The Effect of Spironolactone on the Pathogenesis of Ligature-induced Alveolar Bone Loss in Wistar Rats

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Summary

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that has a straight relationship with tissue destruction in the pathogenesis of periodontitis. Inhibitory effects of TNF production have been attributed to spironolactone. The aim of this study was to evaluate the effect of spironolactone on the pathogenesis of ligature-induced alveolar bone loss in rats. Experimental periodontitis was induced in 38 Wistar rats by ligature placement in the left second maxillary molar. The contra-lateral maxillary molar served as intra-group control. Animals were randomly divided into 4 groups and treated with spironolactone (50, 100, 200 $mg \cdot kg^{-1}$) or saline. Morphometrical registration of maxillary alveolar bone was performed after 28 days of experimental periodontitis. 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 significantly different between groups, independently of the dosage (range: 0.63 – 0.66 mm, one-way ANOVA, $p>0.05$). Although spironolactone has recognized TNF-inhibitory properties, the possibility of its use on modulation of host immune-inflammatory response in periodontal disease was not confirmed.

Introduction

Periodontitis is characterized by an inflammatory process of the periodontal supporting tissues, resulting in alveolar bone resorption and soft tissue destruction (*Page & Kornman, 1997*). The pathogenesis of destructive periodontal disease is based on the host immune inflammatory response to oral microbial challenge (*Kinane, 2001; Madianos et al., 2005*). Some components of the host response are thought to be key factors in this process (*Page, 1991*). Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are pro-inflammatory cytokines able to stimulate matrix metalloproteinases production and induce bone resorption by osteoclasts (*Graves et al., 1998; Graves & Cochran, 2003*). In addition, IL-1 and TNF levels and expression are higher in inflamed human periodontal sites as compared to healthy sites (*Stashenko et al., 1991; Tervahartiala et al., 2001; Figueredo et al., 1999*).

Recent data provided from animal experimental periodontitis models have improved the comprehension of these cytokines function, although the biological role of TNF and IL-1 are not completely elucidated. It has been shown that the in vivo administration of recombinant TNF-alpha (TNF-α) accelerates silk ligature-induced alveolar bone resorption in rats (*Gaspersic et al., 2003*). On the other hand, some agents with inhibitory properties at TNF receptors seem to promote lower periodontal tissue destruction (*Assuma et al., 1998; Oates et al., 2002*). The study of pharmacological modulation of TNF-mediated response is interesting, because it allows better understanding of its biological functions and establish certain drugs as potential tools in periodontal therapy and prevention.

Spironolactone is a competitive aldosterone receptor antagonist, traditionally used as treatment for diseases associated with hyperaldoteronism (*Doggrell & Brown, 2001*). More recently, anti-

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inflammatory properties have been attributed to spironolactone, especially due to its inhibition potential on Interferon-γ (IFN-γ) and TNF (*Bendtzen et al., 2003; Grauballe et al., 2005*). In spite of these inhibitory properties, it has been demonstrated that spironolactone-treated rats did not demonstrate significant less alveolar bone loss compared to non-treated rats in an experimental periodontitis model (*Grauballe et al., 2005*). However, experimental periodontitis was performed in a short length model and dose-response evaluation was not considered. Therefore, the use of spironolactone as modulator of cytokine mediated response in the pathogenesis of periodontal disease remains unclear. The aim of the present study was to evaluate the effect of three different doses of spironolactone on ligature-induced alveolar bone loss in rats.

Materials and Methods

Experimental animals

Forty-four 2-month-old male Wistar were initially used in this study. The animals were bred and housed in previously described conditions (*Björnsson et al., 2003*), including a wire mesh floor, bedding, a finely milled diet (Supralab, SUPRA, São Leopoldo, Brazil) and tap water. These allow one to obtain periodontal disease-free animals at baseline. Animals were kept in a constant temperature (20º C ± 1º C) and light/dark cycle of 12 hours (light: 8:00 am to 8:00 pm). The mean(SD) weight of the rats at the baseline was 239(34) g. The experimental protocol was approved by the Ethical Committee of the Lutheran University of Brazil.

Power calculation

Data from the present study were used for power calculation. Considering a significant difference of 0.2 mm and the variability of our data, the power of this study was estimated as 0.84.

Experimental procedures

A pre-experimental periodontal examination was performed with a manual periodontal probe (PCP 10-SE, Hu-Friedy®, Chicago, IL, USA) according to *Björnsson et al.* (*2003*). If animals presented probing depth greater than 0.5 mm, they should be excluded. Experimental periodontitis was induced by placing 4/0 sterilized silk ligatures (Ethicon, Johnson&Johnson, São José dos Campos, SP, Brazil) around the cervix of the left second maxillary molar under general anesthesia with intramuscular ketamine 5% and xilazine 2% solution (0.2ml/100mg) (Agener, Embu-Guaçu, SP, Brazil). The contra-lateral maxillary molar was considered the intra-group control.

The rats were randomly divided into four groups. At day one, treatment was started by administration of spironolactone (DEG, São Paulo, SP, Brazil) or saline (Basa, Caxias do Sul, RS, Brazil), as described in Table 1. The same operator performed all administrations, by intraperitoneal injection. Body weight was assessed once a week, in order to verify general health. At day 28, animals were killed by an overdose of thiopentone anesthetic (Cristalia, Itapira, SP, Brazil).

Morphometrical registration of bone destruction

The right and left segments of the maxillae were

Table 1. Group distribution, number of animals (n) in each group and treatment

Group	n*	Treatment
SPIR50	10	Spironolactone 50 mg·kg^{-1} every 3rd day
SPIR100	7	Spironolactone 100 mg·kg^{-1} every 3rd day
SPIR200	10	Spironolactone 200 mg·kg^{-1} every 3rd day
CONT	11	Saline solution every 3rd day

* Six rats were lost due to deaths or technical problems during the study (1 in groups SPIR 50 and SPIR 200 and 4 in group SPIR 100).

dissected out manually and then immersed in sodium hypochlorite with 8.5% active chlorine (Mazzarollo, Gravatai, RS, Brazil) during 5 hours to remove soft tissues. After washing, the specimens were stained for 1 minute 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 (Sony®, Tokyo, Japan) camera, with minimal focal distance (*Cavagni et al., 2005*). Pictures were measured with the aid of the ImageTool 3.0 (UTHSCSA ImageTool 3.0, San Antonio, TX, USA) computer software. 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. All registrations were carried out blindly, using coded specimens. Previously, the examiner was trained and calibrated by double measurements of 20 specimens, with a one-week interval. Paired t test statistics were run and no differences were observed in the mean values for comparison ($p=0.330$). Pearson's correlation coefficient was obtained between the two measurements and revealed a very high correlation ($r=0.99$).

Statistical analysis

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

Results

The comparison between groups showed no statistically significant differences on alveolar bone means in sides with ligature ($p=0.726$) and without ligatures ($p=0.448$) (Figure 1). An independent analysis was performed on furcation site and, in the same way, no differences were observed between control and spironolactone-treated rats (Figure 2). The intra-group comparisons between teeth with or without ligature showed that experimental periodontitis always caused statistically significant higher amounts of alveolar bone loss, independently from the experimental group ($p<0.001$) (Figure 1 and Figure 2).

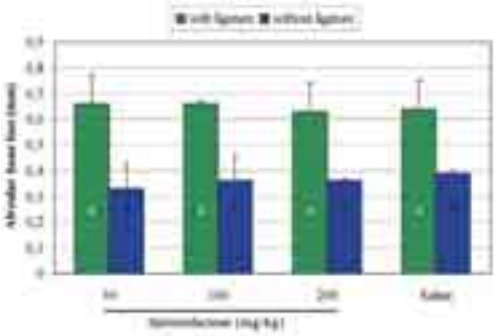

Figure 1. Mean ± SD alveolar bone loss at roots for experimental groups in sides with ligature and without ligature. Different letters indicate statistically significant differences (paired sample t test, $p<0.05$). Inter-group comparisons showed no significant differences on mean alveolar bone loss (one-way ANOVA, $p>0.05$).

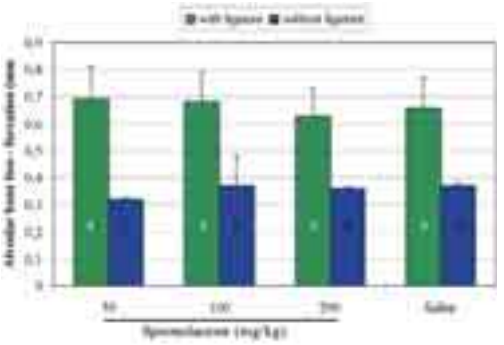

Figure 2. Mean ± SD alveolar bone loss at furcation for experimental groups in sides with ligature and without ligature. Different letters indicate statistically significant differences (paired sample t test, $p<0.05$). Inter-group comparisons showed no significant differences on mean alveolar bone loss (one-way ANOVA, $p>0.05$).

Discussion

The present study evaluated the effect of spironolactone on pathogenesis of alveolar bone loss in an experimental periodontitis model in rats. Our results showed that systemic spironolactone administration had no significant effect on ligature-induced alveolar bone loss progression and no dose-response relationship was observed.

Animal models are useful in the study of the pathogenesis of periodontal disease, especially due to ethical reasons involved in human studies. One of the possible ways to study this subject is via the ligature-induced periodontitis (*Koide et al., 1995; Sallay et al., 1982*). Our results showed that maxillary sides with ligature developed significantly higher alveolar bone loss means than sides without ligature, confirming that it is a suitable model of experimental periodontitis.

However, animal studies have important limitations and should not be directly extrapolated to humans. Additionally, it must be considered that animals used in this study are from the same rat strain (Wistar) and have a very similar genetic profile that establishes no significant differences in immune-inflammatory response. Unlike Wistar rats, humans have a larger variability of immune-inflammatory response profile.

In order to generate better evidence, we observed the principles of "blinding" of the examiner, randomization, utilization of sufficient number of animals and use of comparative groups. The sample power of this study was calculated and a beta error of 0.16 is expected, which indicates the number of animals is sufficient. Loss of animals occurred in our experiment, due to technical problems at anesthetic procedures. However, the results of the study were not directly affected.

It have been demonstrated that the use of a finely milled diet, a wire mesh floor, bedding and tap water drinking are conditions that provide breeding rats with minimal signs of periodontal disease. A pre-experimental examination is also indicated to confirm that animals are periodontal disease-free (*Björnsson et al., 2003*). These cares, related to animal housing and breeding, were all followed in our study, contributing to the reliability of the results.

Rodent periodontitis is caused by a small group of Gram-negative bacteria displaced on to the tooth root surfaces (*Sallay et al., 1982*). Lipopolysaccharides and other bacterial products gain access to the periodontal tissues, initiating and perpetuating an immune-inflammatory response, resulting in high levels of pro-inflammatory cytokines (*Oates et al., 2002*). Consequently, matrix metalloproteinases are released to destroy periodontal connective tissues, as well as prostaglandins, which mediate alveolar bone destruction (*Graves & Cochran, 2003*). TNF has been found to be an important pro-inflammatory mediator of bone destruction in experimental periodontal disease (*Gaspersic et al., 2003*).

In humans, elevated expression of TNF and its p55 receptor are found in sites with periodontitis (*Tervahartiala et al., 2001*). Higher levels and expression of TNF are present in inflamed periodontal sites as compared to healthy sites (*Stashenko et al., 1991; Tervahartiala et al., 2001*). Other clinical data indicate that periodontal therapy also results in a decrease in TNF levels (*Gamonal et al., 2000; Iwamoto et al., 2003*).

Recent data have shown that spironolactone acts as a TNF-inhibitor agent (*Bendtzen et al., 2003*). *Grauballe et al.* (*2005*) reported reduction of TNF serum levels after treatment with spironolactone in an endotoxic shock model (lipopolysaccharide). However, spironolactone-treated rats did not demonstrate significantly less alveolar bone loss when compared to non-treated control rats in a ligature-induced experimental peridontitis. This finding is corroborated by our results. However, it must be considered that there are important methodological differences between the two studies, like the method of administration and the experimental time length.

In our study, spironolactone was administered by intraperitoneal injection in order to allow more efficient control in drug dosage. Spironolactone solutions are difficult to obtain due to their hydrophobic properties. Therefore, spironolactone suspensions

were used based on group dosage and administered as a function of the individual animal body weight. The pharmacological effects of spironolactone are known to start after several days of treatment (*Cook et al., 1988*). We considered the 28 days of experimental periodontitis a sufficient time to provide secure analysis.

Dose-response evaluation was considered in our study. Three spironolactone dosages were administered, but no differences in alveolar bone loss means were observed between them. These findings supply more evidence that spironolactone can not be a useful tool in periodontics, in spite of its TNF-inhibition properties. The failure of the spironolactone treatment may be explained by the incomplete inhibition of TNF (*Grauballe et al., 2005*) and by its fast metabolism (*Kaukonen et al., 1998*). The three days interval of administration performed in our study could significantly contribute to this observation. Moreover, many other inflammatory mediators that are not inhibited by spironolactone (e.g. IL-1) may continue the process of tissue destruction. We believe that the pharmacological modulation of host response in periodontal clinical strategies must include the several mechanisms involved in tissue destruction. Further investigation should be directed in this way.

One particular event found in our study was the occurrence of periodontal abscesses in one animal of the SPIR50 group and two of the SPIR100. All abscesses were developed at the sides with ligature. The very low occurrence of this event does not allow further analysis, but we can associate these animals with higher individual periodontal bone-loss means when compared with their group means. Spironolactone, in spite of its TNF-inhibition properties, had no beneficial effect on the pathogenesis of destructive periodontal disease. Besides, the development of periodontal abscesses should be understood as a sign of no beneficial immune-inflammatory modulation. Additionally, the possibility of tissue destruction mediated by others pro-inflammatory cytokines must be considered. Although anti-TNF treatment has been successfully used in many chronic inflammatory diseases, the same effect seems unlikely to be achieved in periodontal therapy due to the particular pathogenic characteristics of periodontal diseases.

The findings from this study, within the limits of an animal investigation, lead to the conclusion that the use of spironolactone does not seem a useful tool in preventive and therapeutic periodontal strategies. Future studies may elucidate our results and determine whether spironolactone could contribute to periodontal clinical strategies.

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