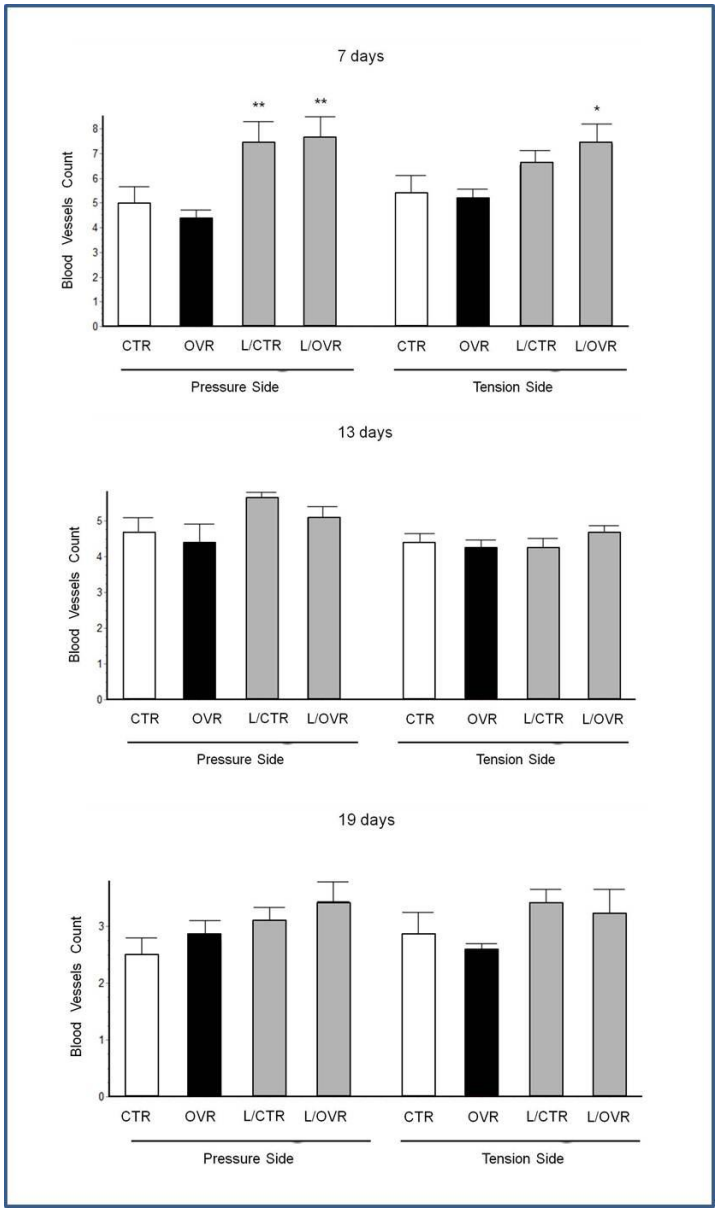

Fig. 6 –Blood Vessels Count (BvC) in both pressure and tension sides of the periodontal ligament in the experimental groups over the time course of the study (mean ± SEM). Significant differences: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Assessment of the hyalinization Contents (HCt)

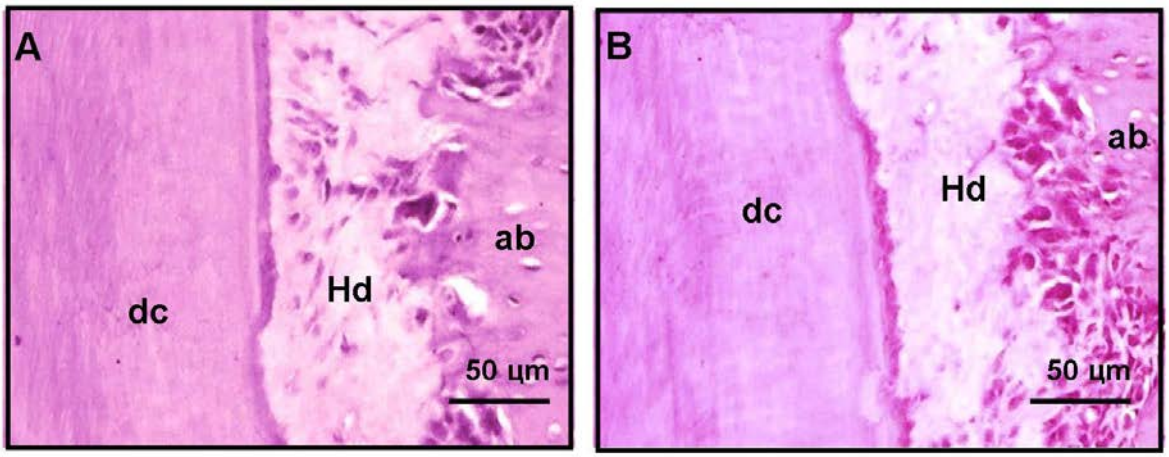

Fig. 7 – Histological sections stained in HE showing intense hyalinization of the connective tissue of the periodontal ligament in (a) CTR and (B) OVR (seven days).

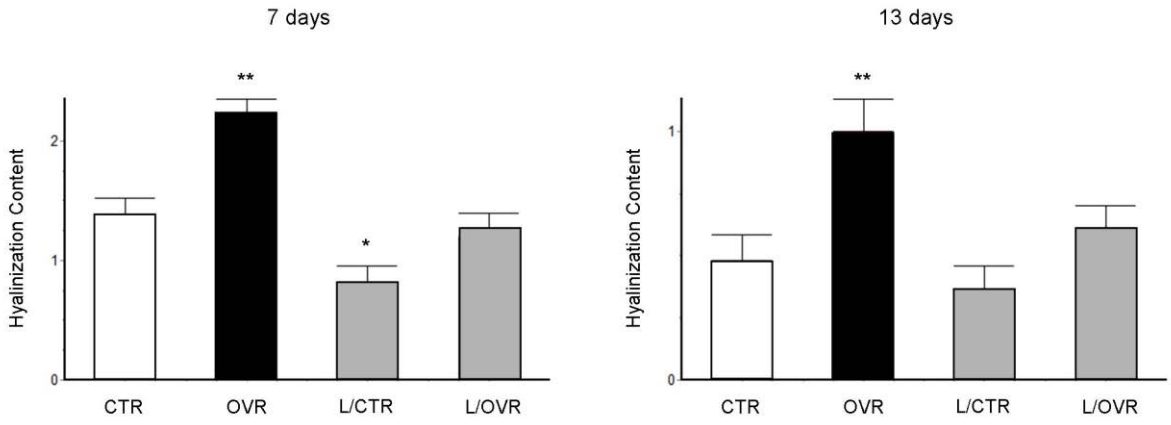

Fig. 8 – Assessment of the Hyalinization Contents (HCt) in the experimental groups in seven and 13 days. Significant differences: * $p<0.05$ and ** $p<0.01$.

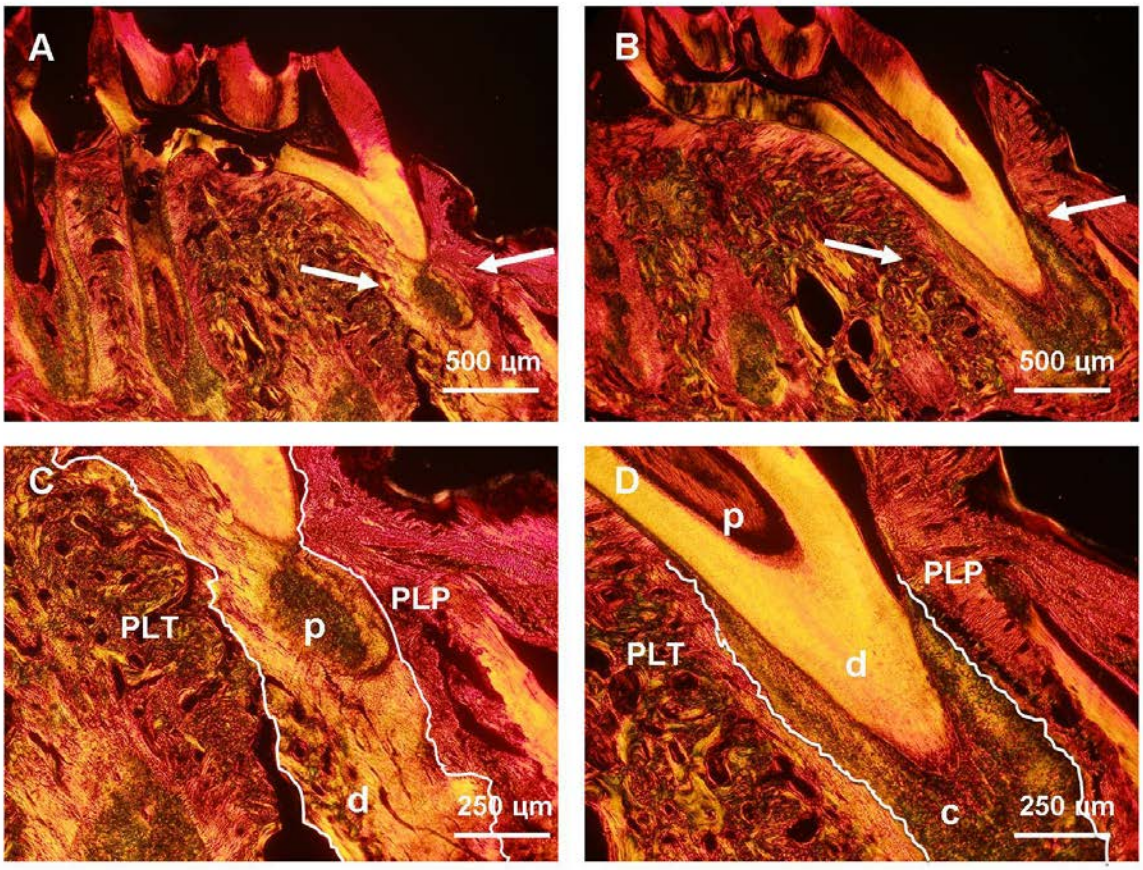

Fig. 9 – Histological sections stained in Sirius Red and analyzed under polarized light. Panoramic view of the mesial root (arrows) in (A) non-irradiated and (B) irradiated ovariectomized rats in seven days. In detail, note that the pressure sides in non-irradiated rats (C) present disorganized fibers, whereas in irradiated ones (D) it is possible to evidence some denser disposed and parallel-arrange fibers in the same side.

PLT – tension side of the periodontal ligament. PLP – Pressure side of the periodontal ligament. d – dentin; c – radicular cementum; p – dental pulp. White line circumvents the root.

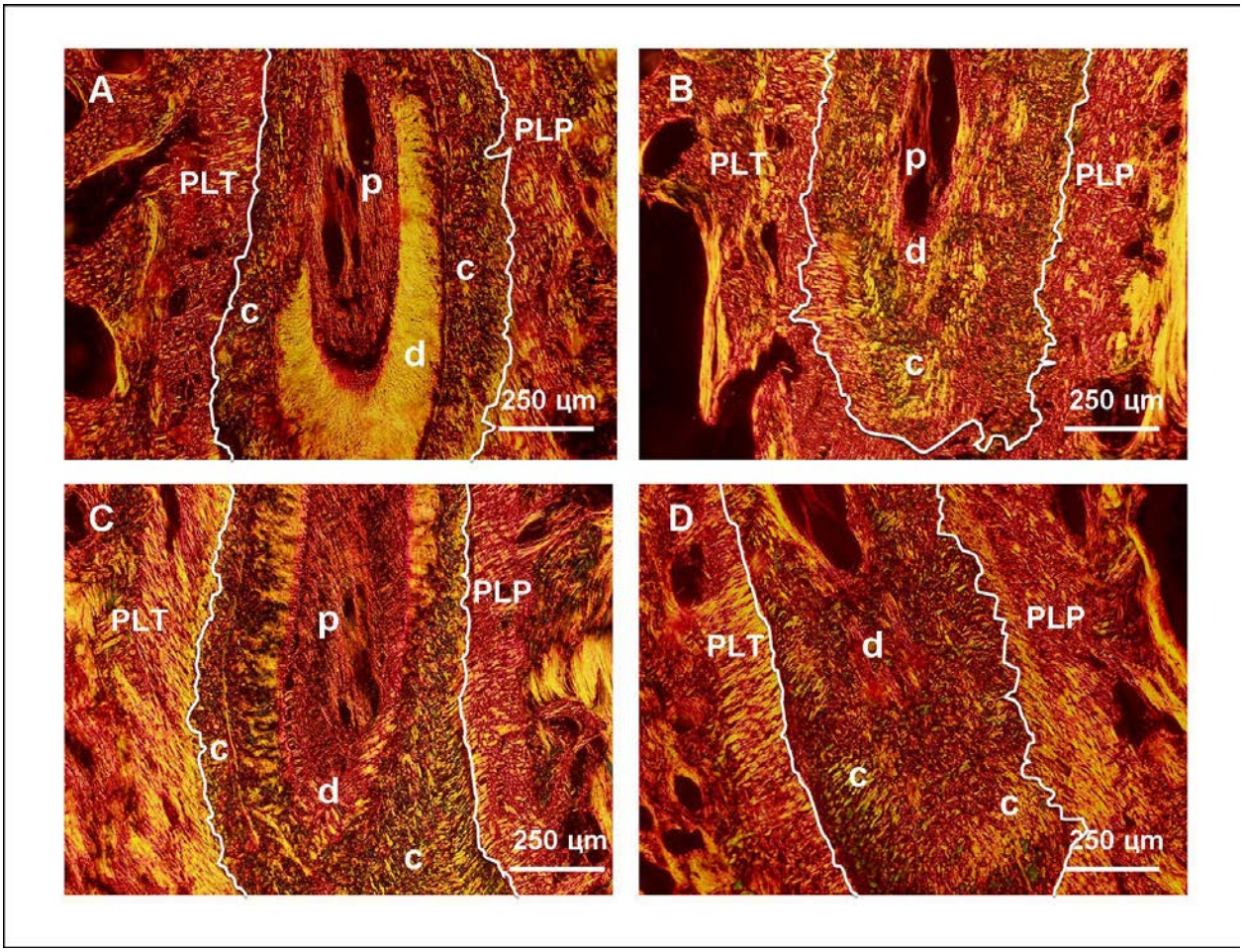

Fig. 10 – Histological sections stained in Sirius Red and analyzed under polarized light. (A) Non-irradiated and (B) irradiated ovariectomized rats in 13 days. Note the denser disposition of the fibers of the periodontal ligament in the irradiated ones. (C) Non-irradiated and (D) irradiated ovariectomized rats in 19 days showing complete recovery of the usual morphological features of the periodontal ligament‟s collagen fibers.

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

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Abstract

Orthodontic treatment in adult diabetic patients is usually complicated by a sort of oral problems, such as periodontal degradation and and bone loss. Orthodontic treatment in adult diabetic patients is usually complicated by a sort of oral problems, such as periodontal degradation and and bone loss. Several studies have indicated that low level laser therapy (LLLT) modulates different biological activities, such as anti-inflammatory action. The present study evaluated the histological changes of the periodontal ligament and alveolar bone during tooth movement in diabetic rats subjected to laser irradiation in Sixty non-pregnant female adult male rats, weighing 250 ± 30 g were randomly divided into four experimental groups (n = 15). The present study allow the suggestion that LLLT provides substantial improvement in the vascularization and collagenization in the pressure side of the periodontal ligament of diabetic rats submitted to experimental tooth movement.

Key words: Tooth Movement, Diabetes Mellitus, Bone Remodeling

Introduction

Diabetes Mellitus (DM) is one of the most common endocrine disorders. It is characterized by persistently raised blood glucose levels (hyperglycemia), resulting from deficiencies in insulin secretion, insulin action, or both. [1] Chronic hyperglycemia 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 in the glucose metabolism is able to directly affect the response of the bone and connective tissues to injury. [3,4] Because of that, the need for orthodontic treatment in adult diabetic patients is usually complicated by a sort of oral problems, such as periodontal degradation and and bone loss. [5] Although there are only few studies assessing the histological changes of the periodontal ligament and alveolar bone during dental movement in diabetic experimental animals, they provide evidence that chronic hyperglycemic status might impair the periodontal ligament response and bone remodeling during orthodontic treatment. [6]

Lasers emit a highly concentrated, non-invasive, non-ionising radiation that, when in contact with different tissues, promote thermal, photochemical and nonlinear effects. [7] Several studies have indicated that low level laser therapy (LLLT) modulates different biological activities, such as anti-inflammatory action, [8,9] angiogenesis [10,11] and collagen synthesis. [12,13] In particular, the acceleration of bone regeneration by laser treatment has been the focus of studies. [14,15]

It has been previously 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 of the periodontal ligament and alveolar bone during dental movement in diabetic rats submitted to laser irradiation.

Material and methods

Ethical Perspectives. 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 nº 341208). The study was carried out at the biotherium and the Laboratory of Morphology and Structural Biology of Tiradentes University (Aracaju/SE, Brazil).

Biological Assay. Sixty adult non-pregnant female male rats (Novergiculs 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 daily replaced wood shavings bedding, under controlled temperature at 22˚C, and 12 h light/darkness scale, with water and food (diet Labina®, Purina, Sao Paulo, Brazil).

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 hypoglycemia. Blood samples were collected from the tail vein of the animals after 72 h in order to assess the plasma glucose levels by the glucose-oxidase enzymatic method, using Accu-Chek Advantage (Boehringer, Germany). Animals presenting glucose levels above 200 mg/ dL were included in the diabetic group. The examinations were repeated every 7 days to confirm maintenance of the glucose levels. The animals presenting 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.

Experimental Tooth Movement. At the end of 2 months, anesthesia was induced by the same method, 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 inch, Orthometric. Marilia, SP, Brazil) was ligated with the maxillary 1st molar cleat by a 0.010-inch 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, by using the same ligature wire (Fig. 1). The orthodontic force exerted by the appliance was 50 g in the beginning of the experiment. Tooth movement was performed for 19 days (day 0 ~ 19).

Low Level Laser Therapy Procedures. Animals were submitted 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 (25 S each point). The output power used was 70 mW, with a focal spot of 0.04 cm2, and power density of 1W/cm^2. The total energy per session was stimated in 4,2 J and the energy density

was 35 J/cm^2 distributed in 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 seven days.

Procedures for the histomorphological analysis of the specimens. After seven, 13 and 19 days, animals were euthanized in a CO2 chamber for post-mortem removal of the maxillas. 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, 10 histological sections (5µm thick) were obtained and stained in hematoxylin-eosin for analysis using a light microscope (Olympus CX31 optic microscope) by three trained observers.

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 profile inflammatory (IP) was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as slighter/absent, moderate or severe.

Quantitative analysis of the osteoblasts (OsTB)/osteoclasts (OsTC) and blood vessels (BvC) count. Counting of osteoblasts, osteoclastos and blood vessels were performed with an image analysis system (Imagelab). All the images were sent to the PC using an analog video camera (PAL system), after being converted to the RGB (red-green-blue) system necessary for digitizing and processing the sections. Five histological fields (200x magnification) of the tension and pressure areas of the periodontal ligament of 1st molar were used to determine the mean of osteoblasts and osteoclasts, respectively. For the assessment of the blood vessels count, images of the tension and pressure areas were analyzed. The images were recorded and automatically processed to find the cell density (CD) in each reference area (RA). Data were expressed as mean ± SD.

Quantitative analysis if the collagenization rates (CR). The quantitative analysis of the area occupied by collagen deposition rates (CR) in the periodontal ligament was determined by optical density in the image analysis system in different randomly selected fields. The system used consists of a CCD Sony DXC-101 camera, applied to a 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 inserted into a computer (Pentium 133 MHz), and processed by a software (ImageLab). A total of ten fields per case were analyzed at a magnification of 1000x. 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 birrefringent (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 in percentage of the periodontal ligament area fraction occupied by the collagen fibers.

Statistical Analysis. Data obtained in the IP and HC analysis were analyzed using the Kruskal-Wallis test, followed by post-hoc Dunn´s test. Data obtained in the OSTB, OsTC, BvC and CR counts were analyzed by Anova followed by post-hoc Tukey's test. Differences among the groups were regarded as significant when $p<0.05$.

Results

*Descriptive analysis of the histological changes of the periodontal ligament***.**

In seven days (Fig. 1), exuberant granulation tissue and multiples erosive alterations, consistent with howship lacunae, were seen along the alveolar wall, most of them containing osteoclasts, were observed in CTR, CTR/LT and DBT/LT, particularly in the cervical thirds of the periodontal ligament. However, intense edematous changes of the connective tissue associated to chronic inflammatory infiltrate, with sparse vascularization, were verified in DBT. It should be emphasized that the collagen fibers appeared more apparent and 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.

In 13 days (Fig. 2), all the groups showed remarkable reduction of the vascular network and substantial increase in the fibrous component. Osteoclastic activity was still present, but it was less expressive than in seven days. The collagen fibers showed gross and thick appearance, as well as disposed in parallel arrangement in CTR, CTR/LT and DBT/LT. In the non-irradiated diabetic animals (DBT), however, the collagen fibers appeared to be thinner and present a not uniform disposition throughout the periodontal ligament.

In 19 days (Fig.3), the periodontal ligament f both CTR and CTR/LT presented similar morphological features, expressed by a cell-rich and moderately vascular connective tissue, associated to thick, but delicate, parallel arranged collagen fibers. In diabetic animals, there were parallel bundles of thin collagen fibers associated to a high content of flat spindle-shaped cells (consistent with fibroblasts), and such fibrous component was quite more expressive in DBT/LT than in DBT.

The analysis of the intensity of the inflammatory response (IR) found throughout the periodontal ligament is expressed in the Table 2. The IR values observed in irradiated non-diabetic animals (CTR/LT) were lower than all the other groups in seven and 13 days ($p<0.05$). Although in seven days diabetic animals (DBT) presented 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. In 13 and 19 days, the leukocyte infiltration progressively decreased, and none of the groups presented significant differences regarding the intensity of the inflammatory response ($p>0.05$).

Assessment of the Mean of Osteoblasts Number (OsTB).

The results of the histomorphometric study of osteoblasts number (OsTB) in the periodontal cortex of the alveolar wall are presented in Fig. 4. he values of OsTB were significantly decreased in DBT in comparison with CTR in seven ($p<0.001$) and 13 days ($p<0.01$), but not in 19 days ($p>0.05$). Irradiated diabetic animals presented a significant increase in OsTB compared to non-irradiated ones in seven and 13 days ($p<0.05$), and although in seven days DBT/LT still showed OsTB values lower than CTR ($p<0.05$), no significant difference was observed between these two groups in 13 days ($p>0.05$). In 19 days, no significant difference in OsTB was observed among the experimental groups ($p>0.05$).

Assessment of the Mean of Osteoclasts Number (OsTC).

The results of the histomorphometric study of osteoclasts number (OsTC) in the periodontal cortex of the alveolar wall are presented in Fig. 5. In seven days, a significant decrease in OsTC was observed in diabetic animals compared with control ($p<0.001$). 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 ones ($p>0.05$). In addition, the increase of OsTC in CTR/LT compared with CTR was not regarded as significant ($p>0.05$). In 13 days, the OsTC in diabetic animals remained significantly lower than in the control group ($p<0.01$), although in laser-irradiated ones (DBT/LT) this difference

was not significant ($p>0.05$). In 19 days, no significant difference in OsTC was observed among the experimental groups ($p>0.05$).

Assessment of the mean number of blood vessels.

The results of the histomorphometric study of blood vessels count (BVC) in the periodontal of the periodontal ligament in the pressure side are presented in Fig. 6. In seven days, the blood vessels count was significantly lower in diabetic animals compared with the control group ($p<0.05$). The LLLT promoted 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 ones ($p>0.05$). Lower rates of blood vessels count were observed in 13 and 19 days, and no significant difference was verified among the experimental groups ($p>0.05$).

Quantitative analysis if the collagenization rates (CR).

Collagen fibers were identified due to their intense golden birefringence under polarized light (Fig. 7), and presented as fibrous structures disposed in parallel arrangement, connected in one side to the dental cement and to the alveolar bone in the other. As shown in Fig. 8., the collagenization rates (CR) were significantly decreased in diabetic animals (DBT) than in controls (CTR) in seven ($p<0.001$), 13 ($p<0.001$) and 19 days ($p<0.01$). Laser irradiation induced a significant increase of CR in CTR/LT and DBT/LT compared to the respective controls (CTR and DBT) in seven ($p<0.01$ and 0.01) and 13 days ($p<0.05$ and 0.001). In addition, the CR values observed in irradiated diabetic animals (DBT/LT) were not significantly different from non-diabetic animals (CTR) during all the experimental period ($p>0.05$).

Discussion

In this study, we found substantial changes in the alveolar bone and periodontal ligament response of diabetic animals to the application of an orthodontic force. Such changes were represented by persistence of inflammatory response, impairment of the granulation tissue formation (development of neoformed capillary vessels and deposition of type I collagen fibers), decrease in both osteoblastic/osteoclastic differentiation and reduced number of howship lacunae. Similar morphological and morphometric findings were recently reported by Villarino et al (2011). [6]

The mechanisms responsible for the enhanced inflammatory response in diabetic animals have not been conclusively established. It has been proposed 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. Thus, the enhanced production of TNF dysregulates the cytokine networks and potentiates the inflammatory responses. 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 the tissues. [20]

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

On the other hand, it has been recently proposed the existence of bone-pancreas endocrine loop through which insulin signaling in osteoblasts ensures osteoblasts differentiation and stimulates osteocalcin production, which in turn regulates insulin sensitivity and pancreatic insulin secretion. [23] Therefore, it is possible to suggest that the significant decrease in osteoblasts number observed in this study might have resulted of the substantial reduction in insulin levels associated to the aloxan-induced pancreatic damages. 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 been recently described that the osteoblasts are one the major sources of the receptor activator for nuclear factor kappa ligand (RANKL), [24] a molecule widely required for osteoclasts formation from the peripheral blood derived mononuclear precursors. Hence, the decrease in osteoblasts differentiation would be ultimately responsible for reduced osteoclasts formation, as observed in this study.

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

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

RANKL release by osteoblasts, resulting in improved osteoclasts formation. [24] Moreover, it has been also suggested that laser irradiation might stimulate the biological events associated to osteoclasts formation, such as bone marrow-derived mononuclear osteoclasts precursors of (preosteoclasts) fusion to mature osteoclasts. [26] As the speed of tooth movement is greatly dependent on the dynamics of bone remodeling, as result of bone resorption and formation balance, it is possible to suggest that LLLT improved tooth movement in diabetic rats, as a result of increased osteoblasts/osteoclasts differentiation. However, it is essential to emphasize that, in spite of the laser-induced histological improvement in the osteoblasts and osteoclasts 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 in seven days, both OsTC and OsTB remained lower than in CTR.

The 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 blood vessels formation was impaired in diabetic rats, LLLT promoted functional recovery of the angiogenesis, and allowed a suitable granulation tissue formation comparable to the non-diabetic rats. The stimulatory role played by LLLT on angiogenesis and granulation tissue during wound healing has been previously reported during wound healing [27,28] and bone repair. [29] 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), molecules able to mediate endothelial proliferation and blood vessels formation, [30] which could support the histological findings regarding the periodontal vascular content observed in this study. The fact that significant results were limited to seven 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. [31]

Finally, improved collagenization and better spatial organization of the collagen fibers were also observed in the periodontal ligament of diabetic rats after laser irradiation. Supporting our findings, [13,28,32] reported that LLLT is able to improve collagen deposition and remodeling during wound healing, likely as a result of biomodulatory effects on the fibroblast function. Therefore, it is possible to suggest that LLLT is able to stimulate the collagen deposition and improve the architectural arrangement of the periodontal fibers in diabetic rats during tooth movement.

In conclusion, the present study allow the suggestion that LLLT provides substantial improvement in the vascularization and collagenization in the pressure side of the periodontal

ligament of diabetic rats submitted to experimental tooth movement. Notwithstanding these findings, the impairment of the osteoblasts and osteoclasts 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 have been shown to be able to cause unwanted effects on bone remodeling.

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Tables and figures Legends

Groups	Pre-treatment	Low Level Laser Therapy (Total Dose)
CTR	Citrat buffer	0 j/cm^2
DBT	Alloxan (150 mg/kg)	20 j/cm^2
CTR/LT	Citrat Buffer	0 j/cm^2
DBT/LT	Alloxan (150 mg/kg)	20 j/cm^2

Table 1. Distribution of the animals in the experimental groups according to treatment

Experimental Period (days)	Experimental Groups			
	CTR	CTR/LT	DBT	DBT/LT
7	2.50 ± 0.5[a]	1.16 ± 0.4 [b]	3.00 ± 0.0 [c]	2.20 ± 0.6 [a]
13	0.75 ± 0.3 [a]	0.70 ± 0.2 [b]	1.00 ± 0.4 [a]	0.90 ± 0.4 [a]
19	0.00 ± 0.0 [a]	0.00 ± 0.0 [a]	0.00 ± 0.0 [a]	0.00 ± 0.0 [a]

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

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

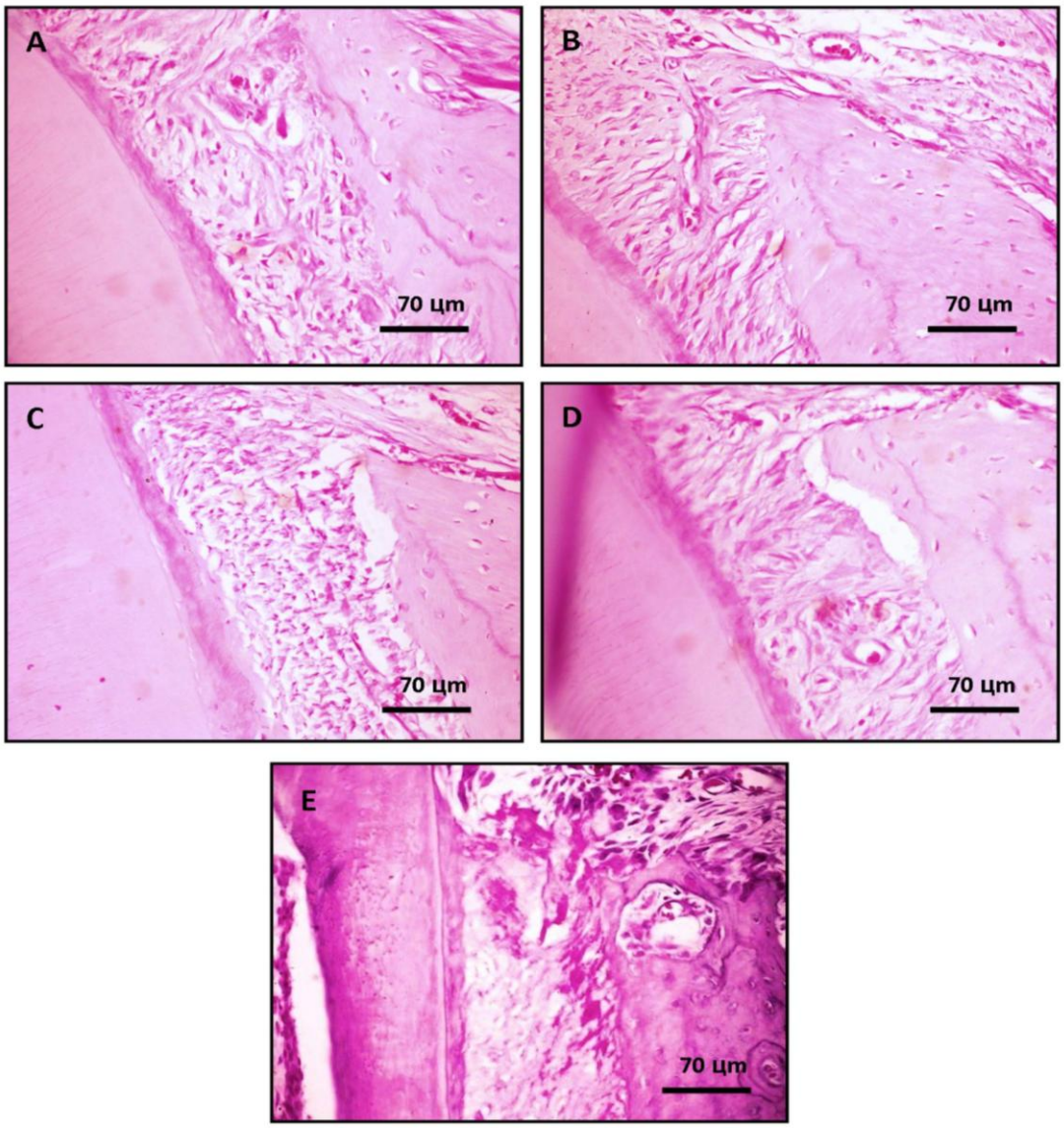

Fig. 1 – Histological changes of the periodontal ligament in the studied groups. (A) CTR, (B) CTR/LT, (C) DBT and (D) DBT/LT. Note the well-developed fibrovascular granulation tissue in CTR, CTR/LT and DBT/LT, and inflammatory infiltrate and edema of the connective tissue in DBT. (E) Coagulative necrosis in DBT (7 days –HE, 200x).

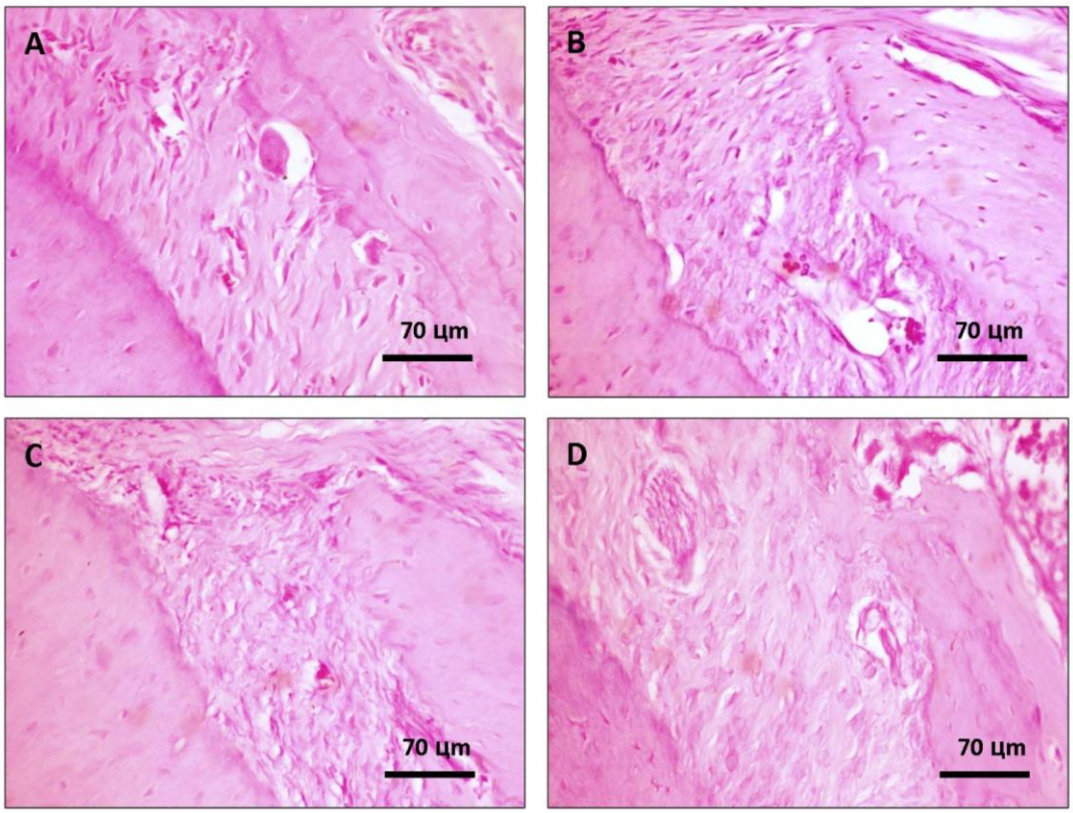

Fig. 2 – Histological changes of the periodontal ligament in the studied groups. (A) CTR, (B) CTR/LT, (C) DBT and (D) DBT/LT. Note the parallel disposition of the collagen fibers in CTR, CTR/LT and DBT/LT, and the disorganization of the collagen architecture in DBT (13 days –HE, 200x).

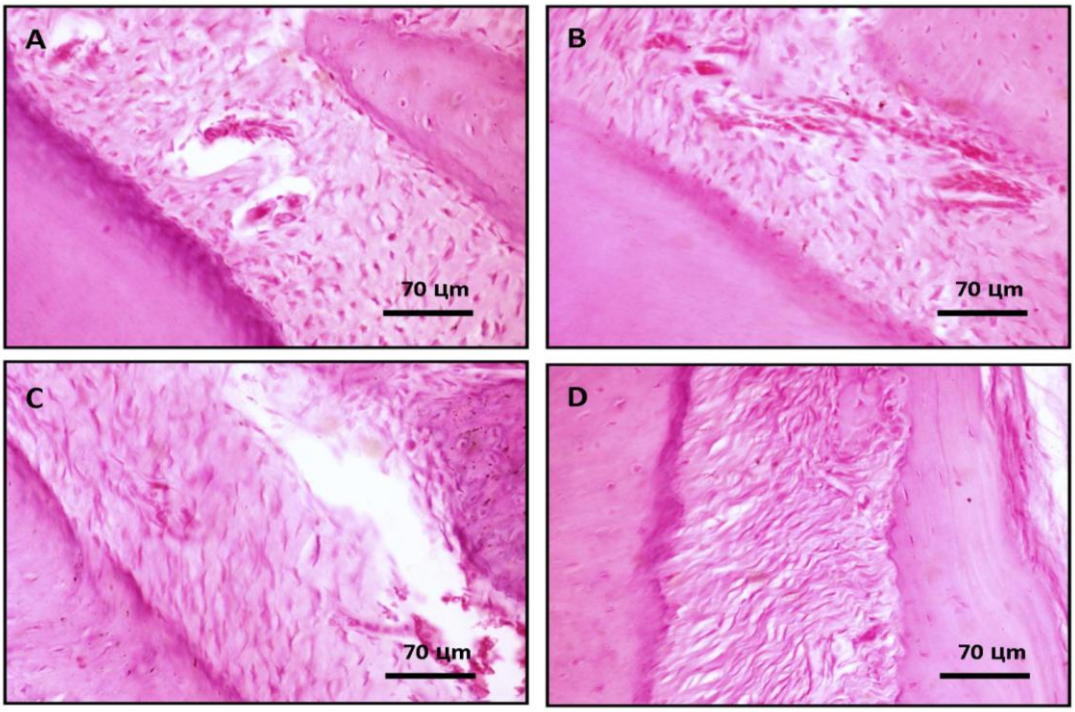

Fig. 3 – Histological changes of the periodontal ligament in the studied groups. (A) CTR, (B) CTR/LT, (C) DBT and (D) DBT/LT. Note the recovery of the normal appearance of the periodontal ligament in CTR and CTR/LT, whereas it appears less remodeled in DBT and fibrotic in DBT/LT, (19 days –HE, 200x).

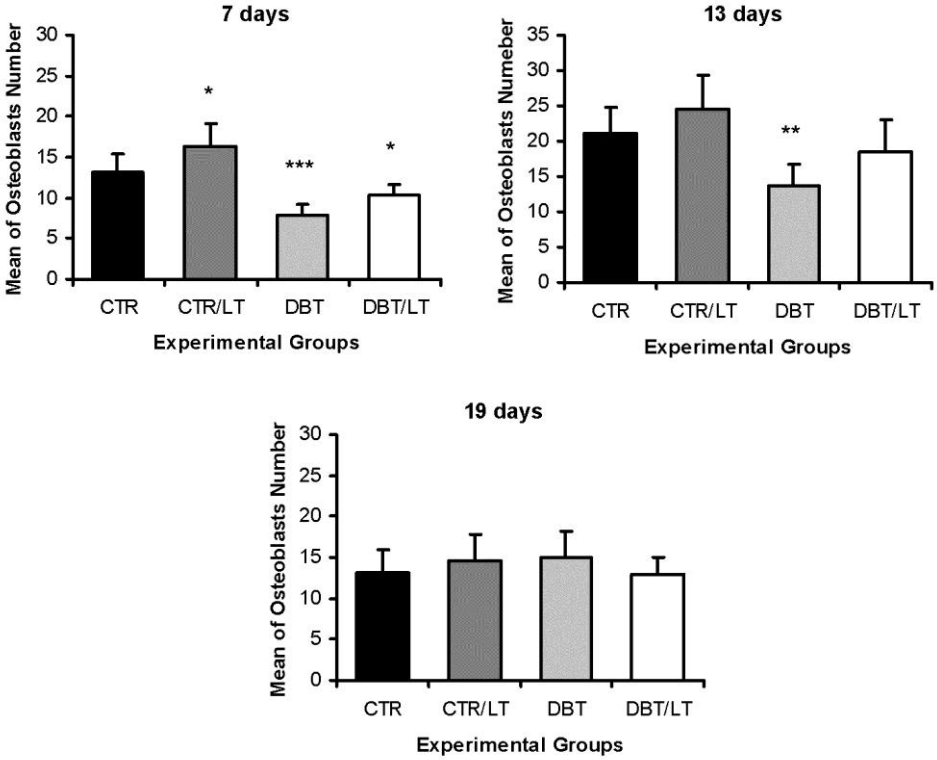

Fig. 4 – Assessment of the mean of osteoblasts number (OsTB) in the studied groups over the time course of the experiment.

* Significantly different from CTR ($p<0.05$); ** Significantly different from CTR ($p<0.01$); *** Significantly different from CTR ($p<0.001$)

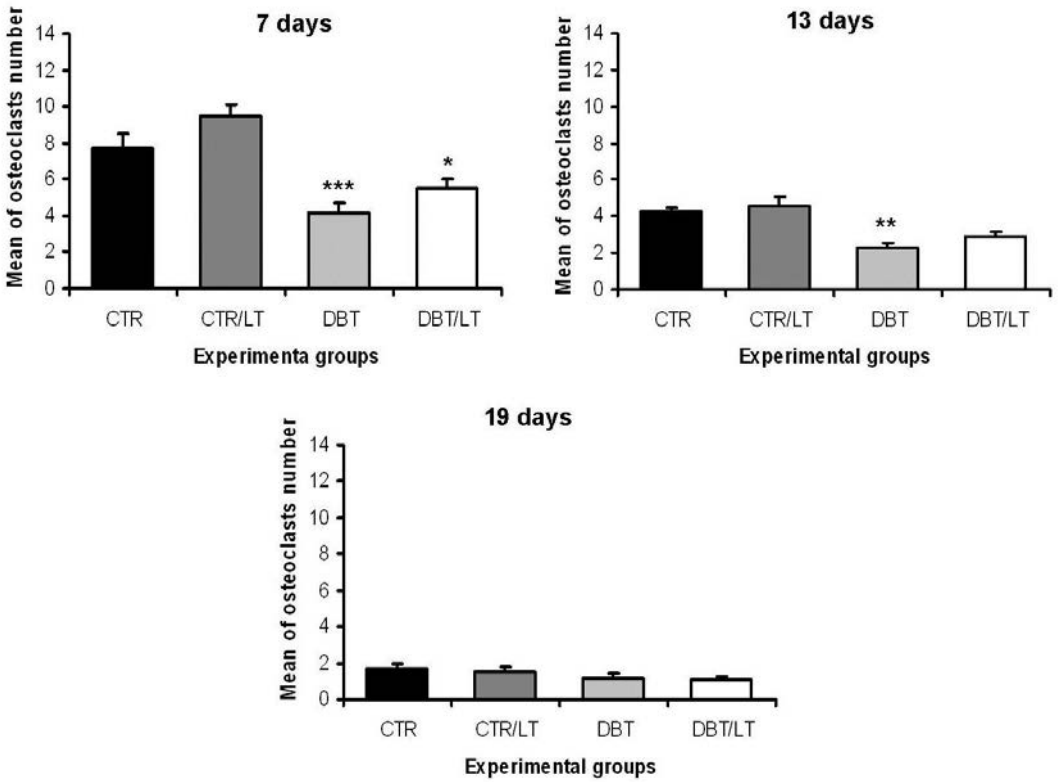

Fig. 5 – Assessment of the mean of osteoclasts number (OsTC) in the studied groups over the time course of the experiment.

* Significantly different from CTR ($p<0.05$); ** Significantly different from CTR ($p<0.01$); *** Significantly different from CTR ($p<0.001$)

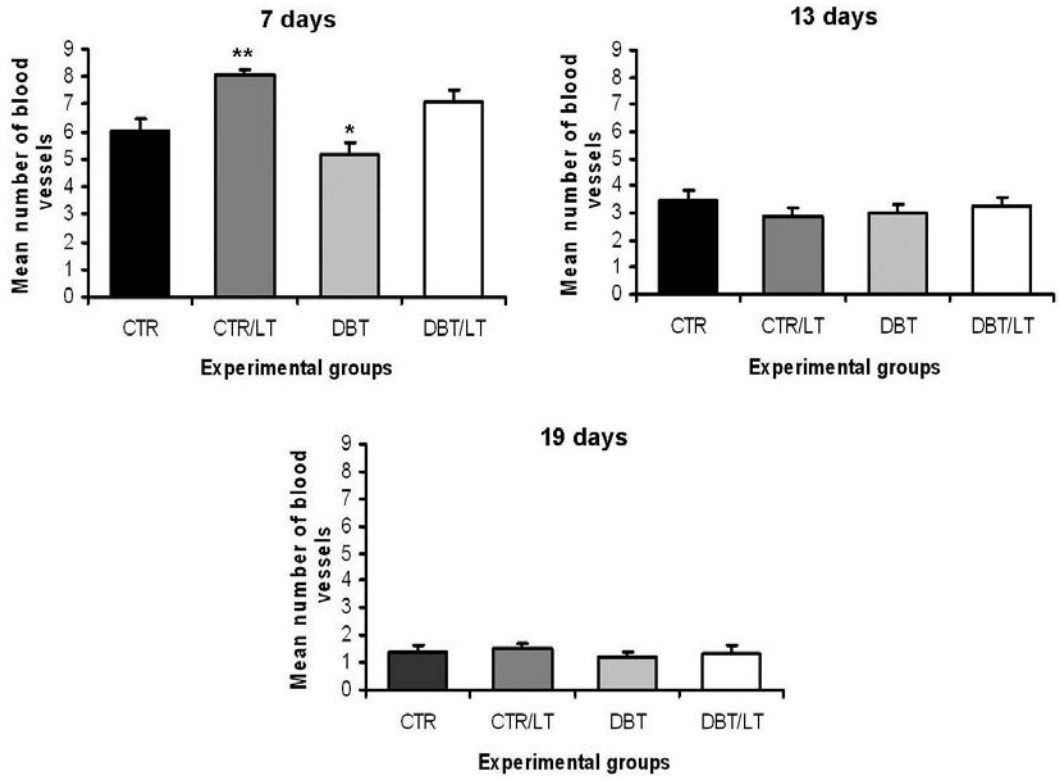

Fig. 6 – Assessment of the mean number of blood vessels (BVC) in the studied groups over the time course of the experiment.

* Significantly different from CTR ($p<0.05$); ** Significantly different from CTR ($p<0.01$)

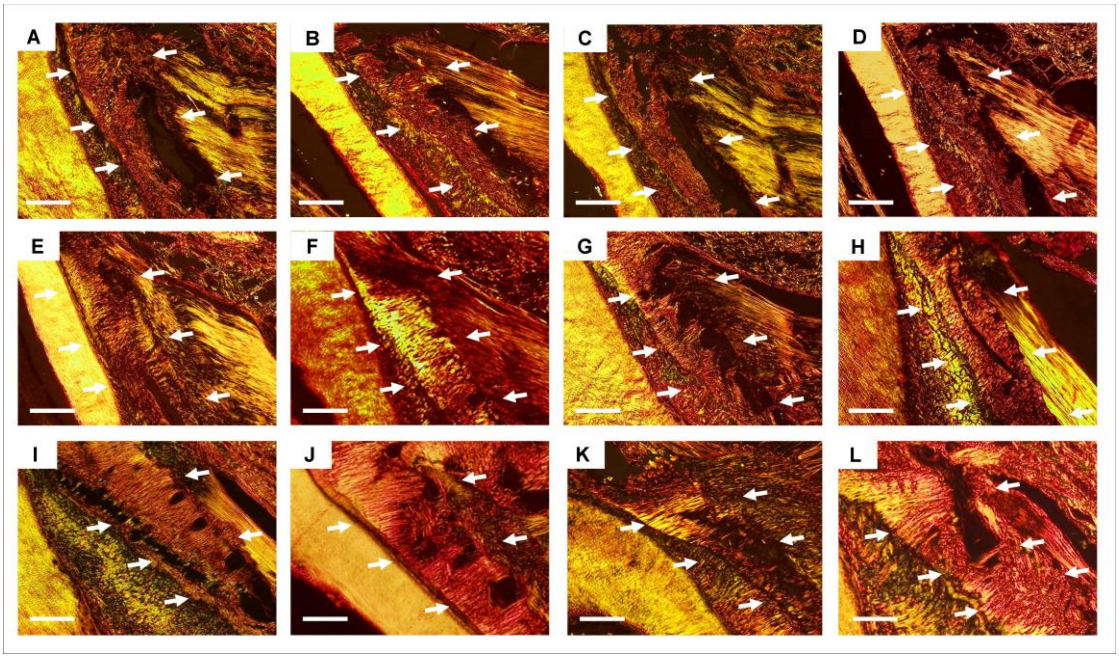

Fig. 7 – Collagen fibers of the periodontal ligament seen under polarized light (arrows) in seven (A-D), 13 (E-H) and 19 days (I-J). Control group (CTR) is expressed in the first column (A, E, I), non-diabetic irradiated group (CTR/LT) in the second (B, F, J), diabetic group (DBT) in the third (C, G, K) and irradiated diabetic group (DBT/LT) in the fourth ones (D, H, L) (Sirius Red, 200x magnification; White Bar – 250 μm).

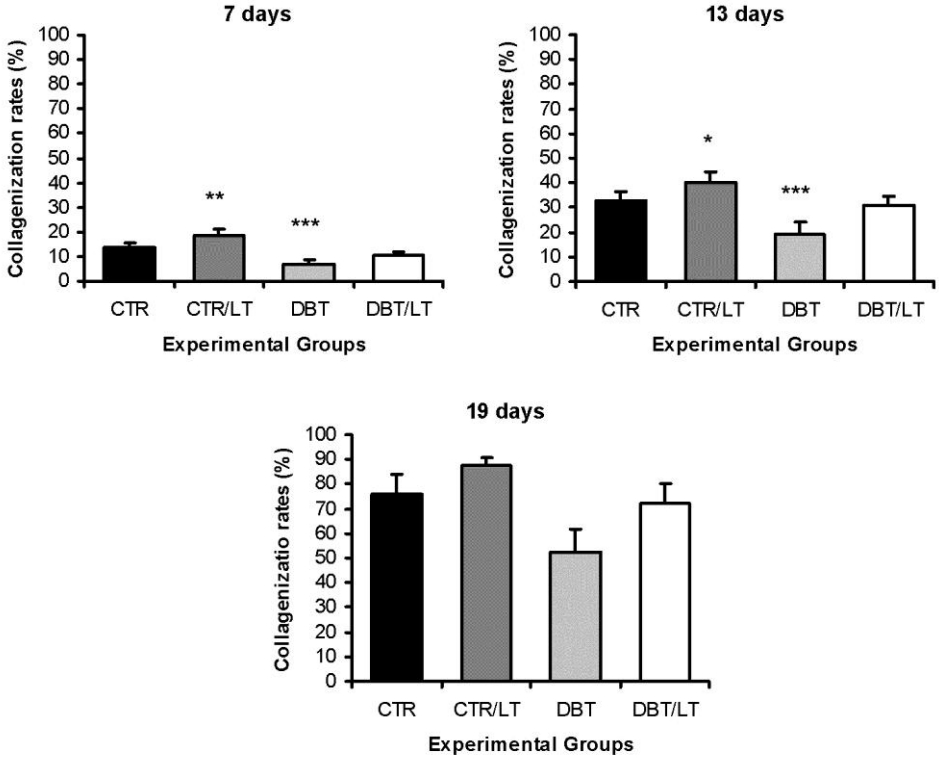

Fig. 8 – Assessment of the mean of collagenization rates (CR) in the studied groups over the time course of the experiment.

* Significantly different from CTR ($p<0.05$); ** Significantly different from CTR ($p<0.01$); *** Significantly different from CTR ($p<0.001$)

Effect of low level laser therapy on the dental pulp histological changes in diabetic rats submitted to orthodontic forces*

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Abstract

It has been suggested that the dental movement provides inflammatory and vascular changes in pulp tissues. Diabetes Mellitus is an endocrine disease resulting from deficiency in insulin production, which is associated to impairment of the healing process. Low level laser therapy (LLLT) has been successfully used to improve dental movement due to its anti-inflammatory, angiogenic and proliferative properties. Therefore, the purpose of this study was to assess the effects of LLLT on the dental pulp during tooth movement in rodents. Thus, 40 rats submitted to dental movement of the 1st molar were assigned into 4 groups (n=5): CTR and DBT – non-irradiated normoglycemic and diabetic animals; CTR/LT and DBT/LT – laser-irradiated (780 nm ; 35 J/cm$^{2)}$ normoglycemic and diabetic animals. The animals were sacrificed in 7 and 13 days, and the first molars pulp tissues were histologically analyzed. DBT group presented significantly enhanced inflammatory response ($p<0.001$ – 7 days) and collagenization rates ($p<0.001$ – 13 days) and reduced content of blood vessels ($p<0.001$ – 7 and 13 days) and stromal cells ($p<0.01$ and $p<0.001$ – 7 and 13 days), as well as thinner odontoblastic layer ($p<0.001$ –13 days), compared to CTR. Most of the histological parameters in DBT/LT were comparable to CTR ($p>0.05$), except for the increased collagenization rates ($p<0.05$ and $p<0.001$ – 7 and 13 days). In conclusion, LLLT was effective in reversing the inflammatory, vascular and cellularity changes, but not the desmoplastic reaction, of the pulp connective tissue of diabetic rats in response to dental movement.

Key words: tooth movement; low-level laser therapy; aloxan diabetes; dental pulp

Introduction

The dental and periodontal components of the stomatognatic system are hard and soft tissues which work together, and present metabolic rates physiologic mechanisms adapted to their functional needs. It has been reported that the homeostasis of such tissues may be influenced by local and/or systemic factors, such as dental movement, whose impact on the morphological or functional depends on intensity, duration of the deleterious stimuli[1,2,3,4,5]. However, other studies have suggested that orthodontic forces applied within a resistance limit do not promote appreciable changes in the dentin-pulp complex[6,7,8].

Diabetes Mellitus (DM) is a common endocrine disorder characterized by persistently raised blood glucose levels (hyperglycemia), as a result of deficiency in insulin secretion, insulin action, or both[9]. Chronic hyperglycemia is associated with long-term damage and dysfunction of various organs and tissues[10], and it has been demonstrated that moderate to severe damage in the glucose metabolism is able to directly affect the response of the bone and connective tissues to injury[11,12] Although there are some few studies providing evidence that chronic hyperglycemic status might impair the vascular and inflammatory response and bone remodeling during orthodontic treatment[13,14], there is no report of the impacts on the dentin-pulp complex in diabetic experimental animals.

Low level laser is a highly concentrated, non-invasive and non-ionising radiation that promotes thermal, photochemical and nonlinear effects when emitted in different hard and soft tissues[15]. Several studies have demonstrated that low level laser therapy (LLLT) modulates different biological activities, such as anti-inflammatory[16,17] and pro-angiogenic action[18,19], as well as stimulatory properties on the collagen synthesis[20,21]. LLLT has shown to accelerate tooth movement, as well as alveolar bone remodeling in experimental studies[22,23] and clinical trials[24,25]. However, little is known about the effect of laser irradiation on the dynamics of dental movement in diabetic subjects, particularly on the morphological and functional features of the dental pulp tissues.

Therefore, the present study was designed to assess the effects of LLLT on the histological changes of the pulp tissues during dental movement in diabetic rats.

Material and methods

Ethical Perspectives. 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 nº 341208). The study was carried out at the biotherium and the Laboratory of Morphology and Structural Biology of Tiradentes University (Aracaju/SE, Brazil).

Biological Assay. Forty adult non-pregnant male rats (Novergiculs 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 daily replaced wood shavings bedding, under controlled temperature at 22˚C, and 12 h light/darkness scale, with water and food (diet Labina®, Purina, Sao Paulo, Brazil).

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 hypoglycemia. Blood samples were collected from the tail vein of the animals after 72 h in order to assess the plasma glucose levels by the glucose-oxidase enzymatic method, using Accu-Chek Advantage (Boehringer, Germany). Animals presenting glucose levels above 200 mg/ dL were included in the diabetic group. The examinations were repeated every 7 days to confirm maintenance of the glucose levels. The animals presenting 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.

Experimental Tooth Movement. At the end of 2 months, anesthesia was induced by the same method, 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 inch, Orthometric. Marilia, SP, Brazil) was ligated with the maxillary 1st molar cleat by a 0.010-inch 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, by using the same ligature wire (Fig. 1). The orthodontic force exerted by the appliance was 50 g in the beginning of the experiment. Tooth movement was performed for 13 days (day 0 ~ 13).

Low Level Laser Therapy Procedures. The **a**nimals were submitted 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 (25 S each point). The output power used was 70 mW, with a focal spot of 0.04 cm^2, and power density of 1W/cm^2. The total energy per session was stimated in 4,2 J and the energy density was 35 J/cm^2 distributed in 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 seven days.

Procedures for the histomorphological analysis of the specimens. After seven and 13 days, animals were euthanized in a CO_2 chamber for *post-mortem* removal of the maxillas. 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, 10 histological sections (5µm thick) were obtained and stained in hematoxylin-eosin and Sirius red (five interspaced histological sections each) for analysis using a light microscope (Olympus CX31 optic microscope) by three trained observers.

Histological analysis of the inflammatory response (IR). The intensity of the inflammatory response was assessed in five coronary and five radicular histological fields of the 1st molar pulp tissue (x 40, 10 ocular, 0.739 mm2 per field), 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 profile inflammatory (IP) was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as slighter/absent, moderate or severe.

Quantitative analysis of the blood vessels count (BvC), stromal cell rates (SCR), vascular perimeter (VsP) and odontoblastic layer thickness (OLT). These parameters were all measured using an image analysis system software (Imagelab®). Therefore, five coronary and five radicular histological fileds of the 1st molar were randomly chosen and photomicrographs (x 40, 10 ocular, 0.739 mm2 per field) were obtained from each histological section. All the images were sent to the PC using an analog video camera (PAL system), after being converted to the RGB (red-green-blue) system necessary for digitizing and processing the sections. In order to assess the BvC and SCR, a network of one hundred

squares was superimposed on the images, and all the cells whose nuclei touched the horizontal and vertical bars. The OLT was measured in three different points at each image. The images were recorded and automatically processed to find the cell density (CD) in each reference area (RA). Data were expressed as mean ± SD.

Assessement of the percentage of collagenization rates (CLR). Histological sections stained in sirius red and analyzed under polarized light were used to the descriptive analysis of the collagenization, according to the birefringence pattern (greenish/yellow-greenish or orange, orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long) and disposition (reticularly disposed, parallel arranged or interlaced) of the collagen fibrils and fibers. The quantitative analysis of the percent area occupied by collagen in the pulp was determined by optical density in the image analysis system in different randomly selected fields. The system used consists of a CCD Sony DXC-101 camera, applied 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 inserted into a computer (Pentium 133 MHz), and processed by a software (ImageTool). A total of five fields per case were analyzed at 400x magnification. 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 birrefringent (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 in percentage of the area fraction occupied by the collagen fibers (mean ± SD).

Statistical Analysis. Data obtained in the IP and HC analysis were analyzed using the Kruskal-Wallis test, followed by post-hoc Dunn´s test. Data obtained in the BvC, VsP, SCR, OLT and CLR counts were analyzed by Anova followed by post-hoc Tukey's test. Differences among the groups were regarded as significant when $p<0.05$.

Results

In seven days (**Fig**. 1), both coronary and radicular pulp tissues presented variable infiltration of lymphocytes and occasional plasma cells, mild interstitial edema and pronounced vasodilatation and congestion (hyperemia). The odontoblastic layers was continuous and presented foci of thickening, In the periapical area, mild chronic inflammatory

infiltrate was seen in CTR and CTR/LT, moderate to intense subacute inflammatory response (due to the presence of neutrophils) was observed in DBT, and intense lympho-hystiocytic inflammation was verified in DBT/LT.

In 13 days (**Fig**. 2), edema and inflammation were mild or absent, and hyperemia was quite less conspicuous, whereas fibrotic areas were evident in all the groups, except for CTR. No inflammatory response was evidenced in the periapical areas, but CTR/LT showed moderate desmoplastic reaction. Foci of dystrophic calcification was seen in the pulp tissues, irrespective of the glycemic status or the laser therapy. The inflammatory response was markedly reduced in the apical region in all the groups, and in one two sample of DBT the apical foramen was hypercalcified.

The analysis of the histological slides stained in sirius red, and observed under polarized light, revealed that the pulp collagen fibers presented a golden birefringence pattern, consistent with type I collagen. Such fibers (or fibrils) were thin, delicate and predominantly parallel-arranged. In 13 days, the fibers were more densely disposed, with less irregular interfibrillar spaces, compared with seven days. The collagen fibers and fibrils were shown to be more concentrated in the central areas, and less conspicuous close to the odontoblastic layer. Moreover, in 13 days, CTR appeared to be less collagenized than the other groups (**Fig.** 3).

As demonstrated in **Fig**. 4, the application of LLLT in non-diabetic animals during dental movement was shown to provide no influence on the dynamics of the inflammatory response, thickness of the odontoblastic layer and stromal cell rates ($p>0.05$) in comparison to non-irradiated animals, in none of the experimental times. However, in seven days, there was a significant increase in the blood vessels count ($p<0.01$) and collagenization rates ($p<0.01$), whereas the perimeter of the vessels lumens was significantly reduced ($p<0.01$). In 13 days, all the histological parameters presented any appreciable alterations and were comparable to the control group ($p>0.05$), except for the collagenization rates, which were significantly higher ($p<0.001$).

The hyperglycemic status promoted significant decrease of the pulp blood vessels count ($p<0.001$) and cell rates ($p<0.01$), whereas the severity of the inflammatory response was increased ($p<0.01$), in seven days of dental movement. In 13 days, the blood vessels count ($p<0.001$), the thickness of the odontoblastic layer ($p<0.001$) and stromal cell rates ($p<0.001$) were significantly decreased, whereas the collagenization rates were increased ($P<0.001$). On the other hand, the diabetic animals treated with LLLT presented only significantly thinner odontoblastic layer ($p<0.001$) in seven days, and increased rates of

collagenization in both seven ($p<0.05$) and 13 days ($p<0.001$), whereas the other changes were utterly reversed.

Discussion

In this study, the response of the dental pulp to mechanic aggression (orthodontic forces) was represented by mild to moderate inflammatory and vascular events (vasodilatation and congestion), which tended to be more expressive in seven than in 13 days. Similar findings were previously observed in other studies[1,2,3,4,26]. And seem to secondary to mast cells degranulation, induced by the noxious external stimulus, and consequent release of several mediators of inflammation (e.g. histamine, bradykinin, neurokine, neuropeptides, prostaglandins and growth factors). Therefore, these mediators are supposed to induce vasodilatation, increase in blood flow and vascular permeability, and edema, as well as inflammation and cell damage[2]. Moreover, as long as the orthodontic force is dissipated, the magnitude of the histological changes is minimized[3,4], which justifies the more expressiveness of the alterations seen in this study concentrated in seven days.

The application of LLLT did not provide appreciable influence in the dynamics of the inflammatory response and vascular changes in the pulp tissues of non-diabetic animals, but promoted fibrosis after 13 days. The anti-inflammatory and pro-angiogenic properties of LLLT are well establish in the literature[13,16,17,18], but the magnitude of such biological effects seems to be related to the severity of the deleterious stimuli and tissue damage. Therefore, our findings are suggestive that the orthodontic forces applied in this study were likely within a resistance range, so that the inflammatory and vascular events were not overinduced. The fibrosis, however, is likely a result of a laser-induced increase of the fibroblast metabolism, and consequent enhanced collagen synthesis[19,27,28]. Since the cell content was not affected, it is possible to hypothesize that such fibrosis is not as noxious to the pulp as the senescent one, which is secondary to the decrease in the fibroblast number and collagen remodeling function, thus resulting in an important reduction in the pulp responsive capability.

The hyperglycemic status enhanced significantly the course and magnitude of the inflammatory response, and reduced the proliferative phenomena occurring in the pulp tissues in response to the dental movement. The precise mechanisms underlying the enhanced inflammatory response in diabetic animals have not been clarified yet. A possible association with higher levels of TNF, which is increased in both humans and animal models of type II

diabetes, has been suggested. Thus, it has been proposed that the high levels of TNF disrupts the cytokine networks, leading to a potentiated inflammatory responses. In addition, advanced glycation end products present at higher levels in diabetic individuals seems to enhance oxidative stress and amplify inflammatory events in the tissues[29]. The impairment of the angiogenesis in diabetic animals has been previously reported[30], and it has been suggested a possible relation with a decrease in the amount of growth factors essential for wound healing, including FGF-2 and PDGF[31]. Surprisingly, the diabetic animals showed reduction in the thickness of the odontoblastic layer, contrary to the assertions of Sasaki et al. (1990)[32], who reported that the odontoblasts are not supposed to be affected by the hyperglycemic status. We hypothesize that such impact on odontoblasts are more likely related to a possible deficiency in the metabolic and proliferative potential of those cells, resulting from the impaired influx of glucose into the cytosolic environment. Accordingly, the desmoplastic appearance of the pulp tissue in diabetic animals might have resulted of impaired proliferation of fibroblasts, which would subsequently lead to reduced number of cells and consequent impairment in the collagen remodeling. That mechanism would ultimately promote histological fibrosis similar to that observed in senescent pulp[33]. However, further investigations are necessary in order to ascertain the validity of these theories.

The diabetic animals treated with LLLT presented inflammatory and vascular response comparable to the control group. Such results are probably associated to the inhibitory properties of LLL on the prostaglandin and interleukin-1 synthesis[34] and lymphoid cell proliferation[21]. These effects could ultimately reduce the magnitude of the inflammation to acceptable levels, as observed in CTR. The reversion of the impairment in the angiogenesis and cell rates might be associated to the photostimulatory properties of LLLT. In fact, it has been reported that LLLT I able to improve the angioblastic differentiation and proliferation in diabetic mice, accelerating the neoformation of capillary blood vessels[35]. In addition, *in vitro* studies demonstrated that diabetic wounded fibroblasts proliferate in response to LLLT[36], which could justify the reversion of the pulp cell rates. It should be noted that LLLT did not reverse the fibrotic changes of the dental pulp. Other studies have previously reported that LLLT is able to induce fibrosis of dental pulp as a result of increased metabolism of the fibroblast[37]. As the cell rates remained significantly lower than in CTR and CTR/LT, it is possible to suggest that the fibrosis seen in DBT/LT is as noxious as in DBT, once the responsive potential of the dental pulp is impaired. Nevertheless, further research is still required to clarify the precise biochemical and molecular mechanisms underlying the effects of LLLT on the dental pulp of diabetic animals submitted to experimental dental movement.

In conclusion, we demonstrated that diabetic animals submitted to dental movement present more expressive histological changes in the pulp tissues than non-diabetic ones. In addition, low level laser therapy was shown to be able to reverse the inflammatory, vascular and cellularity changes, but was ineffective in reversing the desmoplastic reaction of the pulp connective tissue. Notwithstanding the relevance of these experimental findings, it must be noted that the hyperglycemic status may influence a wide range of biochemical and metabolic pathways, so that, even with promising results, the best way to ensure the success of the orthodontic treatment is to assure that the patient´s glycemia is adequately compensated.

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Tables and figures Legends

Groups	Pre-treatment	Low Level Laser Therapy (Total Dose)
CTR	Citrat buffer	0 j/cm^2
DBT	Alloxan (150 mg/kg)	20 j/cm^2
CTR/LT	Citrat Buffer	0 j/cm^2
DBT/LT	Alloxan (150 mg/kg)	20 j/cm^2

Table 1. Distribution of the animals in the experimental groups according to the applied pre-treatment and low level laser therapy.

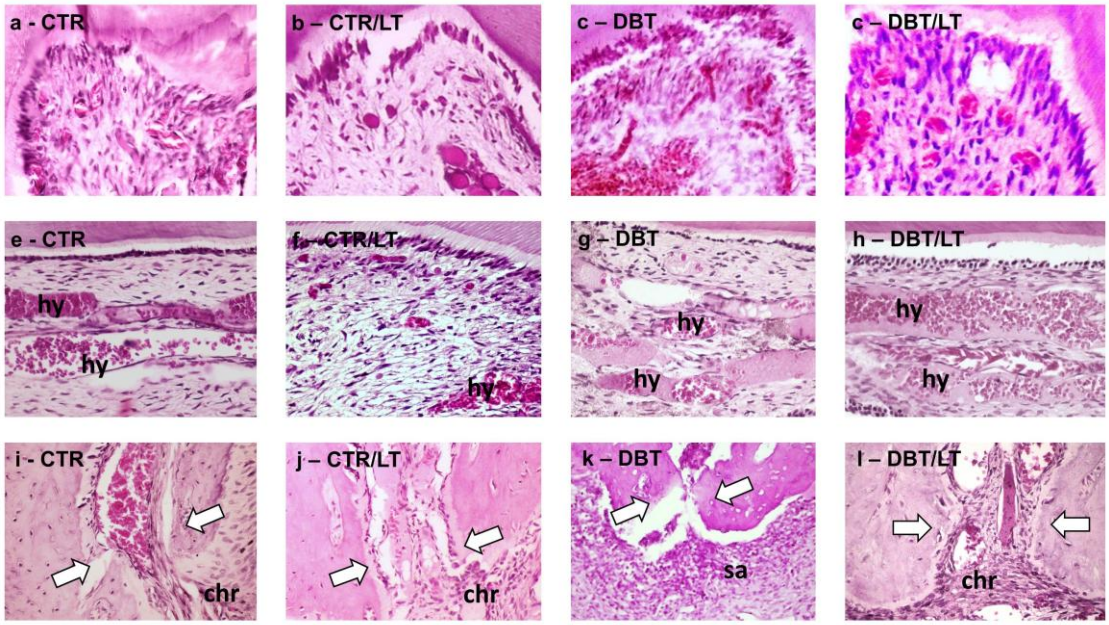

Fig. 1 – Photomicrographs of the pulp tissues of the experimental groups in seven days of dental movement. (a – d) Coronary pulp and (e – f) radicular pulp; observe the intense hyperemia (hy), particularly in the radicular pulp. (I – l) Apical region (white arrows) showing mild chronic inflammatory (chr) in CTR and CTR/LT, intense subacute infiltrate (sa) in DBT and intense chronic infiltrate in DBT/LT (HE, 400 x).

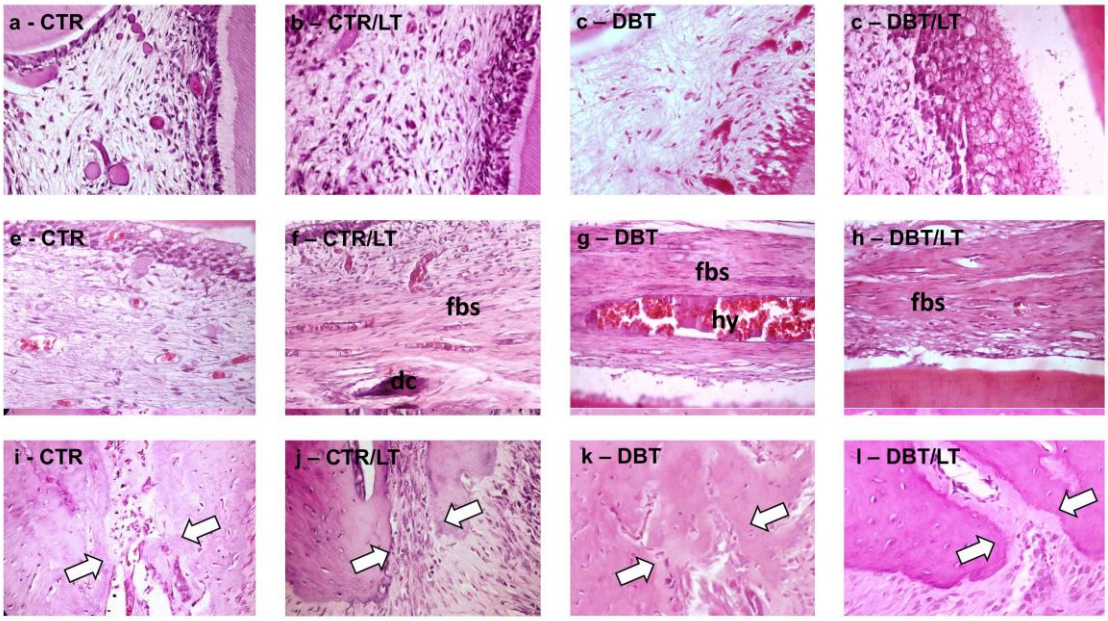

Fig. 2 – Photomicrographs of the pulp tissues of the experimental groups in 13 days of dental movement. (a – d) Coronary pulp and (e – f) radicular pulp; observe the remarkable fibrosis (fbs) in CTR/LT, DBT and DBT/LT, as well as foci of hyperemia (hy), particularly in the radicular pulp. (I – l) Apical region (white arrows) showing mild chronic inflammatory in all the groups, and hypercalcification of the apical foramen in DBT (HE, 400 x).

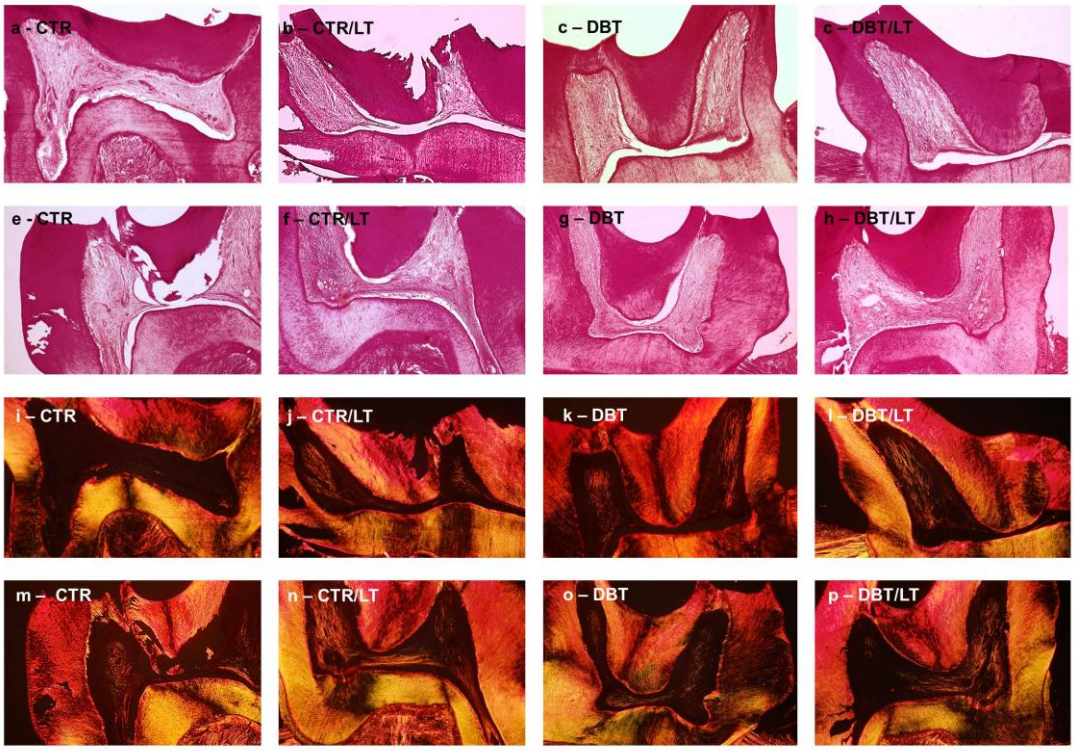

Fig. 3 – Photomicrographs of the pulp tissues of the experimental groups during dental movement. (a – d) Coronary pulp in seven and (e – h) 13 days under conventional light. (I – l) The very same slides observed under polarized light in seven and (m – p) 13 days (Sirius Red, 100x).

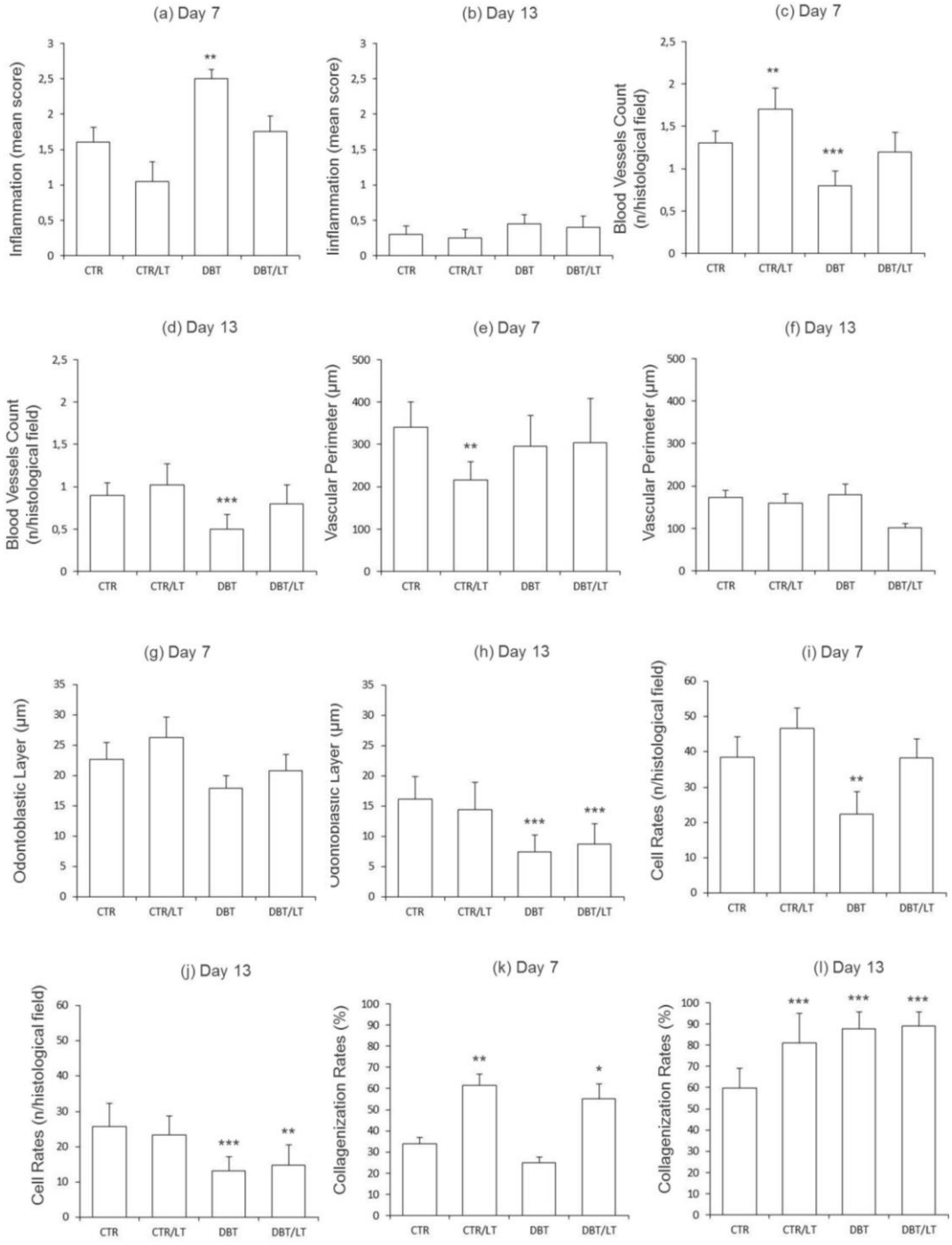

Fig. 4 – Quantitative analysis of the histological parameters assessed in this study. Significant differences (Anova and Tukey post test) are represented by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (in relation to CTR).

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ANEXO 1

Parecer Consubstanciado de Projeto de Pesquisa

Titulo do Projeto: AVALIAÇÃO DAS ALTERAÇÕES TECIDUAIS DECORRENTES DA FOTOBIOMODULAÇÃO À LASER NA MOVIMENTAÇÃO DENTÁRIA EM RATOS DIABÉTICOS

Pesquisador Responsável Luiz Guilherme Martins Maia

Data da Versão 17/12/2008 | Cadastro 341208 | Data do Parecer 10/02/09

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto

Avaliar o efeito biomodulatório da laserterapia de baixa potencia sobre as alterações morfológicas do ligamento periodontal, osso alveolar, cemento radicular e complexo dentina-polpa durante a movimentação ortodôntica em ratos diabéticos

Sumário do Projeto

Quando forças são aplicadas sobre as unidades dentarias, ocorrem reações histologicas no periodonto de inserção, formando-se zona de reabsorção na superfície óssea do lado de pressão e, ao mesmo tempo, neoformação óssea no lado de tração. Em pacientes diabéticos, as alterações do tecido conjuntivo e vascular prejudicam a cicatrização dos tecidos normais. O diabetes tipo I esta associado com a diminuição da integridade dos tecidos ósseos e, por isso, mais propenso a fratura. A terapia com laser de baixa potencia é capaz de acelerar os processos de reparação dos tecidos biológicos traumatizados. Existe alteração na camada de odontoblastos, decorrente de um processo inflamatório e alteração vascular com acumulo de eritrócitos e leucócitos no interior dos vasos, no ligamento periodontal e polpa de dentes que foram movimentados. Sendo assim, teremos como proposta avaliar as alterações histológicas decorrentes da utilização de fotobiomodulação à laser, durante o movimento dentário induzido, em ratos adultos jovens, pesando entre 250 e 300 gramas, machos, hiperglicêmicos, após a aplicação de força de 40g/F, a fim de movimentar os primeiros molares superiores, mesialmente. Utilizaremos 60 animais distribuídos em seis grupos iguais, correspondendo ao tempo de sete, 13 e 19 dias para realização do experimento. Metade dos animais de cada grupo serão submetidos à fototerapia laser durante a movimentação ortodôntica e, posteriormente, mortos para analise histológica semi-quantitativa. A outra metade, não irradiada, servirá como controle.

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Não
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Adequados

Introdução	Adequada

Comentários sobre a Introdução

Adequados

Objetivos	Adequados

Comentários sobre os Objetivos

Adequados e correlacionados com a proposta metodológica.

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 60 Local 60
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção equitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Não se aplica
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Adequados

Cronograma	Adequado
Data de início prevista	01
Data de término prevista	12
Orçamento	Adequado
Fonte de financiamento externa	Não

Comentários sobre o Cronograma e o Orçamento

Cronograma sugere que a pesquisa terá duração de128 meses após aprovação pelo CEP.

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Adequadas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Projeto de alta relevância para comunidade científica e população de maneira geral, e atende aos pressupostos da resolução 196/96 da CONEP sendo considerado aprovado no que se refere a seus aspectos éticos.

ANEXO 2

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: AVALIAÇÃO DAS ALTERAÇÕES TECIDUAIS DECORRENTES DA MOVIMENTAÇÃO ORTODÔNTICA EM RATAS PORTADORAS DE OSTEOPENIA

Pesquisador Responsável Luiz Guilherme Martins Maia

Data da Versão 26/05/2009 | Cadastro 360509 | Data do Parecer 20/06/2009

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto

GERAL

Avaliar a resposta óssea e das estruturas que circundam a estrutura dental durante a movimentação ortodôntica em modelo experimental com ratos osteopênicos.

ESPECIFICOS

Analisar histologicamente as alterações morfológicas da lâmina dura, osso alveolar e ligamento periodontal ocorridas nos lados de pressão e de tração ortodôntica em modelo experimental com roedores.

Avaliar a influência da osteoporose sobre a dinâmica histomorfológica da movimentação dentária ortodôntica.

Estimar a resposta tecidual do periodonto de ratos portadores de osteopenia à movimentação ortodôntica.

Comparar o padrão de resposta histomorfológica ao tracionamento ortodôntico em animais com osteoporose

Comparar o padrão de resposta histomorfológica sobre a movimentação ortodôntica entre ratos portadores de osteopenia induzidos por ovariectomia e por administração de corticóide no tracionamento ortodôntico

Sumário do Projeto

Quando forças são aplicadas sobre as unidades dentárias, ocorrem reações histológicas no periodonto de inserção, formando-se zona de reabsorção na superfície óssea do lado de pressão e, ao mesmo tempo, neoformação óssea no lado de tração. Em pacientes com osteoporose, as alterações do tecido conjuntivo e vascular prejudicam a cicatrização dos tecidos normais. A osteoporose é uma patologia óssea sistêmica caracterizada por um desequilíbrio entre reabsorção e formação óssea, resultando em um aumento da fragilidade óssea. Estudos têm demonstrado que a osteoporose representa fator complicador da dinâmica de turnover ósseo e, portanto, poderia modificar a resposta do osso periodontal à movimentação ortodôntica. Desta forma, este trabalho objetiva analisar histologicamente o comportamento do periodonto e estruturas adjacentes após a movimentação ortodôntica em animais de experimentação osteopênicos. Para tanto, será induzida a osteopenia experimental em roedores por meio de dois modelos: ovariectomia e administração de glicocorticóide (dexametasona). Serão utilizados 60 ratos Wistar (300±50g) distribuídos em quatro grupos (n=15): OVR (ratas ovariectomizadas), GLC (ratas submetidas à corticoterapia), CTR1 (controle submetido à pseudo-ovariectomia) e CTR2 (controle submetido à administração de solução salina). Posteriormente à constatação de quadro osteopênico dos animais, a movimentação ortodôntica será efetuada por mesialização do primeiro molar maxilar com dispositivos de molas helicoidais ancoradas em incisivos, com a aplicação de força de 40g/F. Após os períodos de 07, 13 e 19 dias, cinco animais de cada grupo serão eutanasiados, as hemimaxilas removidas e descalcificadas e

emblocadas em parafina. Serão obtidas seções histológicas, coradas em HE e picrossírius. As variáveis avaliadas serão reação inflamatória, densidade vascular, edema, hemorragia, hialinização, deposição colagênica, análise morfométrica do tecido ósseo neoformado e reabsorvido. A análise estatística será efetuada por meio dos testes Kruskall Wallis (com extensão Dunn), qui-quadrado e Anova (com extensão Tukey), considerando significativo p≤0,05. Espera-se, por meio desta pesquisa, contribuir para o melhor conhecimento do comportamento ósseo e da resposta periodontal a procedimentos de movimentação ortodôntica em animais osteopênicos, obtendo assim informações que possam subsidiar a indicação e prática clínica ortodôntica em indivíduos portadores desta enfermidade.

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Não
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Adequados

Introdução	Adequada

Comentários sobre a Introdução

Adequados

Objetivos	Adequados

Comentários sobre os Objetivos

Adequados e correlacionados com a proposta metodológica.

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 60 Local 60
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção equitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Não se aplica
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Adequados

Cronograma	Adequado
Data de início prevista	01
Data de término prevista	12
Orçamento	Adequado
Fonte de financiamento externa	Não

Comentários sobre o Cronograma e o Orçamento

As atividades terão início no primeiro mês após a aprovação do projeto pelo comitê de ética.

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Adequadas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Projeto bem delineado e de relevância científica, atende aos preceitos éticos envolvidos no uso experimental de naimais..

Araraquara, 19 de março de 2013.

LUIZ GUILHERME MARTINS MAIA