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Effects of glucocorticoid-induced osteoporosis on bone tissue of rats with experimental periodontitis

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ABSTRACT

Objective: To evaluate the effects of osteoporosis induced by glucocorticoid (GIOP) on bone tissue of rats with experimental periodontitis (EP).
Design: 48 male Wistar rats divided into groups: Naïve, EP, GIOP and GIOP + EP. Rats of GIOP and GIOP + EP groups received 7 mg/kg of dexamethasone intramuscularly once a week for 5 weeks. Following, EP and GIOP + EP groups were subjected to ligature-induced periodontitis. Naïve group experienced no manipulation. After 11 days, the animals were euthanized and left maxillae collected for macroscopic, radiographic, micro-tomographic and microscopic analysis of alveolar bone loss (ABL). Blood samples were collected for determination of bone-specific alkaline phosphatase (BALP) levels and the right femurs were removed for radiographic and biomechanical analysis.
Results: EP caused ABL and reduced BALP levels ($p < 0{,}05$), but it did not change the architecture or biomechanics of femur, compared to Naïve. GIOP did not cause ABL, but it significantly decreased alveolar bone mineral density (ABMD), bone percentage and trabecular thickness (Tb.Th) and increased alveolar bone porosity ($p < 0.05$) and significantly reduced BALP serum levels, as well as radiographic density and Young's module of femur, compared to Naïve. There was a greater ABL in group GIOP + EP when compared to EP ($p < 0.05$). GIOP + EP caused a greater decrease on ABMD, Tb.Th, bone percentage and increased bone porosity ($p < 0.05$) and also presented a significant reduction in BALP levels ($p < 0.05$), in radiographic density and in Young's module of femur compared to EP ($p < 0.05$).
Conclusions: GIOP can potentiate the destructive effects of EP on alveolar bone and alter the systemic bone loss, by promoting bone resorption and reducing osteoblast activity.
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1. Introduction

Periodontitis is an infectious-inflammatory and highly prevalent disease, characterized by destruction of connective tissue and alveolar bone loss (ABL), and it is considered the second major cause of tooth loss (Tatakis and Kumar, 2005). This disease is mainly initiated by oral biofilm, however the development of an

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altered host response plays an important role on tissue breakdown (Pihlstrom, Michalowicz, & Johnson, 2005).

Osteoporosis is a common disease characterized by systemic bone loss and impaired bone microarchitecture. It can be a consequence of hormonal imbalance in postmenopausal woman (Jilka, Hangoc, Girasole, Passeri, & Williams, 1992), but it also can present a secondary cause, mainly as a result of the use of some drugs, such as Glucocorticoids (GCs). Glucocorticoid-induced osteoporosis (GIOP) is the most common cause of secondary osteoporosis, the first cause before 50 years of age and the first iatrogenic cause of the disease (Kok & Sambrook, 2009). In addition, considering the continuous raise on the prevalence of GCs use in the community population (Overman, Yeh, & Deal, 2013), it seems interesting to understand the biological mechanisms underlying GIOP.

In the recent decades, numerous studies have focused on the association between osteoporosis and periodontitis at the bone level. The majority of the studies has focused on the effects of postmenopausal osteoporosis on the loss of periodontal attachment (Hernández-Vigueras et al., 2015; Juluri et al., 2015). However, little is known about the effects of GIOP on periodontal tissues. One study showed that GCs can induce an alveolar bone loss in long-term (28 days) treated mice (Bouvard, Gallois, Legrand, Audran, & Chappard, 2013) and another one reported a reduction of bone density in the region of interalveolar septa, insignificant bone tissue loss of the horizontal type and pathological teeth mobility in periodontal tissues of patients with systemic osteoporosis (Dmitrieva, Atrushkevich, & Pikhlak, 2006). There is no study evaluating the effect of GIOP when the periodontal inflammation is present. In this context, we aimed to evaluate the effects of GIOP on bone tissue of rats with experimental periodontitis.

2. Material and methods

2.1. Animals and study design

The experiments were performed on forty-eight young adult male (from two litters) Wistar rats (*Rattus norvegicus*) from central Animal Facility of Federal University of Ceará, weighing 180–220 g, kept in appropriate cages with six animals each. The animals were housed in standard conditions (12 h light-dark cycles and temperature-controlled rooms) with food and water *ad libitum*. The protocols for experimental procedures and animal treatment were approved by Animal Ethics Committee (number 78/2014) of Federal University of Ceará, Brazil.

A power calculation was performed to determine the sample size. The animal was considered the study unit. The sample size was determined to provide 80% power to recognize a significant difference of 20% among groups and the standard deviation of 15% with a 95% confidence interval ($p = 0.05$), considering the change in alveolar bone loss (ABL) as the primary outcome variable. Therefore, a sample size of 6 rats per group was required.

After two weeks of acclimation to the laboratory environment, the rats were divided, in a blind manner, into four groups ($n = 6$): Naïve, Experimental Periodontitis (EP), Glucocorticoid-induced osteoporosis (GIOP) and GIOP + EP. Initially, rats from GIOP and GIOP + EP groups received injections of 7 mg/kg of dexamethasone (Decadron, Aché® – Guarulhos, SP, Brazil) intramuscularly, once a week for 5 weeks (Lucinda et al., 2012), and the ones from EP group received 0.5 ml of 0.9% saline solution. Following, the rats were anesthetized with 100 mg/ml ketamine (Cetamin – Syntec® – Santana de Paraíba, SP, Brazil) and 20 mg/ml xylazine (Xilazin – Syntec® – Santana de Paraíba, SP, Brazil) on the dose of 1 ml/kg intramuscularly. In order to induce periodontitis rats of EP and GIOP + EP groups received a sterile nylon thread ligature (3-0; polysuture NP45330 – São Paulo, SP, Brazil) around the cervical area of their maxillary left second molars (Bezerra et al., 2000; Samejima, Ebisu, & Okada, 1990). After 11 days, all rats were euthanized with 20 mg/kg thiopental (0.5 g Thiopentax; Cristália, São Paulo, SP, Brazil). Rats of Naïve and GIOP groups did not receive the ligature. Rats from Naïve group experienced no manipulation. It was performed 2 studies with parallel experimental groups, one for macroscopic and biochemical analyses and the other one for microscopic evaluation.

2.2. Macroscopic, radiographic and micro-computed tomographic (CT) analyses of alveolar bone

For macroscopic analysis, the excised maxillae were fixed in 10% neutral formalin for 24 h. Hemi-maxillae were then defleshed and stained with 1% aqueous methylene blue in order to differentiate bone from teeth. Then they were placed on microscope slides and photographed. The area of alveolar bone loss (ABL) was measured by a trained and blinded observer (LHTS), using an imaging software (ImageJ® National Institutes of Health, Washington, DC, USA), as previously described (Goes, Lima, Melo, Rego, & Lima, 2010).

The same specimens used for macroscopic study were radiographed using a digital system (Digora Soredex Digital System®, Portslade-East Sussex, UK). The specimens were posed over the sensor and the radiographic images were acquired using 63 kVp, 8 mA, exposure time of 0.06 s and focal distance of 30 mm. Bone mineral density of the maxillae were evaluated using Image J software, as previously described (Goes et al., 2010).

The same non-demineralized specimens were then scanned by a cone beam micro-CT system (Skyscan 1172, Bruker, Kontich, Belgium). The x-ray generator was operated at an accelerated potential of 50 kV with a beam current of 200 μA and an exposure time of 560 ms per projection. Images were produced with a voxel size of $6 \times 6 \times 6\,\mu m$. Using an appropriated software (Data Viewer®, version 1.5.0, Bruker, Kontich, Belgium), the generated 3 dimensional models were rotated into a standard position as described previously (Lisboa et al., 2015). Linear measurements of ABL were performed at 3 different sites: buccal, furcation and interproximal. For the interproximal site, coronal dataset was analyzed using appropriated software (CT-Analyser®, version 1.13.5.1+, Bruker, Kontich, Belgium) (Lisboa et al., 2015). Volumetric analyses were performed using the same software applied for the analysis of the interproximal site, as previously described (Lisboa et al., 2015). Bone mineral density (BMD), bone percentage, bone porosity, and trabecular thickness (Tb.Th) were assessed. All micro-CT analyses were performed by one blinded and calibrated examiner (MRPL).

2.3. Microscopic analysis of alveolar bone

Another set of experiment was performed for histopathological analysis. After the euthanasia of the rats, the maxillae were removed and fixed in 10% neutral buffered formalin and demineralized in 10% EDTA buffered solution. After complete decalcification, the specimens were dehydrated and embedded in paraffin. Serial sections, 4 μm thick, were obtained in a mesio-distal direction. The sections were stained with hematoxylin and eosin. Sections representing the area between the first and second molars were evaluated by light microscopy (×40 magnification). The parameters analyzed were based on the study by Leitão et al. (2005), using scores varying from 0 to 3. The histopathological analysis was performed by a certified histologist (GACB).

2.4. Serum levels of bone-specific alkaline phosphatase (BALP)

Blood samples were collected from the orbital plexus at the 11th day after the induction of periodontitis. Bone-specific alkaline

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