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Impact of *Porphyromonas gingivalis* inoculation on ligature-induced alveolar bone loss. A pilot study in rats

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Background and Objective: Periodontitis is a polymicrobial infection characterized by the loss of connective tissue attachment, periodontal ligament and alveolar bone. The aim of this study was to evaluate the impact of *Porphyromonas gingivalis* inoculation on the ligature-induced alveolar bone loss (ABL) model in rats.

Material and Methods: Forty male Wistar rats were randomly assigned to the following groups: G1, control ($n = 10$); G2, ligature-induced ABL ($n = 15$); and G3, ligature-induced ABL + *P. gingivalis* inoculation ($n = 15$). Rats in G2 and G3 were killed 15, 21 and 30 d after ligature placement, and the following parameters were assessed: microbiological load; ABL; and interleukin (IL)-1β (*Il1beta*)/*Il1ra*, *Il6*/*Il10* and *Rankl*/osteoprotegerin (*Opg*) mRNA ratios in the gingival tissues, as determined by quantitative PCR.

Results: Microbiological analyses demonstrated that rats in G1, G2 and G3 were positive for the presence of bacteria (determined using PCR amplification of the *16S* gene), but that only the treatment sites of rats in G3 were positive for *P. gingivalis* at all time-points investigated. Histometrically, significant bone loss ($p < 0.001$) was observed for both ligated groups (G2 and G3) compared with the nonligated group (G1), with higher ABL observed for G2 at all the experimental time-points. Furthermore, gene-expression analysis demonstrated that the presence of *P. gingivalis* in the dentogingival area significantly decreased the *Il1*β/*Il1ra*, *Il6*/*Il10* and *Rankl*/*Opg* mRNA ratios compared with ligature alone.

Conclusion: Within the limits of this pilot study, it was concluded that inoculation of *P. gingivalis* affected the ligature-induced ABL model by the induction of an anti-inflammatory and antiresorptive host response.

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Periodontal diseases are inflammatory responses triggered specifically by selected microorganisms, such as periodontopathic bacteria, that accumulate in and around the gingival crevice. Animal models offer an opportunity to investigate bacterial etiology and the pathological process of infectious diseases (1). In order to promote alveolar bone loss (ABL) in experimental animals, three methods have been most commonly used in rats: (i) feeding animals with a supragingival plaque-promoting soft diet in order to stimulate the natural accumulation of supragingival plaque on the dentogingival area (2); (ii) inoculation of periodontopathogens that have the

features of a single infection, an occurrence that is somewhat different from infection in humans (3); and (iii) placement of cotton ligatures in the dentogingival area, which enables subgingival microorganisms to accumulate, causing a polymicrobial infection (4–7). The ligature model is by far the most commonly used method to experimentally induce an inflammatory-related alveolar bone loss and, even though the principle is to allow bacterial colonization, very few studies have been designed using this model in a microbiological perspective (8). Rovin *et al*., (8) evaluated the influence of bacteria and irritation in the initiation of periodontal disease in germ-free and conventional rats, and provided evidence that microorganisms were necessary for the occurrence of inflammatory periodontal disease. Furthermore, Duarte *et al*. (7) demonstrated that the composition of the biofilm that accumulated around ligatures in rats, evaluated by checkerboard DNA–DNA hybridization using probes made from human oral bacterial species, includes various bacterial species commonly observed in humans.

Among periodontopathic bacteria, *Porphyromonas gingivalis*, a black-pigmented gram-negative anaerobic rod, has been implicated as a major pathogen of adult periodontitis (9). The pathogenicity of *P. gingivalis* has been ascribed to some cellular and extracellular substances of the organism, which include fimbriae, hemagglutinin, capsules, lipopolysaccharide, serine phosphatase enzyme (SerB), outer membrane vesicles and organic metabolites (such as butyric acid), along with various enzymes such as protease, collagenase, gelatinase and hyaluronidase (10–12). Moreover, *P. gingivalis* has been shown to induce *in vitro* bone resorption in rat calvaria cultures (13).

To date, the interaction between *P. gingivalis* colonization and the host response *in vivo* has been poorly explored owing to the lack of a suitable system. Most of the rodent models used to investigate the role of *P. gingivalis* in the pathogenesis of periodontitis *in vivo* have involved oral inoculation of this microorganism as a single infection. However, a growing body of evidence supports the notion that interactions between different bacterial species are important factors in periodontal disease pathogenesis. Here, it was hypothesized that *P. gingivalis* inoculation would affect the host response to ligature placement in the dentogingival area in rats. The objective was to determine the effect of the association of ligature and *P. gingivalis* on the rat periodontal tissue, at the molecular and histological levels.

Material and methods

Animals

Forty, 4-week-old male Wistar rats were used in the present study. The sample size was estimated using data from previous studies published by our group and calculated using a power of 0.8 and $\alpha = 0.05$ (14). The animals were acclimatized to the housing conditions during the course of 4 wk, with a 12-h light/12-h dark cycle applied. The animals were housed, five per cage, at a permanent temperature of 21°C, and only animals of the same group were housed together. Standard rat chow pellets and water were available *ad libitum*. The animals were randomly assigned to of one of the following experimental groups: G1, control ($n = 10$); G2, ligature-induced ABL ($n = 15$); and G3, ligature-induced ABL plus *P. gingivalis* inoculation ($n = 15$). The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee (#1573-1/2008).

Bacterial strain

P. gingivalis strain W83 was stored frozen at −20°C. For experiments, bacterial cells were cultured at 37°C on blood agar in an oxygen-free atmosphere (80% nitrogen, 10% carbon dioxide and 10% hydrogen) (Mini-Macs Anaerobic Workstation; Don Whitley Scientific, Shipley, UK). After 72 h of growth on blood agar, *P. gingivalis* colonies were selected, inoculated into 25 mL of Brain-Heart infusion (BHI) broth supplemented with 1 mg/L of menadione and 10 mg/L of hemin, and statically cultured until an optical density of 0.050 (at 550 nm) was reached. The culture was maintained under the same growth conditions as described for the culture of *P. gingivalis* on blood agar plates. After 18 h of growth (i.e. during the log phase of growth of *P. gingivalis*), 1×10^9 cells were centrifuged, washed rapidly with a solution of sterile reduced phosphate-buffered saline and resuspended in 1 mL of 2% carboxymethylcellulose solution in sterile phosphate-buffered saline. This inoculum was used immediately after preparation.

Experimental procedures

General anesthesia was obtained by intramuscular administration of ketamine (50 mg/kg) (Francotar; Vibrac Laboratories, Roseira, Brazil). In a parallel single-blinded study, on day 0, cotton ligatures were placed in the dentogingival area of both mandibular first molars of each animal to induce experimental ABL, except for the animals in G1, which were treated with a sham procedure consisting of the same procedures used in the other groups (e.g. animal handling and anesthesia administration), except for ligature placement. The ligatures were kept in place during the entire experimental period and served as a retention device for oral microorganisms. The rats in G3 also received inoculations of *P. gingivalis* on days 0 (immediately after ligature placement), 1 and 3. The inoculation was performed with the aid of micropipettes and a sterile pointer: 100-μL volumes of sterile 2% carboxymethylcellulose sterile solution, containing 1×10^9 colonies of *P. gingivalis*, were inoculated. Rats in G1 and G2, without inoculation, received 100 μL of sterile carboxymethylcellulose solution alone. In addition to this inoculation protocol, two Petri dishes containing blood agar saturated with *P. gingivalis* colonies were placed in the cages of inoculated animals. At the time of death, an inoculation smear was performed with the aid of sterile cotton stick to collected a full-mouth swab, and the bacterial load and *P. gingivalis* load were assessed by PCR using primers for 16S and *P. gingivalis* DNA, respectively (15,16). In G2 and

G3, the animals were subdivided into three subgroups, of five animals per group, which were killed 15, 21 and 30 d after ligature placement and *P. gingivalis* inoculation.

Sample collection

The animals were killed by decapitation. At the end of each experimental period (15, 21 and 30 d), full-mouth swabs were individually collected from all the experimental groups and stored at −20°C in 500 μL of phosphate-buffered saline. Also, the ligatures or supragingival biofilm were collected from ligated and nonligated sites, respectively, and stored individually at −20°C in 500 μL of phosphate-buffered saline. Before use, samples were vortexed for 30s. This final solution was stored at −20°C for subsequent analysis. In addition, the marginal gingival tissues around ligated and nonligated mandibular first molars were harvested (approximately 10 mg, a pooled sample of both lower molars), rinsed with cold sterile saline solution and stored in RNAlater® (Ambion Inc., Austin, TX, USA) at −70°C for future gene-expression analyses. Finally, the jaws were removed and fixed in 4% neutral formalin for 48 h for histological processing in order to determine the area of bone loss in ligated and nonligated teeth for all the experimental groups.

Histological procedures

The fixed jaws were demineralized in a 10% EDTA-buffered solution (pH 7.0) for 45 d at room temperature. Paraffin-embedded serial sections of 6 μm thickness were obtained in a mesial–distal direction and stained with hematoxylin and eosin. After excluding the first and the last sections in which the furcation region was evident, 10 equally distant sections of each tooth were selected for histometric analysis. Using an image-analysis system (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA), the area of bone loss in the furcation region (ABL) or the area of periodontal ligament (PLA) was histometrically determined by a blinded and calibrated examiner (intraclass correlation = 0.92) for ligated and nonligated teeth. The interradicular ABL/PLA area was defined by the area of connective tissue limited by the tooth and bone crest surfaces on ligated and nonligated teeth within the furcation limits.

DNA extraction

The samples from swabs and ligatures that were stored at −20°C were thawed and incubated in a boiling water bath for 10 min (17). After cell lysis, the samples were centrifuged (at 12,000 *g* for 10 s) and the supernatant containing DNA was transferred to a new tube. The DNA samples were stored at −20°C and used subsequently for PCR, to assess total and *P. gingivalis* bacterial loads.

PCR analysis

PCR was performed on DNA samples from swabs and ligatures, using the universal primer, 16S, to detect the presence of nonspecific bacterial DNA species and with *P. gingivalis* primer to detect the presence of *P. gingivalis* (Table 1). The PCR reaction was carried out in a reagent mix containing 2.5 μL of buffer solution (10× Reaction Buffer *Taq* Polymerase; Invitrogen®-Life Technology of Brazil, São Paulo, SP, Brazil), 1.25 μL of $MgCl_2$, 0.5 μL of deoxyribonucleotide triphosphate (dNTP), 0.75 μL of each primer, 0.125 μL of *Taq* DNA polymerase (5 U/μL; Invitrogen®) and ultrapure water to bring the reaction volume to 20 μL. For the reaction with the primer for *P. gingivalis,* an aliquot of 5 μL of each DNA sample from swabs and ligature was added to microtubes with final volume of 200 μL. For the reaction with the universal primer, 16S, an aliquot of 3 μL of each DNA sample from swabs and ligature + 2 μL of water was added to microtubes with 200 μL each. The primers used in this study are shown in Table 1.

RNA extraction

Total RNA from gingival biopsies was extracted using TRIZOL reagent (Gibco BRL, Life Technologies, Rockville, MD, USA) according to the manufacturer's recommendation. The RNA pellet was resuspended in diethylpyrocarbonate-treated water and stored at −70°C. The RNA concentration was determined from the absorbance of the diethylpyrocarbonate-treated water/RNA solution using a Biophotometer (Eppendorf AG, Hamburg, Germany). Samples were quantitatively assessed for the expression of the genes *Il1beta*, *Il1ra*, *Il6*, *Il10*, *Rankl* and *Opg* in all groups, and the following ratios were obtained: *Il1beta*/*Il1ra*, *Il6*/*Il10* and *Rankl*/*Opg*.

Real-time PCR reactions

Reverse transcription — Total RNA was treated with DNase (Turbo DNA-free®; Ambion Inc., Austin, TX, USA) and 1 μg was used for the synthesis of complementary DNA (cDNA). The reaction was carried out using the first-strand cDNA synthesis kit (LightCycler®; Roche Diagnostics Co., Indianapolis, IN, USA), following the manufacturer's recommendations.

Primer design — Primers were designed using probe design software (Roche Diagnostics GmbH, Mannheim, Germany), the details of which are described in Table 2.

Real-time PCR reactions — Real-time PCR was performed (LightCycler®) using the SYBR Green system (Roche Diagnostics Co.). The reaction product was quantified using software (Roche Diagnostics GmbH) with glyceraldehyde-3-phosphate dehydrogenase

Table 1. Primers used for PCR amplification of regions of 16S DNA and *Porphyromonas gingivalis* DNA

Sequence 5′→3′	Target gene	Reference
ACGGCTACCTTGTTACGACTT AGAGTTTGATCCTGGCTCAG	16S DNA	(15)
AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	*P. gingivalis* DNA	(16)

Table 2. Primer details for glyceraldehyde-3-phosphate dehydrogenase (Gapdh), interleukin (IL)-1β (Il1beta), Illra, Il6, Il10, Rankl and osteoprotegerin (Opg) used in the quantitative PCR reaction

Genes	Sequence 5′→3′	Amplification profile Temperature (°C)/time (s)
Gapdh	GCCTTCTCTTCTTGACAAAGTG TGGTGATGGGTTTCCCG	51/9; 72/3
Illbeta	TGTGGATCCCAAACAATACCC TAGGAAGACAGGTCTGTGCTC	55/3; 72/6
Illra	CCTGCAAGATGCAAGCC AACCACATTCCGAAAGTCCAATAGG	55/3; 72/6
Il6	GAGAAGTTAGAGTCACAGAAGGAG TTTAGATACCCATCGACAGGATATATT	55/5; 72/8
Il10	CCTCTGGATACAGCTGCGA TGTCACGTAGGCTTCTATGC	55/3; 72/6
Rankl	AGCGCTTCTCAGGAGTT TACCAAGAGGACAGACTGACTTTA	55/5; 72/6
Opg	AGTGAAGATAAGCTGCTTATAGTTAGG GCTGGAGGATCTTCATTCCC	55/5; 72/6

(*Gapdh*) as the reference gene. Experiments were run twice independently and water was used as the negative control.

Statistical analysis

Mean values and standard deviation of the ABL/PLA and mRNA levels were obtained for intragroup and intergroup analyses using the one-way analysis of variance ($\alpha = 0.05$). If statistical difference was detected, the Student–Newman–Keuls method was used to identify differences against the control group. The parametric Student's *t*-test was used to statistically ($\alpha = 0.05$) compare histometric and gene-expression data from G2 vs. G3.

Results

Histometric findings

The effect of ligature placement and inoculation of *P. gingivalis* on ABL in the inter-radicular area of the furcation region was assessed. Ligature placement (G2) and ligature placement plus *P. gingivalis* inoculation (G3) resulted in significantly higher ABL ($p < 0.001$) compared with nonligated sites (G1). In addition, intragroup analysis showed no significant differences in G2, over time, whereas in G3, significantly higher levels of ABL were found at the 30 d time-point, compared with days 15 and 21 ($p < 0.001$). Intergroup analysis further demonstrated higher ABL in G2 rats at all experimental time-points, compared with G3 rats ($p < 0.001$). Figures 1 and 2 illustrate the histological findings.

Analysis of bacterial loads

A positive PCR reaction with the 16S primer ensures that sufficient amounts of bacterial DNA are present in the sample for molecular detection. All swabs (G1/G2/G3) and ligature (G2/G3) samples were PCR-positive with the 16S primer, indicating not only successful colonization, but correct DNA extraction and storage. Data analysis of *P. gingivalis*-specific reactions further demonstrated that neither G1 nor G2 were colonized by *P. gingivalis* in the assessed sites, whereas the samples from G3 were positive for *P. gingivalis* at all the experimental time-points investigated, indicating that the inoculation procedure efficiently promoted *P. gingivalis* colonization. PCR reactions were performed twice, with similar results obtained each time, and representative findings are illustrated in Fig. 3.

Gene-expression analysis

The results for the mRNA levels in the gingival tissues around the ligated and nonligated teeth, in G1, G2 and G3 were assessed for the ratios *Il1beta*/*Il1ra*, *Il6*/*Il10* and *Rankl*/*Opg*, and are described in the following sections.

Il1beta/*Il1ra* — Intragroup analysis showed that, in G2, the highest *Il1beta*/*Il1ra* ratio was found 21 d after ligature placement ($p < 0.05$), whereas no

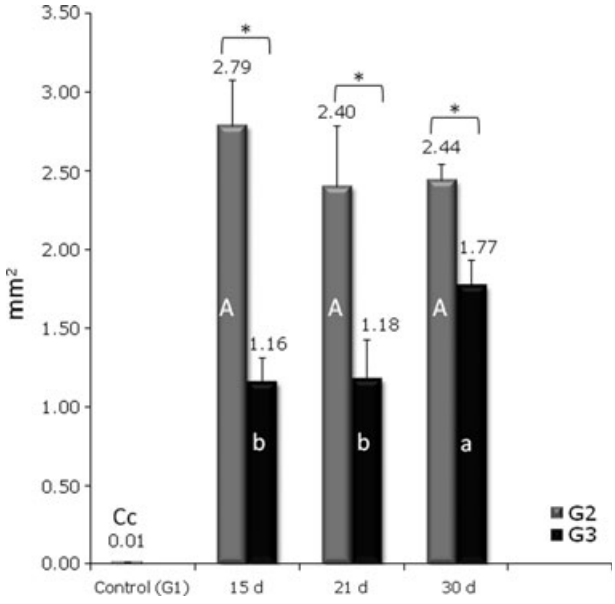

Fig. 1. Bar graph illustrating the histometric findings around the first mandibular molar in all the experimental groups. Capital and noncapital letters represent multigroup statistical comparison, using the one-way analysis of variance, from group 2 (G2; experimental periodontitis) and group 3 (G3; experimental periodontitis + *P. gingivalis* inoculation) in relation to group 1 (G1; control). *Statistical significance (Student's *t*-test; G2 vs. G3) at the same experimental time-point.

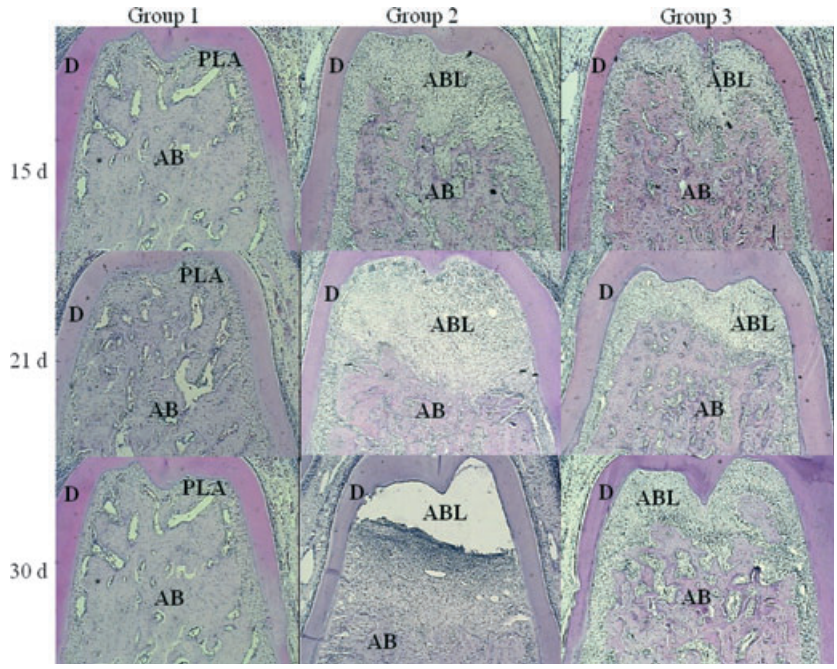

Fig. 2. Representative photomicrographs illustrating the periodontal area in the furcation region of the mandibular molars that was quantitatively assessed. AB, alveolar bone; ABL, alveolar bone loss; D, dentin; PLA, periodontal ligament area.

significant differences were observed in G3 over time. Moreover, intergroup analysis demonstrated that *P. gingivalis* inoculation significantly decreased the *Il1beta*/*Il1ra* ratio on days 15 and 21 after ligature placement and inoculation with *P. gingivalis* ($p < 0.05$) (Fig. 4). Data analysis further demonstrated that inoculation with *P. gingivalis* promoted an increase in the anti-inflammatory cytokine, IL-1ra (data not shown).

Il6/*Il10* — Intragroup analysis demonstrated that the induction of periodontal disease promoted a time-dependent increase in the *Il6*/*Il10* mRNA ratio in the gingival tissues, with the highest values obtained 21 and 30 d after placing the ligatures, compared with the control group ($p < 0.05$). Additionally, for G3, data analysis demonstrated a significant increase in the *Il6*/*Il10* ratio 15 and 21 d after ligature placement ($p < 0.05$), with values similar to the unligated group at 30 d ($p > 0.05$). When the Student's *t*-test was used to compare G2 and G3 data, inoculation with *P. gingivalis* resulted in a trend towards a reduced *Il6*/*Il10* ratio, with significant differences at days 15 and 30 ($p > 0.05$) (Fig. 5).

Rankl/*Opg* — With the aim of investigating the impact of *P. gingivalis* inoculation on the *Rankl*/*Opg* osteoclastogenesis system in the ligature-induced ABL model, we assessed *Rankl*/*Opg* mRNA levels in the gingival tissues around ligated and nonligated sites. As expected, ligature placement led to a time-dependent increase in the *Rankl*/*Opg* ratio, favoring a pro-osteoclastogenic response, with the highest levels found 30 d after ligature placement ($p < 0.05$). Surprisingly, data analysis further demonstrated that *P. gingivalis* inoculation switched the trend towards an antiresorptive response by means of a significant, time-dependent reduction of the *Rankl*/*Opg* ratio, with the lowest values observed 21 and 30 d after ligature placement ($p > 0.05$) (Fig. 6). Individual analysis of the data demonstrated that increased *Opg* mRNA levels were responsible for the inverted ratios (data not shown).

Discussion

The trigger for the initiation of periodontal disease is the presence of

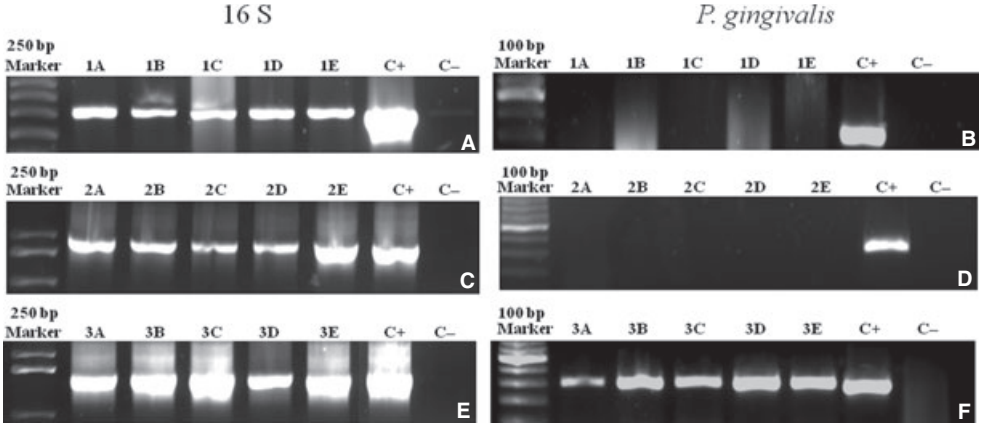

Fig. 3. Representative 1.6% agarose gels showing the results of PCR amplification of DNA extracted from full-mouth and dentogingival SWABs [group 1 (G1:1A-1E)] and ligatures [group 2 (G2:2A-2E) and group 3 (G3:3A-3E)] of five animals in each group (C+, positive control; C–, negative control). In general, the reactions were positive for the presence of bacteria (the *16S* gene) in all the groups, whereas only G3 was positive for *Porphyromonas* gingivalis.

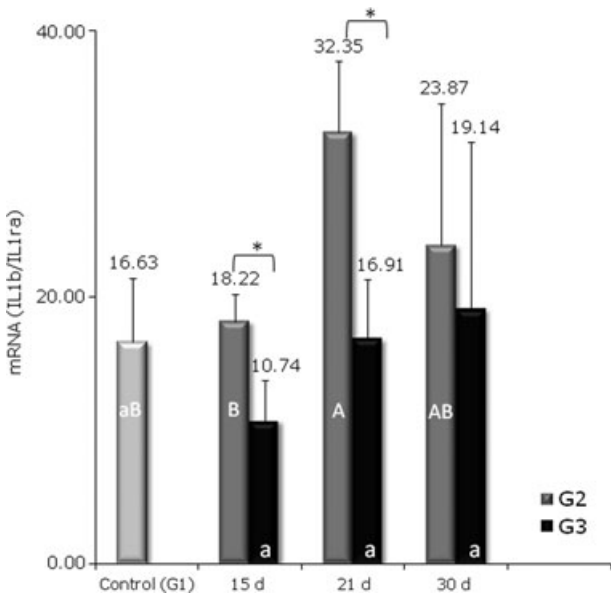

Fig. 4. Bar graph illustrating the interleukin (IL)-1β (*Il1beta*)/*Il1ra* mRNA ratio in the gingival tissues harvested from ligated and nonligated sites around the first mandibular molar in all experimental groups. Capital and noncapital letters represent multigroup statistical comparisons, analyzed using the one-way analysis of variance, from group 2 (G2, experimental periodontitis) and group 3 (G3, experimental periodontitis + *P. gingivalis* inoculation) in relation to group 1 (G1, control), respectively. *Statistical significance, determined using the Student's *t*-test (G2 vs. G3), at the same experimental time-point.

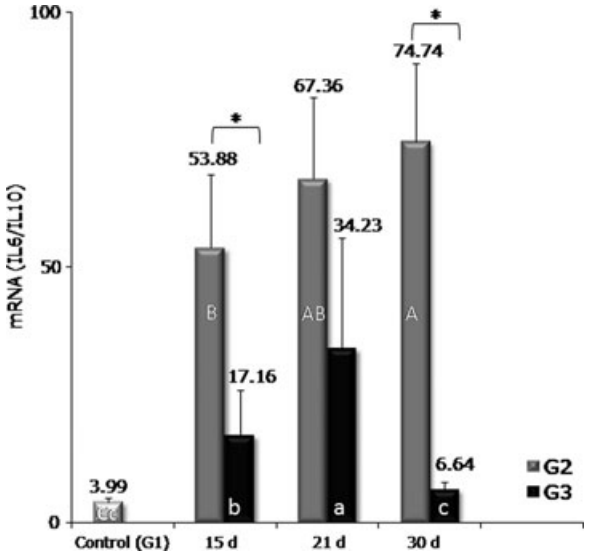

Fig. 5. Bar graph illustrating the interleukin (IL)-6 (*Il6*)/*Il10* mRNA ratio in the gingival tissues harvested from ligated and nonligated sites around the first mandibular molar in all experimental groups. Capital and noncapital letters represent multigroup statistical comparisons, analyzed using the one-way analysis of variance, from group 2 (G2, experimental periodontitis) and group 3 (G3, experimental periodontitis + *P. gingivalis* inoculation) in relation to group 1 (G1, control), respectively. *Statistical significance, determined using the Student's *t*-test (G2 vs. G3), at the same experimental time-point.

complex microbial biofilms (18) that colonize the dentogingival region through specific adherence interactions and accumulation, as a result of architectural changes in the sulcus (i.e. attachment loss and pocket formation). The ligature model has been extensively used over the years, and in rats, although this approach has been proven to induce inflammatory-mediated alveolar bone loss, little attention has been paid to the microbiological aspects of this model. The available literature has reported that *P. gingivalis* colonization, a key factor in the human disease, is not abundantly present in the ligature model in rats (7). Thus, in the present study, it was hypothesized that *P. gingivalis* inoculation, in association with ligature placement (known to lead to a polymicrobial colonization), would affect the host response compared with ligature alone. Data analysis demonstrated that ABL occurred as a result of the presence of ligature around the tooth and, as expected, a more pro-inflammatory and proresorptive environment was found in ligature-treated gingival tissues compared with nonligated sites. Surprisingly, *P. gingivalis* inoculation promoted a switch in the host response towards the anti-inflammatory and anti-resorptive scenario, which may have been responsible for the lower level of ABL, compared with the ligated noninoculated group.

Periodontal disease is a multifactorial, polymicrobial infectious disease characterized by the loss of connective tissue attachment, periodontal ligament and alveolar bone (19). The subgingival biofilm, the major etiologic factor for periodontal disease, harbors up to 500 bacterial species (20), but only a small percentage of these have been shown to be involved in the etiology of periodontal disease (21). Socransky *et al*. (22) examined the relationships among bacterial species in subgingival plaque samples and related the complexes to clinical parameters of periodontal disease. They observed that the complex formed by *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* (known as the red complex), which appears later in biofilm development,

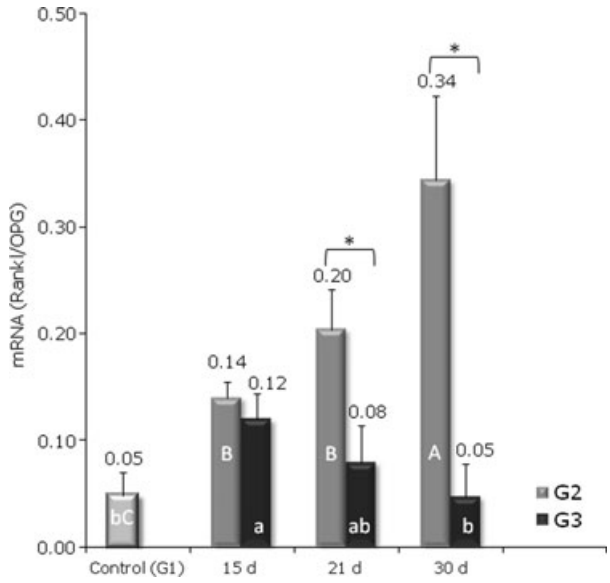

Fig. 6. Bar graph illustrating the *Rankl*/osteoprotegerin (*Opg*) mRNA ratio in the gingival tissues harvested from ligated and nonligated sites around the first mandibular molar in all experimental groups. Capital and noncapital letters represent multigroup statistical comparisons, analyzed using the one-way analysis of variance, from group 2 (G2, experimental periodontitis) and group 3 (G3, experimental periodontitis + *Porphyromonas gingivalis* inoculation) in relation to group 1 (G1, control), respectively. *Statistical significance, determined using the Student's *t*-test (G2 vs. G3), at the same experimental time-point.

was strikingly related to clinical measurements of periodontal disease, particularly pocket depth and bleeding on probing. These bacteria have been suggested to produce a number of virulence factors, as well as to participate in synergistic interactions with other constituents of the biofilm during periodontal infections (23). *In vitro* and *in vivo* studies have been used to determine the mechanisms by which *P. gingivalis* contributes to the destruction of periodontal tissues and, in general, it has been demonstrated that *P. gingivalis* lipopolysaccharide regulates the synthesis and release of cytokines associated with marked osteolytic properties in different models (24–29). However, in the context of periodontal disease, *in vivo* data are limited to the few studies that have used the single infection model. In the present study the hypothesis that *P. gingivalis* inoculation in association with ligature placement in the dentogingival area would affect the host response to ligature alone was confirmed. Additionally, in contrast to previous reports where *P. gingivalis* was used as a single infection model to induce ABL, the findings of the present study demonstrated that the presence of *P. gingivalis*, in association with ligature, leads to a less catabolic environment.

Several methods have been presented for introducing suspected periodontal pathogens into the oral cavity of experimental animals, and a relatively large number of bacteria have been successfully used (30). To our knowledge, this is the first study to include inoculation of *P. gingivalis* in conjunction with ligature placement as an attempt to produce a more polymicrobial-like infection in the presence of a high *P. gingivalis* load, in order to better understand the role that *P. gingivalis* plays in the pathogenesis of periodontal disease. Microbiological analysis demonstrated that *P. gingivalis* inoculation was successfully achieved at all time-points investigated, and that *P. gingivalis* was never detected in noninoculated ligated or nonligated sites (G1 and G2, respectively). Therefore, we assume that the presence of *P. gingivalis* in all animals of G3 was the most relevant differential aspect between the experimental groups, allowing us to investigate its implications.

In the gingival tissues harvested from the dentogingival area around ligated and nonligated teeth, the mRNA levels of key factors modulating periodontal disease were assessed in an attempt to determine the impact of *P. gingivalis* on the host response to ligature placement. It was shown that the presence of ligature and ligature plus *P. gingivalis* may have modulated the expression of these important factors, including *Il1beta*, *Il6*, *Il10*, *Il1ra*, *Rankl* and *Opg*. Data analysis demonstrated that ligature plus *P. gingivalis* (G3) resulted in increased local mRNA levels of anti-inflammatory and antiresorptive factors, when compared to the sites with periodontal disease induction only by ligature placement (G2). In contrast, the presence of only ligature (G2) favored a pro-inflammatory and proresorptive response, as demonstrated by increased *Il1beta*/*Il1ra*, *Il6*/*Il10* and *Rankl*/*Opg* ratios in the gingival tissues harvested from ligated sites vs. nonligated sites. Additionally, histomorphometric analysis demonstrated that ABL was significantly higher in the groups that received ligatures only, whereas *P. gingivalis* inoculation resulted in a decrease in the rate of ABL, possibly owing to a less catabolic type of response, as evidenced by the gene-expression analysis.

Within the limits of the methods used in the present study, it was concluded that the addition of *P. gingivalis* to the ligature placed in the dentogingival area (the classic ligature-induced ABL model) significantly modulated the host response towards a protective response. However, within the constraints of the present study it was not possible to determine the mechanisms by which *P. gingivalis* inoculation resulted in lower alveolar bone destruction compared to the group with ligature alone, other than to identify inclusion of *P. gingivalis* as the parameter associated with a less catabolic type of response. Some studies have suggested that co-aggregation of multiple different bacterial species is important and may guide the impact of bacterial infection on the periodontal tissues (4,7,31). With this in mind, we speculate that the bacterial composition of the biofilm present in the ligatures may have played a critical role

in modulating the host response to *P. gingivalis* inoculation. Further studies are necessary to explore these findings in greater detail, specifically to identify and determine the composition of the biofilm formed around ligatures and to identify the microorganisms that interact with *P. gingivalis* and the type of interactions, in an attempt to elucidate the mechanisms by which *P. gingivalis* modulates the host response *in vivo*.

References

1. Oz HS, Puleo DA. Animal models for periodontal disease. *J Biomed Biotechnol* 2011, (in press).
2. Tani-Ishii N, Minamida G, Saitoh D *et al*. Inhibitory effects of incadronate on the progression of rat experimental periodontitis by *Porphyromonas gingivalis* infection. *J Periodontol* 2003;**74:**603–609.
3. Ranney RR. Classification of periodontal diseases. *Periodontol 2000* 1993;**2:**13–25.
4. Kimura S, Nagai A, Onitsuka T *et al*. Induction of periodontal experimental periodontitis in mice with *Porphyromonas gingivalis*-adhered ligature. *J Periodontol* 2000;**71:**1167–1173.
5. Nociti FH Jr, Nogueira-Filho GR, Primo MT *et al*. The influence of nicotine on the bone loss rate in ligature-induced periodontitis. A histometric study in rats. *J Periodontol* 2000;**71:**1460–1464.
6. Peruzzo DC, Benatti BB, Antunes IB *et al*. Chronic stress may modulate periodontal disease: a study in rats. *J Periodontol* 2008;**79:**697–704.
7. Duarte PM, Tezolin KR, Figueiredo LC, Feres M, Bastos MF. Microbial profile of ligature-induced periodontitis in rats. *Arch Oral Biol* 2010;**55:**142–147.
8. Rovin S, Costich ER, Gordon HA. The influence of bacteria and irritation in the initiation of periodontal disease germfree and conventional rats. *J Periodontal Res* 1966;**1:**193–204.
9. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. *Periodontol 2000* 1997;**14:**12–32.
10. Bainbridge BW, Coats SR, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide display functionally diverse interactions with the innate host defense system. *Ann Periodontol* 2002;**7:**29–37.
11. Yoshimura F, Murakami Y, Nishikawa K, Hasegawa Y, Kawaminami S. Surface components of *Porphyromonas gingivalis*. *J Periodontal Res* 2009;**44:**1–12.
12. Bainbridge B, Verma RK, Eastman C *et al*. Role of *Porphyromonas gingivalis* phosphoserine phosphatase enzyme SerB in inflammation, immune response, and induction of alveolar bone resorption in rats. *Infect Immun* 2010;**78:**4560–4569.
13. Heijil L, Wennstrom J, Lindhe J, Socransky SS. Periodontal disease in gnotobiotic rats. *J Periodontal Res* 1980;**15:**405–419.
14. Benatti BB, Nogueira Filho GR, Diniz MC, Sallum EA, Sallum AW, Nociti FH Jr. Stress may enhance nicotine effects on periodontal tissues. An in vivo study in rats. *J Periodontal Res* 2003;**38:**351–353.
15. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173:**697–703.
16. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11:**266–273.
17. Millar BC, Jiru X, Moore JE, Earle JAP. A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. *J Microbiol Methods* 2000;**42:**139–147.
18. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*: ''the red complex'', a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* 2005;**38:**72–122.
19. Listgarten MA. Pathogenesis of periodontitis. *J Clin Periodontol* 1986;**13:**418–430.
20. Paster BJ, Boches SK, Galvin JL *et al*. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.
21. Moore WE, Moore LV. The bacteria of periodontal diseases. *Periodontol 2000* 1994;**5:**66–77.
22. Socransky SS, Hafajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
23. Stefanopoulos PK, Kolokotronis AE. The clinical significance of anaerobic bacteria in acute orofacial odontogenic infections. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;**98:**398–408.
24. Choi BK, Lee HJ, Kang JH, Jeong GJ, Min CK, Yoo YJ. Induction of osteoclastogenesis and matrix metalloproteinase expression by the lipooligosaccharide of *Treponema denticola*. *Infect Immun* 2003;**71:**226–233.
25. Nociti FH Jr, Foster BL, Barros SP, Darveau RP, Somerman MJ. Cementoblast gene expression is regulated by *Porphyromonas gingivalis* lipopolysaccharide partially via toll-like receptor-4/MD-2. *J Dent Res* 2004;**83:**602–607.
26. Darveau RP, Pham TT, Lemley K *et al*. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 2004;**72:**5041–5051.
27. Kesavalu L, Chandrasekar B, Ebersole JL. In vivo induction of proinflammatory cytokines in mouse tissues by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 2002;**17:**177–180.
28. Baker PJ, Howe L, Garneau J, Roopenian DC. T cell knockout mice have diminished alveolar bone loss after oral infection with *Porphyromonas gingivalis*. *FEMS Immun and Med Microbiol* 2002;**34:**45–50.
29. Kesavalu L, Bakthavatchalu V, Rahman MM *et al*. Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in *Porphyromonas gingivalis*-induced experimental periodontal disease. *Oral Microbiol Immunol* 2007;**22:**232–239.
30. Klausen B. Microbilogical and immunological aspects of experimental disease in rats: a review article. *J Periodontol* 1991;**62:**59–73.
31. Polak D, Wilensky A, Shapira L *et al*. Mouse model of experimental periodontitis induced by *Porphyromonas gingivalis*/*Fusobacterium nucleatum* infection: bone loss and host response. *J Clin Periodontol* 2009;**36:**406–410.