Evaluation of the Host Response in Various Models of Induced Periodontal Disease in Mice

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Background: The aim of this study was to characterize and evaluate the host response caused by three different models of experimental periodontitis in mice.

Methods: C57BL/6 wild-type female mice were distributed into 5 experimental groups and sacrificed at 7, 15 and 30 days after the induction of periodontal disease: Group C - no treatment control group; Group L - periodontal disease induced by ligature; Groups G-Pg and G-PgFn – oral gavage with Porphyromonas gingivalis (P.g.) and Fusobacterium nucleatum (F.n.) + Porphyromonas gingivalis, respectively; Group I-Pg – heat-killed Porphyromonas gingivalis injected into the palatal mucosa between the molars; and Group I-V - PBS injected into the palatal mucosa. The samples were used to analyze the immune-inflammatory process in the gingival tissue via descriptive histological and real-time polymerase chain reaction (qPCR) analyses. The alveolar bone loss was evaluated using micro-computed tomography (μCT). The data were analyzed using the Kruskal-Wallis test followed by a post-test Dunn test and ANOVA followed by a Tukey test using a 5% significant level.

Results: Only the ligature model displayed significant alveolar bone loss in the initial period (7 days), which was maintained over time. The injection of heat-killed P.g. model displayed significant alveolar bone loss starting from day 15 day, which continued to progress over time (p<0.05). A significant increase (p<0.05) in the gene expression of pro-inflammatory cytokines (IL-6, IL-1β) and proteins involved in osteoclastogenesis (RANKL and OPG) was observed in the ligature group on day 7.

Conclusions: The ligature and injection of heat-killed Porphyromonas gingivalis models were the most representative of periodontal disease in humans, whereas the oral gavage models were not effective at inducing the disease under the experimental conditions.

KEYWORDS
Cytokines; Host-parasite interactions; Porphyromonas gingivalis; Mice; Periodontitis; Periodontal disease.

Periodontitis is an infectious disease characterized by a chronic inflammation of the periodontium and is mediated and modulated by the host immune system. The disease involves the apical migration of epithelium and alveolar bone loss. It is considered the most prevalent form of bone pathology in humans. Several microorganisms play a pivotal role in periodontitis pathogenesis. Considerable attention has been given to Porphyromonas gingivalis, a Gram-negative black-pigmented bacterial known to be associated with chronic periodontitis, and its
damage to periodontal tissues.\textsuperscript{6,7} Previous studies\textsuperscript{4,8} demonstrated that \textit{Fusobacterium nucleatum} is also commonly observed in deep periodontal pockets and is positively correlated with the progression of periodontal disease. This bacteria participates in both adhesion and co-aggregation with many other oral bacteria, such as \textit{P. gingivalis}, acting as a bridge between early and late colonizers\textsuperscript{8} that elicit bone destruction.\textsuperscript{9}

Various animal models have been used to investigate the host-bacteria interaction and to evaluate the pathogenesis of periodontitis.\textsuperscript{1,10-13} Animal models of periodontal disease have contributed new knowledge to the biological sciences. An important feature of the experimental models used to study human infectious diseases is the ability to simulate an infectious process similar to that observed in humans while mimicking the pathogenesis of the natural disease.\textsuperscript{14} Animal models allow the study of defined aspects of periodontitis, such as the etiology, the role of specific virulence factors, the effect of cells and mediators on tissue responses, the colonization mechanisms and the role of other infections.\textsuperscript{14} Mice models of periodontal disease play an important role compared with rats models, especially given the ability to manipulate their genetic characteristics and increase the potential for study interpretations and allowing the study of specific cells or molecules in the development and progression of periodontal disease.\textsuperscript{1} Longitudinal studies of periodontitis in humans present some limitations related to the mechanisms of disease due to many variables that are difficult to control among patients, such as activity level, progression, susceptibility and duration of the disease.\textsuperscript{15} Thus, human studies are limited by the difficulty of elucidating the pathogenesis of periodontal disease.

To initiate experimental periodontal disease in rodents, ligature is one of the most widely used models in periodontal research. In rats, alveolar bone loss occurs predictably after 7 days.\textsuperscript{16,17} This model is dependent on the presence of bacteria because it has been demonstrated that in germ-free rats, ligatures do not induce significant alveolar bone destruction.\textsuperscript{18} A limitation of this model is the mechanical injury caused during the placement of a ligature that could aggravate periodontal tissue destruction and physiological bone remodeling.\textsuperscript{19,20} Another used model involves the localized injection of bacteria or a pathogen component (e.g., LPS) into the palatal gingival tissue. This method promotes significant periodontal inflammation that is characterized by an increased expression of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α; apical migration of the junctional epithelium; and osteoclastogenesis activation, resulting in tissue destruction.\textsuperscript{21-23} Oral gavage models have been used in an attempt to reproduce the chronic bone loss observed in periodontal diseases. An experimental model of periodontitis was developed that involves introducing \textit{P. gingivalis} into rats to induce the destruction of alveolar bone.\textsuperscript{24-27} This model has also been used with other bacterial strains\textsuperscript{22} and with mixtures of several bacteria species to evaluate polymicrobial periodontal disease.\textsuperscript{12,13} In this model, significant bone loss typically takes longer than 4 weeks after the last inoculation.

The variability of the models found in the literature often prevents a direct comparison between the results and conclusions of the studies. Therefore, studies that comparatively assess the disease progression as well as the cellular and molecular characteristics of different periodontitis models can be useful in selecting the appropriate model to be used and in the interpretation of the results obtained in previous studies. Thus, the aim of this study was to characterize and evaluate the host response caused by three different models of experimental periodontitis in mice. To
our knowledge, no previous studies have compared these three methods together at a molecular and morphometric level in periodontal tissues.

MATERIALS AND METHODS

Animals

The experimental group comprised six 8-week-old female C57BL/6 wild-type (WT) mice weighing 30 g, and they were maintained in the animal facilities of the School of Dentistry at Araraquara with a controlled temperature (23±2°C) and a 12-hour light-dark cycle. Throughout the experimental period, mice were housed in plastic cages, fed a standard laboratory diet and given water ad libitum. The study protocol was conducted according to the recommendations of the National Council for Control of Animal Experimentation (CONCEA). The protocol was approved by the local Institutional Experimentation Committee for Animal Care and Use (protocol 12/2010), and the protocol followed all recommendations of the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments) for the execution and submission of studies in animals.28

Experimental Protocol

A total of 108 animals were randomly assigned into six groups: a control group (C), in which no treatment was performed, and groups representing three different experimental periodontal disease models, including ligature-induced periodontal disease (group L), oral gavage models with Porphyromonas gingivalis (group G-Pg) and Fusobacterium nucleatum + Porphyromonas gingivalis (group G-PgFn) and heat-killed Porphyromonas gingivalis (Group I-Pg) injected into the palatal mucosa between the molars. Negative controls included sham-infected mice, which received PBS injections without heat-killed P. gingivalis.

Ligature-Induced Periodontal Disease

The animals underwent general anesthesia with a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) administered via intramuscular injection. The ligature model was obtained by placing a sterilized 6.0 nylon thread around the first maxillary molar and bilaterally knotting mesiobuccally. The ligatures were inspected twice a week and repositioned if necessary to maintain the ligature during the entirety of the experimental period. In all animals, the ligature was maintained in position throughout the duration of the experiment without the necessity of replacement.

Bacterial Strains and Culture Conditions

Porphyromonas gingivalis strain (ATCC 33277) and Fusobacterium nucleatum (ATCC 25586) were used in this study. Briefly, Porphyromonas gingivalis were grown on blood agar medium supplemented with 1 μL/mL menadione, 5 μL/mL hemin and 40 μL/mL of kanamycin to supply their nutritional needs and were maintained in an anaerobic jar containing 90% N2 and 10% CO2 at 37°C for 10 days. Fusobacterium nucleatum was grown on blood agar plates (5% defibrinated sheep blood) at 35°C under anaerobic conditions (90% N2 and 10% CO2). Gram’s method and stereoscopic visualization were used to confirm the purity of the colonies. Then, the microorganisms were transferred to a micro-centrifuge tube with 100 μl of...
phosphate-buffered saline (PBS). In addition, 100 μl of the sample was diluted in peptone water and plated on petri dishes. The dilutions were performed in triplicate. After incubation, the plates were examined under a stereomicroscope for morphological examination and to count the total number of colony-forming units (CFU). Plates that contained 30 to 300 CFUs were chosen for the counting. The solution was diluted to achieve a concentration of $10^9$ CFU in 100 μl of PBS.

**Monomicrobial and Polymicrobial Oral Gavage Model**

For oral monomicrobial infection, animals were subjected to a protocol consisting of oral inoculation of $1 \times 10^9$ CFU of *P. gingivalis* in 100 μl of VMGA with sterile 2% carboxymethylcellulose†† (CMC) placed directly into the oral cavity of mice with a micropipette. For oral polymicrobial infection, *P. gingivalis* ($1 \times 10^9$ CFU cells per ml) was mixed with an equal volume of *F. nucleatum*, and the microorganisms were allowed to interact for 5 min. An equal volume of 2% CMC was added to the consortium and mixed thoroughly. Mice were infected three times in 2-day intervals for the 7-day experimental period, and five times in 2-day intervals for the 15- and 30-day experimental periods.

**Heat-killed *P. Gingivalis* Injection Model**

After sedation with isoflurane, ‡‡ the animals received a bilateral direct injection of 0.5 μl of $1 \times 10^{10}$ CFU/ml of heat-killed *P. gingivalis* (ATCC 33277) diluted in PBS into the palatal gingival tissue between the molars, using custom-designed 0.375 in × 12 gauge needles attached to a 1-μL syringe. §§ Previously, the *P. gingivalis* were inactivated by incubation in suspension at 60°C for 5 minutes. The bilateral injections were repeated 3 times weekly throughout the duration of the experiment. Negative controls included sham-infected mice, which received PBS injections without heat-killed *P. gingivalis*.

**Animal Sacrifice and Analysis**

After 7, 15, and 30 days of periodontal disease induction, 6 animals from the control and the experimental groups were sacrificed per period via anesthetic overdose. The maxillary jaws were hemi-sectioned, and half of the block samples (n=6) were submitted to routine histological processing for histological descriptive analysis. In the other half block, the gingival tissues were excised for the extraction of total RNA for RT-qPCR. After dissection of the gingival tissues, the block samples were fixed in 4% paraformaldehyde for 24 hours, stored in 70% ethanol and used to evaluate alveolar bone resorption via micro-computed tomography.

**Histological Analysis**

The samples for histological analysis were decalcified in a solution of 10% EDTA (0.5 M, pH 8.0) for 4-5 weeks (with 3x solution changes per week) and then embedded in paraffin. Serial sections of 4-μm thickness were obtained in the buccal-palatal direction, mounted on slides and stained with hematoxylin and eosin (H/E). Using an optical microscope¶¶ at 100x or 200x magnification, a blinded and calibrated examiner (C.R.A.) assessed the inflammatory reactions of the connective tissue and periodontal ligament present in the palatal area.

The histological analysis included the area corresponding to the periodontal tissues in the palatal side of the first molar palatal root, corresponding to the area
closest to the injection site from the cemento-enamel junction to the alveolar bone crest. The presence and intensity of the inflammatory infiltrate were evaluated in two specific regions: the sub-epithelial region, near to the gingival sulcus/periodontal pocket, and in the supra-crestal region, above the alveolar bone crest. The severity of the inflammatory process was classified in each region using polymorphonuclear leukocyte and mononuclear cell inflammation scoring, as previously described.\textsuperscript{29, 30} Severity was ranked as follows: (0) no inflammatory cells, (1) mild inflammation (a few inflammatory cells), (2) moderate inflammation (remarkable inflammatory cells scattered throughout the connective tissue above the bone crest), or (3) severe inflammation (predominance of inflammatory cells). We also counted the number of blood vessels according to the method described by de Souza et al.\textsuperscript{31} Briefly, the region of interest for the analysis, as represented by an area involving the palatal side of the first molar palatal root and involving the connective tissue subjacent to the gingival sulcus and also, was examined for the presence of any other tissue morphological changes.

**Real-time Polymerase Chain Reaction**

Total RNA from the gingival palatal tissues between the mesial of the first molar and the distal site of the second molar was extracted using a kit according to the manufacturer's instructions as previously described.\textsuperscript{31} Briefly, the quantity and purity of total RNA were determined using a spectrophotometer by evaluating the absorbance at 260 nm and the 260/280 nm ratios, respectively. The integrity of the total RNA was confirmed by electrophoresis of 0.5 mg of total RNA in 1% formaldehyde–agarose gels, followed by visualization of the bands corresponding to 18S and 28S ribosomal RNA in the appropriate ratio (1:2). Complementary DNA was synthesized by reverse transcription of 400 ng of total RNA in the presence of Oligo (dT), reverse transcriptase enzyme, MgCl\textsubscript{2}, dNTPs, and RNAse inhibitor, according to the manufacturer’s protocol.

Real-time polymerase chain reaction (qPCR) was performed using a thermocycler. The reaction included 1 \( \mu \)L of the RT reaction product in a 20 \( \mu \)L total volume PCR reaction mix that included 8 \( \mu \)L of nuclease-free water, 10 \( \mu \)L of master mix and 1 \( \mu \)L of gene expression assay reagents, including forward, reverse primers and fluorophore-conjugated probe for mice genes (see Table 1). The cycling conditions used for all primers were pre-optimized: 50°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The determination of the relative levels of gene expression was performed using the cycle threshold method and normalized to the housekeeping gene GAPDH. The results are represented as the mean mRNA expression from duplicate measurements normalized using the internal control GAPDH and expressed as a fold change over the levels determined in cDNA samples prepared from healthy control gingival tissues.

**Micro-Computed Tomography Analysis (\( \mu \)CT)**

After the sacrifice of the animals, the hemi-maxilla samples of six mice per group were carefully harvested, fixed in 4% paraformaldehyde for 24 hours, and stored in 70% ethanol at room temperature until they were scanned using the MicroCT system. The x-ray generator was operated at 50 kVp and the beam current at 500 \( \mu \)A with a 0.5-mm aluminum filter at an image resolution of 12.45 \( \mu \)m. The images were reconstructed with specific software in all three spatial dimensions, and then all the images were orientated and saved in sagittal slices (2000 x 1336) using specific
The linear measurements were determined from the cemento-enamel junction (JCE) to alveolar bone crest (ABC) in the distal region of the first molar palatal root and the mesial region of the second molar palatal root. The two linear samples measurements resulted in an average value, which was used to define the distance from the JCE to ABC in μm. The volumetric measurements were performed after the selection of a 3D ROI (region of interest) with an appropriate program.

During the drawing of the ROI, the examiner was guided by morphological landmarks. The ROI was delimited from the distal root of the second molar to the distal root of the first molar, which served as the endpoint landmark borders, as the experimental periodontal disease and the most expressed bone loss were evident on the first molar around the furcation and interproximal area. The delimited landmark borders and the contours of the ROI were drawn at regular intervals with a slice-based method every 10 planes. All contours were drawn beginning immediately below the cemento-enamel junction and moving 3 mm in the apical direction. Thus, the entire bone area of the interproximal region and the furcation area of the first molar were included in the ROI. Finally, on the original three-dimensional image, the indices were calculated through the binarized ROI. The architectural parameter evaluated was bone volume (BV), which represents the volume of the mineralized tissue around the defect.

Data Analysis

Statistical analysis was performed using specific software. The results obtained for the different groups for all parameters were comparatively evaluated for bone volume and mRNA expression in each period using one-way analysis of variance (ANOVA) followed by a post-test of multiple comparisons Tukey's test to determine the presence of any significant differences between groups. The histological analysis results were evaluated using the nonparametric Kruskal-Wallis test followed by a Dunn post-test. Differences were considered significant when p<0.05.

RESULTS

Histological Analysis

The results of the histological descriptive analysis of all groups in all experimental periods are presented in figures 1A to 1I and 2A to 2I. The clinical examination of periodontal tissues did not reveal tissue ulceration or dilacerations. The ligature model for 7, 15 and 30 days induced an inflammatory response consistent with periodontal disease as observed in humans. The changes included an intense infiltration of inflammatory cells, loss of connective tissue attachment, and alveolar bone resorption characterized by an increase in the distance between the JCE to ABC (Figures 1D, 1E and 1F). Consistent with these results, the heat-killed P. gingivalis model displayed an increased influx of inflammatory cells and alveolar bone loss, which was evident from day 15 and gradually increased over time (Figures 2D, 2E and 2F) as further indicated by the intense presence of inflammatory cells, especially leukocytes and macrophages (Figure 2F). Graphs with the results of tissue inflammation and blood vessels in all experimental periods are shown in figures 3A to 3F. Significant differences in tissue inflammation were observed at 7, 15 and 30 days after disease induction in the ligature model, whereas in the heat-killed P. gingivalis model, tissue inflammation was evident from day 15 and continued over time, as shown in figures 3A, 3B, and 3C. Blood vessels were also analyzed, and the results indicated a significant
difference in the heat-killed \textit{P. gingivalis} and ligature model at 15 and 30 days (Figures 3E and 3F). Images 3G to 3I illustrate, respectively, what was considered mild, moderate and severe inflammation.

\textit{Micro-Computed Tomography}

The bi-dimensional and three-dimensional sagittal micro-computed tomography views of the maxillary molars from each group at 30 days are shown in figures 4A to 4F and 4G to 4L, respectively. Alveolar bone loss was obvious in the ligature and heat-killed \textit{P. gingivalis} injection models (Figures 4E, 4F, 4K and 4L). Graphs of all experimental periods with the results of bone volume and linear measurements over a 30-day period are shown in figures 4A to 4D. The relevant effect on bone loss was observed in the ligature model on days 7 and 15 with a decrease in the intensity of bone loss over the 30-day period (p<0.05). The heat-killed \textit{P. gingivalis} injection model displayed continuous bone loss starting from day 15 day (p<0.05) compared with the oral gavage models and the control group.

\textit{Quantitative Analysis of mRNA Expression}

At 7 days, the mRNA expression of RANKL, OPG and the cytokines IL-1\(\beta\) and IL-6 was more pronounced in the ligature model with a consequent reduction by day 15. In the heat-killed \textit{P. gingivalis} model, there was a tendency for increased expression in all of the cytokines analyzed, RANKL, OPG, IL-1\(\beta\) and IL-6, over the 15-day period, but there was no significant difference between the groups (Figure 5A to 5H).

\textbf{DISCUSSION}

Experimental models of periodontal disease are used to obtain a better understanding of physiology, the pathogenesis of diseases and the action of new drugs. Elucidating the periodontal disease pathophysiology and developing targeted therapies is the main purpose of experimental models. To this end, we focused our efforts on characterizing and evaluating the host response caused by three different models of experimental periodontitis in mice at a histologic, molecular and radiographic level, aiding in the selection of the model to be used in preclinical experiments according to their purpose.

In our study, similar to other published reports, we utilized an oral gavage model to induce periodontal disease. Our results indicated that monomicrobial (\textit{P. gingivalis}) or polymicrobial (\textit{P. gingivalis} + \textit{F. nucleatum}) oral infection was not effective at eliciting significant periodontal bone destruction compared with the control group after a period of 30 days. Our results are in agreement with another report where the animals infected with \textit{P. gingivalis} had no significant alveolar bone loss compared with the control group. However, our results are not consistent with other studies that have shown significant bone destruction following oral inoculation with \textit{P. gingivalis} 42 days after the last infection. Previous studies using polymicrobial infection with \textit{P. gingivalis}/\textit{F. nucleatum} in BALB/c mice displayed more destructive alveolar bone loss and a stronger inflammatory response compared with the monomicrobial infection. However, Kesavalu et al., using this same model, noted that although the polymicrobial infection with \textit{P. gingivalis}, \textit{T. forsythia}, \textit{T. denticola} and \textit{F. nucleatum} results in considerable alveolar bone loss compared with mono-infections, when \textit{F. nucleatum} was excluded from the experimental group, there was no significant difference in bone destruction. Our findings were consistent
with the above work as the association of *F. nucleatum* with *P. gingivalis* did not aggravate the alveolar bone loss over an experimental period of 30 days.

These inconsistent results can be explained by different concentrations of microorganisms, mice strains, time of the experimental period and bacterial strains. Although *P. gingivalis* has been shown to induce periodontal disease in experimental models, previous studies have suggested that the strains ATCC 52977, 381 and W50 are more aggressive than those used in our study (ATCC 33277). Furthermore, the same research groups used different bacterial concentrations that were higher than the concentrations used in the present study. According to Baker et al., C57BL/6 mice are more resistant to experimental disease induction than the BALB/c mice used in previous studies. Baker et al. assessed the genetic susceptibility among different strains of mice to induce experimental periodontal disease. An oral gavage model with human strains of *P. gingivalis* was used. Increased alveolar bone loss in AKR/J, DBA/2J and BALB/c mice was observed, whereas no bone loss was observed in A/J, A/Hej, SJL/J and C57BL/6 mice. Our findings are similar to the abovementioned findings, as periodontal disease induction by oral gavage in C57BL/6 mice resulted in no significant difference in alveolar bone loss compared with the control group.

The ligature model developed here was effectively used to induce alveolar bone resorption in C57BL/6 mice. Our results revealed in the histological sections the apical migration of the junctional epithelium, connective attachment loss and bone destruction, especially in the interproximal area between the first and second molar, which is consistent with several studies in the literature that have used the same method using rats or using C57BL/6 mice. These results suggest that the mice strain C57BL/6 is method sensitive. In other words, these mice are susceptible to periodontal disease depending on the method of disease induction. The principle of this model is based on the adherence of microorganisms around the ligature, which serves as a niche for bacterial colonization, leading to the initiation of the periodontal injury.

Some authors consider this model to be the most representative of human periodontitis. The disadvantages of the ligature model are related to the mechanical trauma caused during the ligature placement and with the decrease in disease severity over time. As a result of alveolar bone destruction, periodontal tissues tend to migrate to an apical position in an attempt to recover the biological space, as corroborated by our research group. For maintenance of the disease intensity over time, some authors have used ligature incubated with *P. gingivalis* and repositioned the thread daily in an apical position to maintain the ligature in intimate contact with the marginal tissues. In our study, although the ligature was nylon and was not repositioned daily around the tooth, the results indicated evident bone loss in accordance with previous studies. Ligature-induced bone loss is a model of acute periodontal disease in which bone loss occurs primarily during the first 7 to 15 days. We are in agreement with this statement because our results indicate that significant bone loss occurred in the initial period and a decrease in bone loss could be observed for the longer period of 30 days. The monofilament characteristic of the nylon thread did not influence the periodontal bone destruction, even in the C57BL/6 mice.

The induction of experimental periodontal disease by injections of bacterial LPS or inactivated bacteria into the maxillary palatal mucosa has been widely used to evaluate gene expression and the induction of bone resorption in animal models.
Unlike the ligature model, which allows the accumulation of viable microorganisms, the bacterial injection model is not initiated by the host response to biofilm. The results of the histological and μCT analyses demonstrate that this model displays some characteristics of human periodontitis, especially in the area where the injections were made, including the connective attachment loss and bone destruction observed at 15 and 30 days and that increased over time.

In the present study, the mRNA of different pro-inflammatory cytokines and molecules involved in osteoclastogenesis were evaluated to demonstrate the inflammatory nature at a molecular level in the different models of periodontal disease induction. During the periodontitis, the interplay between cytokines and their antagonists will determine the extent and severity of bone destruction. Several biological mediators released during the tissue destruction can be induced by increased levels of cytokines, especially RANKL, IL-1β and IL-6. The binding between RANKL to RANK expressed on osteoclast precursors, is the main event for stimulatory differentiation and subsequent osteoclasts activation. The effects of RANKL are regulated by the osteoprotegerin (OPG) that, in turn, inhibits bone resorption by preventing the interaction of RANK and RANKL. Changes in the balance between protein expression of RANKL and OPG define the pathogenesis of various bone diseases, including periodontitis. During the progress of periodontal disease, high levels of RANKL can be found in periodontal tissues explaining alveolar bone resorption. These proteins are regulated by the production of pro and anti-inflammatory cytokines in the periodontal tissues that determine the course and/or severity of disease. On the other hand, IL-1β and IL-6 have been shown to be key molecules that contribute to induce the differentiation factors expression and osteoclasts activators, which results in connective tissue degradation and, ultimately, bone resorption. Also, IL-1β can induce regulation of adhesion molecules on leukocytes and endothelial cells and stimulate the production of chemokine’s necessary to recruit circulating leukocytes. These cytokines can induce the expression of new mediators such as prostaglandins that increase or maintain the inflammatory response. Thus, the ligature and bacterial injection models were the most representative models of experimental periodontitis and were characterized by an increased intensity of the inflammatory process, increase in the expression of pro-inflammatory mediators and bone loss. In our results, the ligature model induced an upregulation in the mRNA expression of RANKL, OPG and the cytokines IL-1β and IL-6 on day 7 after disease induction with a consequent reduction by day 15. These results corroborate previous reports in which the ligature model was characterized by an intense bone resorption and increased mRNA expression in the initial periods (7 days) with a consequent decrease in mRNA expression and alveolar bone loss over time. The reason of decrease on the inflammation severity at 15-days period could be attributed to a protective feature of periodontal tissues that migrated apically from the aggression located on the gingival margin, in an attempt to recover the biological space. This possibility is supported by the fact that once placed, ligatures were kept throughout the 30-day experimental period. However they were not displaced further apically even if the gingival margin had retreated. On the other hand, in the heat-killed P. gingivalis injection model, although the difference was not significant, the peak expression in all the cytokines analyzed, RANKL, OPG, IL-1β and IL-6, as well as the intensity of inflammation occurred at 15 days after disease induction. These results can be justified by the repeated bacterial challenge, which was performed 3 times per week for the entire duration of the experiment. Similarly,
both oral gavage models displayed a tendency to increase the expression of pro-inflammatory mediators over time.

CONCLUSIONS

The results obtained in this study suggest that the ligature and heat-killed *P. gingivalis* injection models of periodontal disease, as generated using C57BL/6 mice, are the most representative models of periodontitis in humans for short-term experiments. These experimental models can provide a basis for future interventional studies that contribute to the understanding of the disease pathogenesis and the complex host response to microbial challenge. Furthermore, animal models can provide hypothesis validation and demonstrate the effectiveness of new treatment therapies for periodontal disease in humans.

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**Figure 1**
Histologic views (H/E, 100X) of the sections of the palatal root frontal aspect of the first maxillary molars in the C, L, G-Pg groups at 7 (A, D and G), 15 (B, E and H) and 30 (C, F and I) days. The histomorphological aspect of the periodontium was conserved, and evident ulcerations of the gingival tissues, influx of inflammatory cells and bone loss from ligature model were observed by days 7, 15 and 30 (D, E and F). The distances from the CEJ (yellow line) to the alveolar crest (white line) were demonstrated in all experimental groups in which the epithelium apical downgrowth is more noticeable in the ligature group in all the experimental periods, as demonstrated in figures D, E and F compared with the control group and the G-Pg model. R: root, EPI: oral epithelium, CT: connective tissue, PL: periodontal ligament, AB: alveolar bone.

**Figure 2**
Histologic views (H/E, 100X) of the sections of the palatal root frontal aspect of the first maxillary molars, in G-PgFn, I-Pg and I-V groups at 7 (A, D and G), 15 (B, E and H) and 30 (C, F and I) days. The I-Pg model (D, E and F) displayed an evident influx of inflammatory cells in the connective tissue and bone loss by day 15 with an increase over time (Figure 2E and 2F), which can be observed by comparing the distance between the CEJ (yellow line) and the alveolar crest (white line). The levels of attachment were evaluated, and the pockets were observed in the I-Pg group. R: root, EPI: oral epithelium, CT: connective tissue, PL: periodontal ligament, AB: alveolar bone.

**Figure 3**
The graphs show the tissue inflammation scores and blood vessels in the 7-, 15- and 30-day experimental periods. Note the increased tissue inflammation in all experimental periods in the ligature group (A, B and C). Significant differences were also observed at days 15 and 30 in the heat-killed P. gingivalis injection model (A, B and C). The blood vessels were also evaluated in all experimental periods, indicating an increase in the number of blood vessels at days 15 and 30 day the heat-killed P. gingivalis injection model and ligature induced disease (D, E and F). All the results are expressed as the median. *Significant difference compared to all the other groups: (*p < 0.05, Kruskal–Wallis followed by Dunn multiple comparisons test). Figures G, H and I demonstrate, respectively, what was considered mild (few inflammatory cells), moderate (remarkable inflammatory cells scattered throughout the connective tissue above the bone crest) and severe (predominance of inflammatory cells) tissue inflammation.

**Figure 4**
Comparison of bone volume percentage measured in a selected ROI of the maxillary first molars using micro-computed tomography in all experimental groups in a 30-day period. Bi-dimensional (A to F) and three-dimensional (G to L) sagittal micro-computed tomography views of the maxillary molars of different animals from each group at 30 days. Figures E, F, K and L show evident alveolar bone loss, mainly in the interproximal area between the molars. A and G: control group; B and H: G-Pg group; C and I: G-PgFn group; D and J: I-V group; E and K: I-Pg group, F and L: ligature model.

**Figure 5**
The graphs show the bone volume fraction in all experimental periods (A to C) and linear bone loss measured from the cemento-enamel junction to alveolar bone crest in a 30-day period (D). All the results are expressed as the mean ± standard deviation. * Significant difference compared to all the other groups: p<0.05. (One-way ANOVA and Tukey’s post hoc tests).
Figure 6
mRNA expression of the pro-inflammatory cytokines IL-1β (A and B) and IL-6 (C and D), the proteins involved in osteoclastogenesis RANKL (E and F) and OPG (G and H) during the course of the experimental periodontal disease in all experimental groups (7- and 15-day time points) accomplished by reverse transcription real-time PCR. Cytokines expression levels were normalized to the expression of the house-keeping gene GAPDH. *Significant differences compared with the other groups (p<0.05). (One-way ANOVA and Tukey’s post hoc tests).

Table 1 -
Inventoried Primers and probe.

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<th>Target Gene</th>
<th>Assay ID</th>
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<th>Amplicon Lenth (bp)</th>
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<td>75</td>
</tr>
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</table>

1 Francotar; Virbac do Brasil Ind. e Com. Ltda, Roseira, SP, Brazil
¶ Rompun; Bayer S.A. Saúde Animal, São Paulo, SP, Brazil
§ Ethicon, Somerville, NJ, USA
** Sigma-Aldrich, St. Louis, MO, USA
†† Sigma, St. Louis, MO, USA
‡‡ Baxter Healthcare Corp., Deerfield, IL, USA
§§ Hamilton Company, Reno, NV, USA
ǁ LEICA microsystem GmbH, Wetzlar, Germany
**** RNAqueous-4PCR, Foster City, CA, USA
††† Biomate3, Thermo Electron Corporation, Rochester, NY, USA
*** Step One Plus, Applied Biosystems, Foster City, CA, USA
†††† TaqMan, Applied Biosystems, Foster City, CA, USA
‡‡‡ Skyscan, 1176, Aartselaar, Kontich, Belgium
§§§ NRecon 1.6.1.5 – Skyscan, Kontich, Belgium
ǁǁǁ Data Viewer 1.4.3.1 - Skyscan, Kontich, Belgium
*** CT Analyser 1.10.1.0 - Skyscan, Kontich, Belgium
**** GraphPad Software Inc., San Diego, CA, USA
***** TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA, USA