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ORIGINAL ARTICLE

Effect of methotrexate on alveolar bone loss in experimental periodontitis in Wistar rats

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Abstract

Objective. Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are pro-inflammatory cytokines directly related with tissue destruction in the pathogenesis of periodontitis. Inhibitory effects on IL-1 and TNF production have been attributed to the folate analog methotrexate. The purpose of this study was to evaluate the effect of methotrexate on the pathogenesis of alveolar bone loss in experimental periodontitis in rats. ***Methods.*** Ligature-induced experimental periodontitis was created in 44 Wistar rats. The animals were randomly divided into four groups and treated with methotrexate (0.1, 0.5, and 1.0 $mg \cdot kg^{-1}$) or saline. Morphometrical registration of alveolar bone loss was carried out after 28 days of ligature placement to determine the effect of methotrexate on the progression of experimental periodontitis. ***Results.*** Intra-group comparisons showed significantly higher alveolar bone loss mean values in maxillary sides with ligature (paired sample *t*-test; $p < 0.05$). Mean alveolar bone loss was not different between groups and was independent of the dosage (range 0.63–0.67 mm, one-way ANOVA; $p > 0.05$). ***Conclusion.*** Although methotrexate has important cytokine-inhibitory properties, its possible use in modulating the host immune-inflammatory response in periodontal disease was not confirmed.

Key Words: *Cytokines, interleukin-1, periodontal disease, tumor necrosis factor*

Introduction

Periodontitis is currently understood to be a consequence of an immune inflammatory response to oral microbial challenge. Lipopolysaccharides and other bacterial products elicit the host response, i.e. a response that involves several mechanisms of the immune-inflammatory system [1–3]. Certain components of this process are thought to be key factors in the pathogenesis of periodontal diseases, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) have been given special attention owing to their direct relationship with tissue destruction [4,5]. Both cytokines stimulate fibroblasts to produce matrix metalloproteinases and induce bone resorption by osteoclasts [5]. Additionally, level and expression of TNF and IL-1 are higher in inflamed periodontal sites than in healthy sites, as demonstrated by clinical studies [6–8].

Although several studies involving experimental periodontitis models in animals have improved our understanding of this process, the biological roles of TNF and IL-1 are not completely known. It has been shown that the *in vivo* administration of recombinant human IL-1β accelerates silk-ligature-induced alveolar bone resorption in rats [9]. The same effect has been reported after administration of recombinant TNF-α [10]. However, some agents with inhibitory properties at TNF and IL-1 seem to promote lower periodontal tissue destruction, as demonstrated in models of experimental periodontitis in primates [11,12] and in rats [13,14].

Methotrexate is a folate analog widely indicated in antineoplastic and antirheumatoid therapies because of its cytotoxic, immunosuppressive and anti-inflammatory properties [15]. Inhibition of IL-1 and TNF production, in particular, has been reported [16,17]. Rising of anti-inflammatory cytokine expression (IL-4 and IL-10) is also related to methotrexate treatment, resulting in anti-inflammatory effects [17]. Based on these properties, it could be assumed that methotrexate is a drug potentially modulating

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periodontal immune-inflammatory host response. Modulation would occur by inhibited production of these cytokines. It can be speculated that inhibition would have an anti-inflammatory role, i.e. diminishing alveolar bone loss during pathogenesis of periodontitis.

To the best of our knowledge, there is only one study in which the effect of systemic administration of methotrexate on experimental periodontal disease has been evaluated [18]. However, in this study a high dose of methotrexate was used to promote neutropenia, which is one of its recognized side effects [19]. Higher alveolar bone destruction has been found in rats exposed to methotrexate-induced neutropenia [18].

When adequate dosage is established, the possibility of minimizing the side effects caused by administration of drugs can be considered. In this way, low doses of methotrexate could promote a slight immunosuppression, especially in IL-1 and TNF expression and production. Our hypothesis is that modulation of these cytokines by methotrexate might inhibit the progression of bone destruction, resulting in lower amounts of alveolar bone loss. The aim of the present study was to evaluate the effect of three different doses of methotrexate on alveolar bone loss in experimental periodontitis in rats.

Material and methods

Animals

Forty-four 2-month-old male Wistar rats were bred and housed as described previously [20] to ensure periodontal disease-free animals at baseline. These conditions included wire mesh floor bedding, a finely milled diet (Supralab, SUPRA, São Leopoldo, RS, Brazil) and tap water *ad libitum*. The animals were kept in a constant temperature ($20^{\circ}C \pm 1^{\circ}C$) and at a light/dark cycle of 12 h (light: 0800 to 2000 h). Mean (SD) weight of the rats at baseline was 262 (32) g. The experimental protocol was approved by the Ethics Committee of the Lutheran University of Brazil.

Experimental procedures

A pre-experimental examination was performed to exclude animals with periodontal probing depths exceeding 0.5 mm (PCP 10-SE, Hu-Friedy, Chicago, Ill., USA), according to Björnsson et al. [20], with slight modifications, to ensure that the animals were periodontally disease-free. Experimental periodontitis was induced by placing 4/0 silk ligatures (Ethicon; Johnson & Johnson, São José dos Campos, SP, Brazil) around the cervix of the left 2nd maxillary molar under general anesthesia with intramuscular ketamine 5% (Ketamina Agener; Agener, Embu-Guaçu, SP, Brazil) and xilazine 2% (Calmiun; Agener, Embu-Guaçu, SP, Brazil) 1:1 solution (0.2 ml/100 mg). The contralateral maxillary molar was considered as the internal control.

The rats were randomly divided into four groups; on day 1 receiving methotrexate (Genix; Anápolis, GO, Brazil) or saline (Basa; Caxias do Sul, RS, Brazil) by intraperitoneal injection, and on every 3rd day. Three different doses of methotrexate were used (0.1, 0.5, and 1.0 $mg \cdot kg^{-1}$), based on previous studies [18,21,22]. The same operator performed all administrations. Bodyweight was assessed once a week in order to assess general heath. Presence of the ligature was verified in the same interval. After 28 days of experimental periodontitis [23], the animals were killed with an overdose of thiopentone anesthetic (Thiopentax; Cristalia, Itapira, SP, Brazil).

Sample size calculations

Sample size was calculated using data from a previous study [18]. Taking into consideration a mean difference in alveolar bone loss of 0.2 mm, accepting alpha and beta errors of 0.05 and 0.20, respectively, groups of 9 animals were considered necessary.

Morphometrical registration of bone destruction

The right and left segments of the maxillae were dissected out manually and then immersed in sodium hypochlorite (Mazzarollo, Gravatai, RS, Brazil) with 8.5% active chlorine for 5 h to remove soft tissues. After rinsing, the specimens were stained for 1 min in methylene blue 1% (Sigma-Aldrich, Saint Louis, Mo., USA) to delineate the cemento-enamel junction.

Standardized digital pictures were taken from the buccal and palatal aspects of each specimen using a Sony DSC-F828 camera (Sony®, Tokyo, Japan) with minimal focal distance [24]. Measurements were performed with the aid of the ImageTool 3.0 computer software (UTHSCSA ImageTool 3.0, San Antonio, Tx., USA). Periodontal bone loss was defined as the distance between the cemento-enamel junction and the alveolar bone crest. Buccal and palatal measurements were made at five points. Two measurements were taken in each root (mesial and distal) and one on furcation. The locations of the measurements are illustrated in Figure 1. All registrations were carried out blindly.

Reproducibility

Before the analysis, the examiner was trained and calibrated by double measurements of 20 specimens at 1-week intervals. Paired *t*-test statistics revealed no statistically significant differences in the mean values for comparison. Additionally, Pearson's correlation

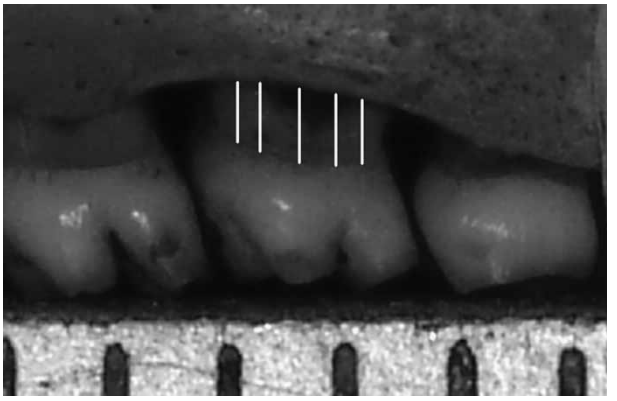

Figure 1. Digital photograph of the maxillae. The lines indicate the five measurement sites.

coefficient obtained between the two measurements reflected a very high correlation ($r = 0.99$).

Statistical analysis

After checking for normality, mean alveolar bone loss was calculated. Intra-group comparisons (sides with and without ligature) were performed by paired sample *t*-test and inter-group comparisons by one-way ANOVA. The animal was the unit of analysis and the alpha level was set at <0.05.

Results

One animal excluded after pre-experimental periodontal examination was substituted by another one showing no signs of periodontal disease (probing depths not exceeding 0.5 mm). One ligature lost during the experimental period was observed at the last verification (day 28) in one animal of the MTX0.1 group.

The main outcome of the study is given in Table I. The comparison between experimental groups showed no statistically significant differences on alveolar bone loss mean values. An independent analysis was performed on furcation sites. In the same way, no differences were observed between control-treated and methotrexate-treated rats (Table I). Intra-group comparison between teeth with and teeth without ligature showed that the experimental periodontitis model always caused statistically significantly higher amounts of alveolar bone loss independently of the experimental group ($p < 0.05$).

Discussion

In the present study, we evaluated the effect of methotrexate on the pathogenesis of alveolar bone loss in experimental periodontitis. Our results show that systemic methotrexate administration does not modulate the progression of alveolar bone loss. We also confirmed that ligature-induced periodontal disease is a suitable experimental model for promoting alveolar bone destruction.

The possibility of dose–response relationship evaluation was present in our study. Low doses of methotrexate (0.1 and 0.5 $mg \cdot kg^{-1}$) aiming at a slight anti-inflammatory and immunosuppressive [22] effect on experimental periodontitis were evaluated. It might be expected that this pharmacologic modulation would result in a different IL-1 and TNF profile response and thus contribute to lower tissue destruction. However, no significant differences on alveolar bone loss were observed between the experimental and control groups. This finding suggests the possibility of weak methotrexate modulation of cytokine production in the periodontium of the host, which should be confirmed by further study. Another hypothesis for the lack of effect of methotrexate treatment can be based on the continuation of periodontal tissue destruction by alternative or complementary ways that are not inhibited by methotrexate, such as several other pro-inflammatory mediators. Moreover, the enzymatic environment in periodontal disease sites may destroy the cytokine antagonist agents prior to their peak activity. Although the initial studies [13,14] in rats have shown that cytokine inhibition provides better experimental periodontitis outcome, characterized by lower tissue destruction, other studies have demonstrated no benefits of cytokine-antagonist administration [25].

A previous study has reported higher tissue destruction in rats exposed to methotrexate (1.0 $mg \cdot kg^{-1}$ three times per week) due to induced neutropenia [18]. In order to verify this relationship, a similar dosage was reproduced in our study, but with no additional alveolar bone loss compared to

Table I. Alveolar bone loss (mean (SD)) in rats treated with methotrexate and saline

	ABL roots, mm		ABL furcation, mm	
Group	With ligature*	Without ligature*	With ligature*	Without ligature*
MTX 0.1 $mg \cdot kg^{-1}$ ($n = 11$)	0.67 (0.10)	0.36 (0.11)	0.66 (0.11)	0.37 (0.11)
MTX 0.5 $mg \cdot kg^{-1}$ ($n = 11$)	0.63 (0.11)	0.34 (0.01)	0.66 (0.12)	0.33 (0.01)
MTX 1.0 $mg \cdot kg^{-1}$ ($n = 11$)	0.67 (0.12)	0.33 (0.10)	0.72 (0.14)	0.32 (0.01)
Saline ($n = 11$)	0.64 (0.11)	0.39 (0.01)	0.66 (0.11)	0.37 (0.01)

ABL: alveolar bone loss. MTX: methotrexate.
Inter-group comparisons showed no significant differences on mean alveolar bone loss (one-way ANOVA, $p > 0.05$).
*Statistically significant difference between sides with and without ligature (paired *t*-test, $p < 0.05$).

controls. One possible explanation for these conflicting results could be the longer experimental periodontitis period performed in the first study (8 weeks) [18], as well as methodological differences such as assessment of periodontal tissue destruction.

Periodontal abscesses were observed in three animals that received methotrexate. This particular event can be attributed to a possible systemic immunosuppression mediated by methotrexate, i.e. impairing an adequate response. All abscesses were developed at sides with ligature. Higher individual periodontal bone loss means were found in these animals when compared with their group means.

Blinding of the examiner, randomization, utilization of sufficient numbers of animals and use of comparative groups were principles followed by this study in order to generate better evidence [26]. Our sample size calculation indicated a minimum of 9 animals in each group, i.e. an adequate sample in terms of quantity. Additionally, methodological cares related to breeding and housing of the rats were observed [20], thereby further improving the reliability of the results.

Despite its IL-1 and TNF inhibition properties, methotrexate had no beneficial effect on the pathogenesis of destructive periodontal disease. Besides, the development of periodontal abscesses has to be understood as a sign of no beneficial immune-inflammatory modulation.

Within the limits of an animal investigation, the findings from this study lead to the conclusion that the use of methotrexate is not a useful tool in preventive and therapeutic periodontal strategies.

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