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Local administration of Tiludronic Acid downregulates important mediators involved in periodontal tissue destruction in experimental periodontitis in rats

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ABSTRACT

Objective: The purpose of this study was to evaluate whether local administration of TIL could influence the expression of the inflammatory mediators IL-1β, TNF-α, MMP-8 and COX-2 in rats with experimental periodontitis (EP).

Methods: Twenty-four adult male rats (*Rattus norvegicus, albinus*, Wistar) were assigned to groups C, EP, EP-TIL (C—Control group, EP–Periodontitis groups). On EP groups, a ligature was placed around maxillary 2nd molars on day 1. On group EP-TIL, 20 µL of TIL solution (1 mg/kg body weight) was injected into the subperiosteal palatal area adjacent to the maxillary 2nd molar every other day until euthanasia (day 11). Alveolar bone loss was morphometrically analyzed. mRNA expressions of IL-1β, TNF-α, MMP-8 and COX-2 were assessed by qPCR. IL-1β, TNF-α, MMP-8 and COX-2 were immunohistochemically analyzed. Data were analyzed statistically.

Results: Group EP-TIL presented reduced alveolar bone loss when compared with group EP ($p < 0.05$). Group EP-TIL presented decreased mRNA expressions of IL-1β, TNF-α, MMP-8 and COX-2 and reduced immunolabeling of IL-1β, TNF-α and MMP-8 when compared with group EP ($p < 0.05$). No differences regarding the immunolabeling of COX-2 were found when group EP-TIL was compared with the other groups ($p > 0.05$).

Conclusion: Within the limits of this study, it can be concluded that local administration of TIL downregulates important mediators involved in periodontal tissue destruction in ligature-induced periodontitis in rats.

1. Introduction

The host modulation of matrix metalloproteinase (MMP) or the blockage of inflammatory cytokines are approaches to block the progression of inflammatory bone loss observed in periodontitis (Kirkwood, Cirelli, Rogers, & Giannobile, 2007). Therefore, inflammatory cell signaling pathways that generate these inflammatory and tissue destruction proteins have become promising therapeutic targets (Kirkwood et al., 2007). Bisphosphonates (BPs) are one of the categories of host-modulating agents that have been investigated in the periodontal therapy (Kirkwood et al., 2007).

BPs are synthetic chemical drugs that inhibit bone resorption by selective adsorption to mineral surfaces and subsequent internalisation by osteoclasts substantially affecting genesis and resorptive activity of these cells (Russell et al., 2007). BPs can also promote osteogenesis by stimulating osteoblastic differentiation (D'Aoust, McCulloch, Tenenbaum, & Lekic, 2000). In fact, increases in osteoid matrix formation and in collagen synthesis were observed with the use of BPs (Guenther, Guenther, & Fleisch, 1981; Reddy et al., 2005). Other effect attributed to a bisphosphonate (BP) was the reduction on bacterial

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growth in experimental periodontitis in rats (Menezes et al., 2005). Some BPs also present anti-inflammatory effects by reducing the influx of neutrophils to the inflamed gingiva and/or the inhibition of pro-inflammatory mediators (Buduneli et al., 2007; Menezes et al., 2005). It has been reported that BPs may be an appropriate adjunctive treatment in the clinical management of periodontitis (Lane et al., 2005; Sharma & Pradeep, 2012).

Tiludronic Acid (chloro-4-phenyl-thiomethylene-1,1-bisphosphonate) (TIL) is a non-nitrogen-containing BP mainly indicated for the treatment of Paget's disease of bone (Silverman, 2008). It inhibits osteoclast-mediated bone resorption (Rogers et al., 1999), disrupts cytoskeleton resorption of osteoclasts (Murakami et al., 1997), inhibits activity of these cells on the proton pump (David, Nguyen, Barbier, & Baron, 1991) and leads to osteoclast apoptosis (Rogers et al., 1999). TIL also has anti-inflammatory effects, since it can dose-dependently restrain interleukin (IL)-6 production by osteoblasts (Tokuda, Kozawa, Harada, & Uematsu, 1998) and the secretion of tumor necrosis factor (TNF)-α, IL-1β, IL-6 and nitric oxide (NO) by activated macrophages (Mönkkönen, Simila, & Rogers, 1998). It was demonstrated that TIL can inhibit MMP-1 and −3, important enzymes for the degeneration of extracellular matrix constituents in periodontal diseases, in human periodontal ligament cells (Nakaya et al., 2000).

Recently, we demonstrated that local administrations of TIL solutions influenced osteoclasts, reduced alveolar bone resorption and the intensity of the local inflammatory response in experimental periodontitis (EP) in rats (Furlaneto et al., 2014). However, it is necessary to analyze how TIL influences periodontal tissues, examining the regulation of important inflammatory mediators involved in periodontal tissue destruction. The purpose of this study was to evaluate the influence of local administration of TIL on the genic expression and the immunolabeling of IL-1β, TNF-α, MMP-8 and cyclooxygenase (COX)-2 in rats with ligature-induced periodontitis.

2. Materials and methods

2.1. Sample

This study was conducted according to the ethical principles of animal experimentation, as well as standards for the didactic-scientific practice of vivisection and the Universal Declaration of Animal Rights by United Nations Educational, Scientific and Cultural Organization. The present research was performed after acceptance by the Ethics Committee on Animal Research at Federal University of Ceara-UFC, Fortaleza, CE, Brazil (protocol 028/2011).

A power calculation was executed to establish the sample size. The animal was considered the study unit. The sample size was calculated to provide 80% power to recognise significant differences among groups with a 95% confidence interval (a = 0.05), considering the means and standard deviations of the genic expression of IL-1β of the study by Lisboa et al. (2015). Thus, a sample size of eight animals per group was needed.

2.2. Experimental model

Twenty-four 3- to 4-month-old male Wistar rats (*Rattus norvegicus, albinus*, Wistar) weighing 250 to 300 g were used (Central Animal Facility of the UFC) in this study. The animals were maintained in a room with a 12-h light/dark cycle with temperatures from 22 °C to 24 °C. All through the experiment, the rats were kept in plastic cages and fed with selected solid diet and water *ad libitum*. Before the study began, the rats were identified by a numeric code. According to a random numeric table generated by a computer software, the study coordinator (F.A.C.F.) allocated each rat into one of the following groups (n = 8): 1) C (control): EP was not induced and TIL was not administered; 2) EP: EP was induced with a ligature and TIL was not administered; 3) EP-TIL: EP was induced with a ligature and TIL solutions at a dosage of 1 mg/kg body weight were locally administered. The allocation sequence was unknown to the investigators of the study (operators, outcome assessors and biostatistician).

2.3. Induction of periodontitis with ligature

Animals were anesthetized by an intramuscular injection of xylazine.[1] (6 mg/kg body weight) and ketamine[2] (70 mg/kg body weight) on day 1. After general anesthesia, each animal was placed on the operating table, which allowed keeping the rat's mouth open, facilitating access to posterior teeth of the maxillae. In groups EP and EP-TIL, a nylon ligature[3] was placed around the cervical area of the maxillary second molars of each rat, remaining supragingivally at both buccal and lingual faces. The knots were positioned at the buccal face of the teeth. In group EP-TIL, 20-μL TIL[4] solutions (1 mg/kg body weight) were injected into the subperiosteal palatal area adjacent to the maxillary second molars on days 1, 3, 5, 7, and 9 in the morning. Throughout the experimental period, the animals were weighed every other day, and the doses of TIL were adapted accordingly (Furlaneto et al., 2014).

The animals were euthanized under anesthesia with a final solution of xylazine (30 mg/kg body weight) and ketamine (240 mg/kg body weight) 11 days after the placement of the ligatures. The hemimaxillae were excised and fixed in 4% formaldehyde for 24 h (Furlaneto et al., 2014). Samples of gingival tissues around maxillary second molars of each animal were collected and stored at −80 °C.

2.4. Morphometric analysis

The hemimaxillae were chemically defleshed and stained with 1% aqueous methylene blue in order to enhance the visibility of the cementoenamel junction (CEJ). Standardized photographs of the buccal aspects of the hemimaxillae were obtained using a digital camera[5] connected to a stereomicroscope[6] and a computer, with an original magnification of x16. The images were evaluated with appropriated software.[7]

The linear distances between alveolar bone crest and CEJ (ABC-CEJ – mm) were measured in the interproximal area between the 1st and the 2nd molar, in the interproximal area between the 2nd and the 3rd molar and also at the long axes of the mesial and distal roots of the 2nd molar. The average of these linear measurements obtained from each animal was used to express the ABC-CEJ value, which represented the alveolar bone loss. Morphometric analysis was performed by a masked and calibrated examiner (N.P.R.F.).

2.5. Quantitative reverse transcription-polymerase chain reaction (qPCR)

The gingival samples were macerated under freezing with liquid nitrogen. Total RNA was extracted by guanidinium isothiocyanate-phenol-chloroform method, using TRIzol™[8] and RNA was resuspended in diethyl pyrocarbonate (DEPC)[8]–treated water. RNA concentration was evaluated by spectrophotometry using the ratio of the absorbance at 260 and 280 nm, and its integrity was analyzed by 1% agarose-gel electrophoresis with ethidium bromide (10 mg/mL). Complementary DNA (cDNA) was reverse transcribed from 5.0 μg of total RNA by using an oligo (dT) primer[8] and SuperScript III reverse transcript,[8] according to manufacturer's instructions (Coura et al., 2015). The primers used are described in Table 1. Quantitative Real Time PCR (qPCR) for gene

[1] Rompam®, Bayer Saúde Animal, São Paulo, SP, Brazil.
[2] Dopalen, Agribands, Paulínia, SP, Brazil.
[3] 3-0, Technofio, Goiânia, GO, Brazil.
[4] Tildren, Ceva Saúde Animal, Paulínia, SP, Brazil.
[5] Leica DFC 310 FX, Leica Microsystems CMS GmbH, Wetzlar, Germany.
[6] Leica MZ6, Leica Microsystems GmbH, Wetzlar, Germany.
[7] Image J – National Institutes of Health, Washington, DC, USA.
[8] Invitrogen, Life Technologies, Carlsbad, CA, USA.