Strontium Ranelate Elevates Expression of Heme Oxygenase-1 and Decreases Alveolar Bone Loss in Rats

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ABSTRACT

Objectives: The purpose of this study was to determine the effects of strontium ranelate on ligature-induced periodontitis in rats and assess the putative involvement of heme oxygenase-1 (HO-1) pathway in these effects.
Material and Methods: Male Wistar rats underwent nylon ligature placement around maxillary molars and were treated (v.o.) with strontium ranelate (20 or 100 mg/kg) for 7 days. After that, rats were euthanized and histomorphometric/histopathological analyses and RT-PCR for HO-1 expression were performed.
Results: Strontium ranelate (20 or 100 mg/kg) prevented bone resorption by 28% and 38%, respectively. Strontium ranelate treatment (100 mg/kg) up-regulated ($P < 0.05$) heme oxygenase-1 mRNA levels in the gingival tissues in comparison to control groups.
Conclusions: Strontium ranelate prevented periodontal bone loss in experimental periodontitis in rats while heme oxygenase-1 mRNA levels increased after treatment.

Keywords: bone resorption; diphosphonates; heme oxygenase; periodontitis.

INTRODUCTION

Periodontitis leads to bone loss [1]. Its onset mechanisms require investigation, but large amounts of eicosanoids/cytokines are involved [2]. Mechanical treatments are standard approaches, but cannot stop inflammatory responses. Therefore, immunomodulation can attenuate them.

Thus, heme oxygenase (HO) may be involved in this process as it provides a negative feedback signal. HO-1 is inducible by inflammatory stimuli, while the others are constitutively expressed [3,4].

Albeit HO-1 modulates inflammation, its role in periodontitis is unclear. Strontium ranelate (SR) enhances bone growth and reduces bone loss [5]. Thus, since HO-1 appears to regulate bone loss, we aimed to assess whether SR effects depend on the HO-1 pathway.

MATERIAL AND METHODS
Animals

Forty-eight male Wistar rats (180 - 250 g) were housed in a temperature-controlled room under a 12/12 h light/dark cycle and had free access to food and water. Animal numbers were kept to a minimum and efforts were made to minimize animal suffering. The study protocol complied with the European Communities Council Directive (86/609/EEC), the local guidelines on the welfare of experimental animals, and was approved by the local Ethics Committee of the Faculty of Medicine - Federal University of Ceará (protocol number 54/10).

Experimental periodontitis

Periodontitis was reproduced in a rodent model (n = 6) as previously described [2]. These animals were allotted to the following groups:
- Group 1: unchallenged-group receiving vehicle (0.9%, w/v, NaCl);
- Groups 2, 3, 4, and 5: periodontitis-challenged groups receiving vehicle (0.9%, w/v, NaCl), and euthanized at different time points (3, 7, 11 or 14 days);
- Groups 6, 7, and 8: groups of rats treated with SR (20 or 100 mg/kg) or vehicle (0.9%, w/v, NaCl) 1 hour before periodontitis induction, and once a day until euthanasia on day 7.

Rats were anaesthetized with tribromoethanol (Sigma, St Louis, MO, USA) (1 mL/100 g, i.p.), a 3.0-nylon ligature was placed around maxillary left second molars. The contralateral right side was used as negative control. The unchallenged group (n = 6) included animals that were not subjected to periodontitis. Animals were weighed daily. Three groups of rats (n = 6) were treated (v.o.) with SR (Protos® 2g, Les Laboratoires Servier Industrie, 45 520 Gidy, France) (20 or 100 mg/kg) or vehicle (0.9%, w/v, NaCl) 1 hour before periodontitis induction, and once a day until sacrifice on day 7. SR administration occurred at the same time during the experimental days and food was removed 2 hours before treatment to avoid pharmacokinetics interference. On day 7, animals were euthanized under anaesthesia and the maxillae and gingival tissues were excised to assess alveolar bone resorption, and to perform histopathological (haematoxylin and eosin stain) and molecular analyses.

Assessment of periodontal bone loss

Animals were euthanized under anaesthesia on day 7. Maxillae were removed and fixed in 10% neutral formalin. The maxillary halves were prepared for staining with 1% aqueous methylene blue to differentiate the alveolar bone from teeth as the enamel is not stained. The maxillae were then photographed (Sony Cyber-shot Dsc-h2). The obtained image was transferred to the Image J® Software (National Institutes of Health, Bethesda, USA). The alveolar bone loss was assessed by the uncovered molars root surface (mm^2) on the buccal aspect of the maxillary by subtracting the total area of the left maxillary tooth and bone resorption from the right maxillary tooth area (negative control) [2]. All acquired images were compared with a known area (1×1 mm^2).

Histopathological analysis

Additional experiments were performed to assess histological parameters. Maxillae were demineralized in 10% EDTA and included in paraffin. Six-micrometres thick sections were obtained and later stained for haematoxylin and eosin stain analysis. The area between the 1st and 2nd right molars was examined under light microscopy (original magnification x100). To evaluate SR effects on the periodontal tissues, histopathological scores (range 0 to 3) were used to assess the periodontal damage as previously described [6].

Heme oxygenase-1 expression

To evaluate the expression of HO-1, the gingival

tissues were excised on days 3, 7, and 11 for quantitative real-time PCR (qRT-PCR). Total RNA was extracted by guanidinium isothiocyanate phenol-chloroform extraction method [7]. Primers were designed by PrimerBlast using a GenBank (National Centre for Biotechnology Information NCBI). Reference sequences of HO-1 (NM_012580.2) to *Rattus norvegicus* are shown in Table 1. Contamination by genomic DNA was avoided by positioning the primers at the exon-exon junction. The application efficiency of all genes was examined in accordance with literature data [8]. qRT-PCR for gene expression was performed using Mastercycler® ep realplex4 (Eppendorf AG, Hamburg, Germany), and Power SYBR Green Master Mix® (Applied Biosystems, Foster City, CA, USA). Each sample was analysed in total reaction volume 20 μL, consisting of 0.1 μg of cDNA, 10 μL of 2 X SYBR Green Master Mix® and 300 nM of forward and reverse primers. Three replicates of each sample were amplified. The $E^{(\Delta\Delta Ct)}$ method was used to calculate both the HO-1 mRNA relative expression levels [9]. The expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous control (housekeeping).

Analysis of strontium ranelate safety

Body mass, organ weight changes, and creatinine, alanine aminotransferase (AST), and aspartate aminotransferase (ALT) blood levels were evaluated 7 days after SR treatment. Blood samples were obtained from the orbital plexus for biochemical analysis (creatinine, ALT, AST, alkaline phosphatase - LabtestÒ, Lagoa Santa, Brazil). After euthanasia, the liver, kidney, and heart were excised and weighed, and any ulcerative lesions were quantified and macroscopically measured.

Statistical analysis

Parametric data were expressed as mean and standard deviation (M [SD]). One-way ANOVA followed by Bonferroni's test were used to compare means, and Kruskal-Wallis test followed by Dunn's test was used to compare medians. The normal distribution of PCR parameters was studied by Lilliefors (Kolmogorov-Smirnov [K-S]) test. Statistical differences determined if P < 0.05.

RESULTS
Effect of strontium ranelate on the alveolar bone loss

The nylon ligature on maxillary second molars caused significant periodontal bone loss, root exposition and furcation in comparison with the unchallenged group. SR-treatment (100 mg/kg) decreased (P < 0.05) bone loss preserving the periodontal architecture when compared with the periodontitis group (Figures 1 and 2).

Histopathological scores

The histopathological analysis depicted inflammatory infiltration, severe cementum breakdown, and periodontal bone loss on day 7. In SR-treated groups (100 mg/kg), less infiltration of inflammatory cells, alveolar process, and cementum maintenance occurred (Table 2 and Figure 3).
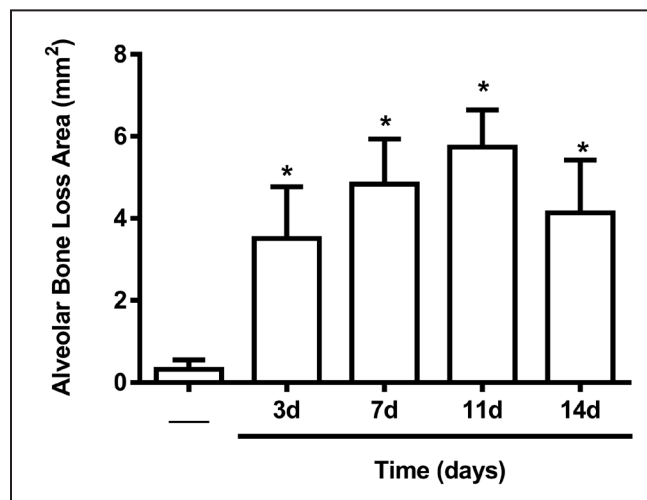

Figure 1. Morphometric analyses of the alveolar bone loss in maxillae until day 14 of periodontitis challenge.
*P < 0.05 versus unchallenged group.
Data are shown as the mean (SD) for each group of six rats (one-way ANOVA followed by Bonferroni's test).

Table 1. Category and description of primer sequences from both heme oxygenase-1 (HO-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), according to National Center for Biotechnology Information (NCBI)

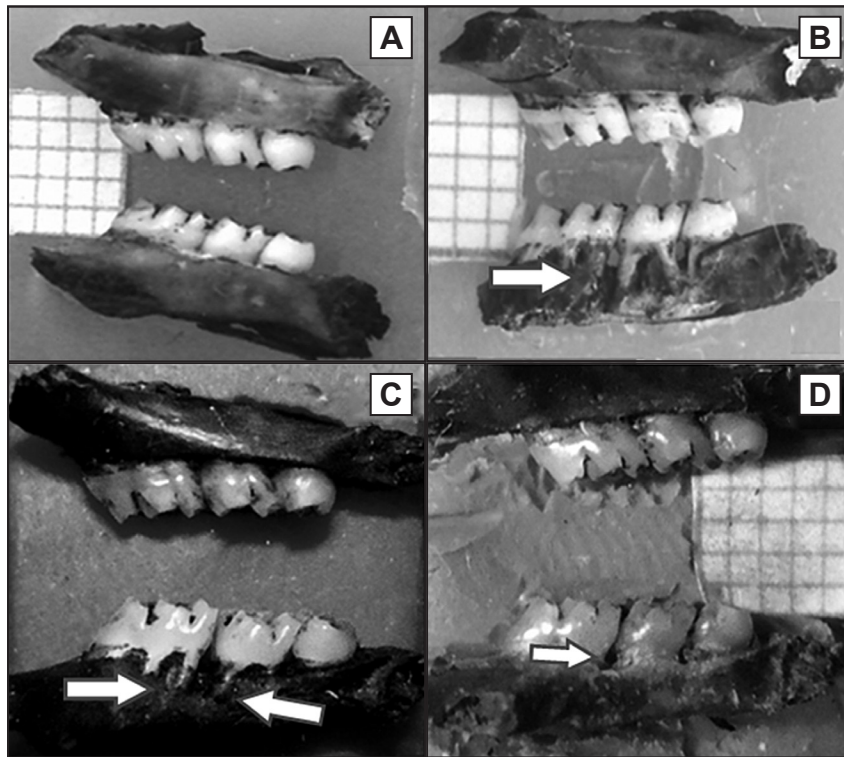| Symbol	Sequence	Access number	Amplicon size	Primer efficiency
HO-1	F 5' ACAGCATACGTAAAGCGTCTCCA-3' R 5'CATGGCCTTCTGCGCAATCTTCTT-3'	NM_012580.2	136	1.974
GAPDH	F5'-GGGGGCTCTCTGCTCCTCCC-3' R 5'-CGGCCAAATCCGTTCACACCG-3'	NM_017008.3	108	1.932

Figure 2. Macroscopic aspect of the hemi-maxillae from rats subjected to periodontitis and treated with strontium ranelate (20 or 100 mg/kg) for 7 days. Upper hemi-maxillae corresponds to the contralateral side used as negative control whilst lower hemi-maxillae corresponds to the following groups: A = maxilla from the unchallenged group; B = maxilla from rats 7 days after periodontitis; C and D = maxilla from strontium ranelate-treated (20 or 100 mg/kg) rats 7 days after periodontitis induction.
White arrows indicate the alveolar bone total area in maxillae (1% methylene blue staining).
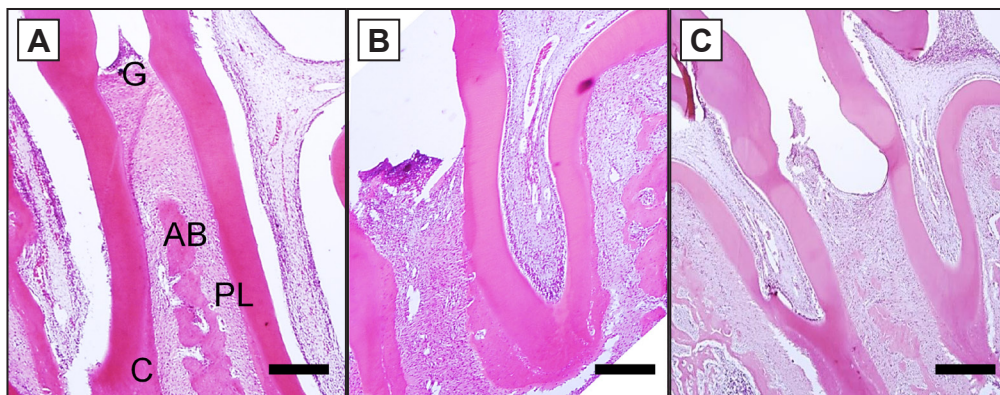
Figure 3. Representative histology of the periodontal tissues: A = rats not subjected to periodontitis (cementum [C], alveolar bone [AB], gingiva [G], and periodontal ligament [PL]); B = maxilla from rats 7 days after periodontitis and not treated; C = treated with strontium ranelate at 100 mg/kg (haematoxylin and eosin stain, original magnification x40).

Table 2. Effect of SR on histopathological analysis of rat maxillae subjected to periodontitis

Groups	Scores
	Median values (range)
Naive	0 (0 - 0)
NT	3 (2 - 3)[a]
SR	1 (1 - 2)[b]

[a] P < 0.05 compared with the naive group.
[b] P < 0.05 compared to NT group.
Data are reported as median scores (range) for six rats per group (Kruskal-Wallis and Dunn's tests).
SR = strontium ranelate; NT = non-treated.

Heme oxygenase-1 mRNA expression profile by quantitative real-time PCR

mRNA samples were examined before the cDNA synthesis for purity, integrity, and concentration. The absorbance ratio 260/280 was between 1.8 and 2, which indicates that all samples were proteins or phenol free. Efficiency values obtained for qRT-PCR amplification of three genes were 1.932 (GAPDH) and 1.974 (HO-1), respectively, which indicates that amplification efficiency was near the theoretical optimum level of 2 (Table 1) [9].
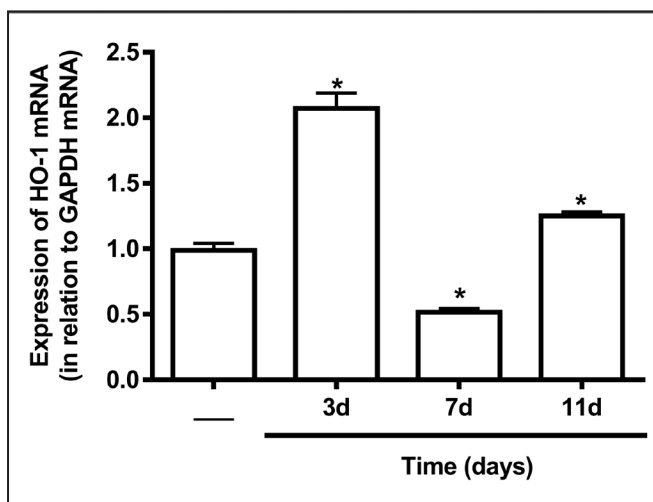
Figure 4. Time-course of HO-1 mRNA expression in gingival tissues after periodontitis challenge.
*$P < 0.05$ versus unchallenged group.
Data are shown as the mean (SD) for each group of six rats (one-way ANOVA followed by Bonferroni's test).

To evaluate the time-course of HO-1 mRNA expression in gingival tissues after periodontitis challenge, rats were euthanized under anaesthesia on days 3, 7, and 11 after periodontitis. The qRT-PCR analysis using on days 3, 7 or 11 revealed that HO-1 mRNA expression achieved its lowest levels on day 7 compared with the unchallenged group (Figure 4). Further, SR (100 mg/kg) up-regulated ($P < 0.05$) the HO-1 expression levels in the gingival tissues compared with the challenged vehicle-treated group (Figure 5).

Strontium ranelate safety

Repeated administration of SR (100 mg/kg) over 7 consecutive days produced no sign of toxicity. The overall body mass and the wet weights of the liver, kidneys, and heart were normal (not shown). Serum levels of creatinine and enzymatic markers of hepatic function (ALT, AST, and alkaline phosphatase) did not differ from their respective controls (Table 3).

DISCUSSION

This study demonstrated the HO-1 involvement in periodontitis using a rat ligature-induced periodontitis. Some authors showed *in vitro* assays that HO-1 can be a potential target for the treatment of periodontitis [10]. To our knowledge, this is the first report that attempts to identify *in vivo* the participation of HO-1 in periodontitis. Further, our study reported the effects of SR on the alveolar bone loss, finding evidence that SR upregulates HO-1 gene expression in the gingival
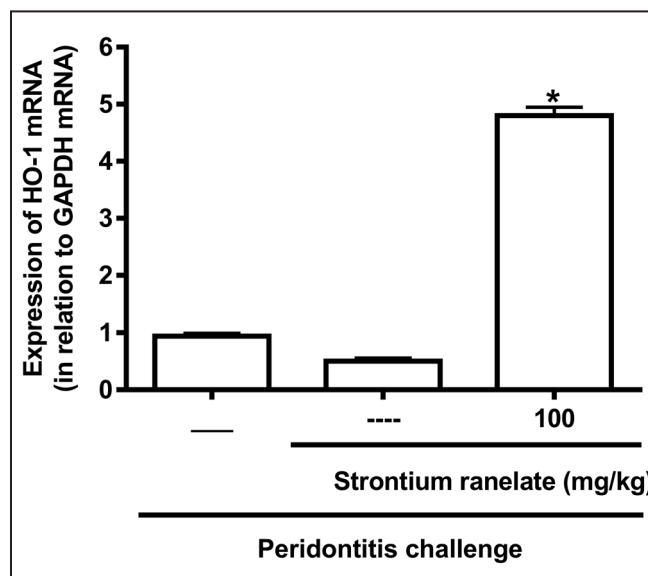

Figure 5. Effects of strontium ranelate on the relative expression of heme oxygenase-1 mRNA levels in gingival tissues.
*$P < 0.05$ versus challenged vehicle-treated group (----).
Data are shown as the mean (SD) for each group of six rats (one-way ANOVA followed by Bonferroni's test).

Table 3. Blood levels of the biochemical parameters of rats subjected to periodontitis and treated with strontium ranelate (SR)

Parameters	Non-treated	SR (100 mg/kg)
	Mean (SD)	Mean (SD)
Creatinine (mg/dL)	0.47 (0.03)	0.55 (0.04)
Alanine aminotransferase (U/L)	134.5 (5.88)	137.4 (12.6)
Aspartate aminotransferase (U/L)	417.5 (1.56)	429.5 (29.3)
Alkaline phosphatase (U/L)	417.5 (26.6)	429.5 (29.3)

tissues. HO-1 contributes to a proper function of the antioxidant defence system and downregulates cellular activation and production of inflammatory mediators, thus, modulating the inflammatory process [3].

A large amount of studies have reported that the HO-1 expression and its metabolites carbon monoxide and biliverdin exert anti-inflammatory response. In this regard, our research showed that HO-1 inhibition favours inflammatory events [11]. Similarly, HO-1 activation resulted in a reduction of cell migration and pro-inflammatory mediators release [3].

The present study demonstrates that the HO-1 mRNA expression achieved its lowest levels on day 7. It has been shown that alveolar bone loss begins in 3 days after periodontitis induction and reaches maximum levels from days 7 to 11 [2]. Considering the evidence in favour of the HO-1 mediated anti-inflammatory effects, we assume that the alveolar bone loss on days 7 and 11 might be associated with low levels of HO-1 mRNA expression. The literature has also shown a relationship between HO-1 expression and receptor activator of nuclear factor-kappa β ligand

(RANKL)/RANK/osteoprotegerin (OPG) signalling pathway. RANKL/RANK signalling regulates the formation of multinucleated osteoclasts from their precursors as well as their activation and survival in normal bone remodelling and in a variety of pathologic conditions. OPG protects the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. Thus, RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity [12]. Additionally, this is the first report demonstrating the HO-1 signalling during periodontitis *in vivo*.

SR effectively reduced bone loss, increased bone mass, and bone resistance [13-14]. Albeit the SR mechanism of action is not completely understood, there is evidence showing that it acts on the bone surface, promoting the osteoblastic differentiation via stimulation of calcium sensor receptor, inhibition of osteoclastic differentiation by RANKL suppression, and promotion of osteoprotegerin (OPG) activity. It was also demonstrated that SR can reduce osteoclast numbers in rats with periodontitis [15], which can support the preventing effect of SR on alveolar bone seen in this study.

Our research showed that SR also up-regulated HO-1 mRNA expression in gingival tissues. Several reports demonstrated in murine macrophage-like RAW264.7 cells stimulated with LPS isolated from *Prevotella intermedia* and *Porphyromonas gingivalis* that the anti-inflammatory activity of various promising therapeutic agents in periodontitis occurs via HO-1 expression [16-18].

Phase III trials showed that SR was tolerated, and its safety profile was like that in the youngest participants. Even rarely occurring, SR can induce drug rash with eosinophilia and systemic symptoms (DRESS) [19]. Herein, we also evaluated the safety of SR administration and observed that SR at 100 mg/kg would be safe when administered to rats over seven consecutive days.

CONCLUSIONS

In summary, the present study demonstrated that the strontium ranelate efficacy in ligature-induced periodontitis in rats is partly dependent on the heme oxygenase-1 up-regulation. Therefore, validating novel therapeutics is very encouraging, broadening our current pharmaceutical choices.

ACKNOWLEDGMENTS AND DISCLOSURE STATEMENTS

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