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

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Background: The aim of this study is 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 six experimental groups and sacrificed at 7, 15, and 30 days after the induction of periodontal disease: 1) group C: no treatment control group; 2) group L: periodontal disease induced by ligature; 3) group G-Pg: oral gavage with *Porphyromonas gingivalis* (*Pg*); 4) group G-PgFn: oral gavage with *Fusobacterium nucleatum* + *Pg*; 5) group I-Pg: heat-killed *Pg* injected into the palatal mucosa between the molars; and 6) group I-V: phosphate-buffered saline injected into the palatal mucosa. The samples were used to analyze the immune-inflammatory process in the gingival tissue via descriptive histologic and real-time polymerase chain reaction analyses. The alveolar bone loss was evaluated using microcomputed tomography. The data were analyzed using the Kruskal-Wallis test, followed by a post hoc Dunn test and analysis of variance, followed by a Tukey test using a 5% significance level.

Results: Only the ligature model displayed significant alveolar bone loss in the initial period (7 days), which was maintained with time. The group injected with heat-killed *Pg* displayed significant alveolar bone loss starting from day 15, which continued to progress with time ($P < 0.05$). A significant increase ($P < 0.05$) in the gene expression of proinflammatory cytokines (interleukin-6 and -1β) and proteins involved in osteoclastogenesis (receptor activator of nuclear factor-κB ligand and osteoprotegerin) was observed in the ligature group on day 7.

Conclusion: The ligature and injection of heat-killed *Pg* 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. *J Periodontol 2014;85:465-477.*

KEY WORDS

Cytokines; host-parasite interactions; mice; periodontal diseases; periodontitis; *Porphyromonas gingivalis*.

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Periodontitis is an infectious disease characterized by a chronic inflammation of the periodontium and is mediated and modulated by the host immune system.[1] The disease involves the apical migration of epithelium and alveolar bone loss. It is considered the most prevalent form of bone pathology in humans.[2] Several microorganisms play a pivotal role in periodontitis pathogenesis. Considerable attention has been given to *Porphyromonas gingivalis* (*Pg*), a Gram-negative black-pigmented bacterium known to be associated with chronic periodontitis[3-5] and its damage to periodontal tissues.[6,7] Previous studies[4,8] demonstrated that *Fusobacterium nucleatum* (*Fn*) is also commonly observed in deep periodontal pockets and is positively correlated with the progression of periodontal disease. This

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bacterium participates in both adhesion and coaggregation with many other oral bacteria, such as *Pg*, acting as a bridge between early and late colonizers[8] that elicit bone breakdown.[9]

Various animal models have been used to investigate the host–bacteria interaction and to evaluate the pathogenesis of periodontitis.[1,10-13] Animal models of periodontal disease have contributed new knowledge to the biologic 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.[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.[14] Mice models of periodontal disease play an important role compared to rat 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.[1] Longitudinal studies of periodontitis in humans present some limitations related to the mechanisms of disease because of many variables that are difficult to control among patients, such as activity level, progression, susceptibility, and duration of the disease.[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.[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 breakdown.[18] A limitation of this model is the mechanical injury caused during the placement of a ligature that could aggravate periodontal tissue breakdown and physiologic bone remodeling.[19,20] Another model used involves the localized injection of bacteria or a pathogen component (e.g., lipopolysaccharide [LPS]) into the palatal gingival tissue. This method promotes significant periodontal inflammation that is characterized by an increased expression of inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α, apical migration of the junctional epithelium, and osteoclastogenesis activation, resulting in tissue breakdown.[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 *Pg* into rats to induce the breakdown of alveolar bone.[24-27] This model has also been used with other bacterial strains[22] and with mixtures of several bacteria species to evaluate polymicrobial periodontal disease.[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 is to characterize and evaluate the host response caused by three different models of experimental periodontitis in mice. To the best of the authors' knowledge, no previous studies have compared these three methods together at a molecular and morphometric levels in periodontal tissues.

MATERIALS AND METHODS

Animals

The experimental groups comprised six 8-week-old female C57BL/6 wild-type mice weighing 30 g, and they were maintained in the animal facilities of the School of Dentistry at Araraquara, São Paulo State University, Araraquara, São Paulo, Brazil, with controlled temperature (23°C ± 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. 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 (Animal Research: Reporting In Vivo Experiments) guidelines for the execution and submission of studies in animals.[28]

Experimental Protocol

A total of 108 animals were randomly assigned into six groups: 1) group C: no treatment control group; 2) group L: periodontal disease induced by ligature; 3) groups G-Pg: oral gavage with *Pg*; 4) group G-PgFn: oral gavage with *Fn* + *Pg*; 5) group I-Pg: heat-killed *Pg* injected into the palatal mucosa between the molars; and 6) group I-V: phosphate-buffered saline (PBS) injected into the palatal mucosa. Negative controls included sham-infected mice, which received PBS injections without heat-killed *Pg*.

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 mesio-buccally. The ligatures were inspected twice a week and repositioned if necessary to maintain the ligature during the entire 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

Pg (ATCC 33277) and *Fn* (ATCC 25586) strains are used in this study. Briefly, *Pg* was grown on blood agar medium supplemented with 1 μL/mL menadione,** 5 μL/mL hemin,†† and 40 μL/mL kanamycin to supply nutritional needs and was maintained in an anaerobic jar containing 90% N_2 and 10% CO_2 at 37°C for 10 days. *Fn* was grown on blood agar plates (5% defibrinated sheep blood) at 35°C under anaerobic conditions (90% N_2 and 10% CO_2). Gram's method and stereoscopic visualization were used to confirm the purity of the colonies. Then, the microorganisms were transferred to a microcentrifuge tube with 100 μL PBS. In addition, 100 μL of the sample was diluted in peptone water and plated on cell culture dishes. The dilutions were performed in triplicate. After incubation, the plates were examined under a stereomicroscope for morphologic examination and to count the total number of colony-forming units (CFU). Plates that contained 30 to 300 CFU were chosen for counting. The solution was diluted to achieve a concentration of 10^9 CFU in 100 μL PBS.

Monomicrobial and Polymicrobial Oral Gavage Model

For oral monomicrobial infection, animals were subjected to a protocol consisting of oral inoculation of 1×10^9 CFU of *Pg* in 100 μL viability medium Gothenburg anaerobically with sterile 2% carboxymethylcellulose‡‡ (CMC) placed directly into the oral cavity of mice with a micropipette. For oral polymicrobial infection, *Pg* (1×10^9 CFU cells per mL) was mixed with an equal volume of *Fn*, and the microorganisms were allowed to interact for 5 minutes. 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 Pg *Injection Model*

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

Animal Sacrifice and Analyses

After 7, 15, and 30 days of periodontal disease induction, six animals from the control and experimental groups were sacrificed per period via anesthetic overdose. The maxillary jaws were hemisected, and half of the block samples (N = 6) were submitted to routine histologic processing for histologic descriptive analysis. In the other half block, the gingival tissues were excised for the extraction of total RNA for reverse transcription-qualitative polymerase chain reaction (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 microcomputed tomography (micro-CT).

Histologic Analyses

The samples for histologic analysis were decalcified in a solution of 10% EDTA (0.5 M, pH 8.0) for 4 to 5 weeks (with ×3 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 ×100 or ×200 magnification, a masked and calibrated examiner (CRA) assessed the inflammatory reactions of the connective tissue (CT) and periodontal ligament present in the palatal area.

The histologic 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 (CEJ) to the alveolar bone

‖ Francotar, Virbac of Braz, Roseira, Brazil.
¶ Rompun, Bayer Animal Health, São Paulo, Brazil.
# Ethicon, Somerville, NJ.
** Sigma-Aldrich, St. Louis, MO.
†† Sigma-Aldrich.
‡‡ Sigma-Aldrich.
§§ Baxter Healthcare, Deerfield, IL.
‖‖ Hamilton Company, Reno, NV.
¶¶ Leica Microsystems, Wetzlar, Germany.

crest (ABC). The presence and intensity of the inflammatory infiltrate were evaluated in two specific regions: 1) the subepithelial region, near the gingival sulcus/periodontal pocket; and 2) in the supracrestal region, above the ABC. The severity of the inflammatory process was classified in each region using polymorphonuclear leukocyte and mononuclear cell inflammation scoring, as described previously.[29,30] Severity was ranked as follows: 0 = no inflammatory cells; 1 = mild inflammation (a some inflammatory cells); 2 = moderate inflammation (remarkable inflammatory cells scattered throughout the CT above the bone crest); or 3 = severe inflammation (predominance of inflammatory cells). The number of blood vessels were counted according to the method described by de Souza et al.[31] Briefly, the region of interest (ROI) for the analysis, as represented by an area involving the palatal side of the first molar palatal root and involving the CT subjacent to the gingival sulcus, was examined for the presence of any other tissue morphologic changes.

RT-qPCR

Total RNA from the gingival palatal tissues between the mesial aspect of the first molar and the distal site of the second molar was extracted using a kit[##] according to the instructions of the manufacturer as described previously.[31,32] 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 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 (cDNA) was synthesized by RT of 400 ng total RNA in the presence of oligo-dT, RT enzyme, $MgCl_2$, deoxynucleotide triphosphates, and ribonuclease inhibitor, according to the protocol of the manufacturer.

qPCR was performed using a thermocycler.[†††] The reaction included 1 μL of the RT reaction product in a 20-μL total volume PCR reaction mix that included 8 μL nuclease-free water, 10 μL master mix, and 1 μL gene expression assay reagents, including forward and reverse primers and fluorophore-conjugated probe[‡‡‡] for mice genes (Table 1). The cycling conditions used for all primers were preoptimized: 50°C for 2 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. 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 compared to the levels determined in cDNA samples prepared from healthy control gingival tissues.

Micro-CT

After the sacrifice of the animals, the hemimaxilla 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 a micro-CT system.[§§§] The x-ray generator was operated at 50 kVp and the beam current at 500 μA with a 0.5-mm aluminum filter at an image resolution of 12.45 μm. The images were reconstructed with specific software[||||] in all three spatial dimensions, and then all the images were orientated and saved in sagittal slices (2,000 × 1,336) using specific software.[¶¶¶] The linear measurements were determined from the CEJ to 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 CEJ to ABC in micrometers. The volumetric measurements were performed after the selection of a three-dimensional ROI with an appropriate program.[###] During the drawing of the ROI, the examiner was guided by morphologic 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 because the experimental periodontal disease and the most bone loss expressed 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 CEJ 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.

RNAqueous-4PCR, Applied Biosystems, Foster City, CA.
*** BioMate 3, Thermo Electron Corporation, Rochester, NY.
††† StepOne Plus, Applied Biosystems.
‡‡‡ TaqMan, Applied Biosystems.
§§§ Model 1176, SkyScan, Kontich, Belgium.
|||| NRecon v.1.6.1.5, SkyScan.
¶¶¶ Data Viewer v.1.4.3.1, SkyScan.
CT-Analyser v1.10.1.0, SkyScan.

Table 1.

Inventoried Primers and Probe††††

Target Gene	Gene Expression Assay No.	RefSeq Accession No.	Amplicon Length (bp)
GAPDH	Mm 99999915_gl	NM_008084.2	107
IL-1β	Mm 01336189_ml	NM_008361.3	63
IL-6	Mm 00446190_ml	NM_031168.1	78
RANKL	Mm 00441906_ml	NM_011613.3	66
OPG	Mm 01205928_ml	NM_008764.3	75

Data Analyses

Statistical analysis was performed using specific software.**** The results obtained for the different groups for all parameters were comparatively evaluated for BV and mRNA expression in each period using one-way analysis of variance (ANOVA), followed by a post hoc test of multiple comparisons and Tukey test to determine the presence of any significant differences among groups. The histologic analysis results were evaluated using the non-parametric Kruskal-Wallis test, followed by a Dunn post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Histologic Analyses

The results of the histologic descriptive analysis of all groups in all experimental periods are presented in Figures 1 and 2. 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, CT attachment loss (AL), and alveolar bone resorption characterized by an increase in the distance between the CEJ to ABC (Figs. 1D through 1F). Consistent with these results, the heat-killed *Pg* model displayed an increased influx of inflammatory cells and alveolar bone loss, which was evident from day 15 and gradually increased with time (Figs. 2D through 2F) as further indicated by the intense presence of inflammatory cells, especially leukocytes and macrophages (Fig. 2F). Graphs with the results of tissue inflammation and blood vessels in all experimental periods are shown in Figures 3A through 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 *Pg* model, tissue inflammation was evident from day 15 and continued with time, as shown in Figures 3A through 3C. Blood vessels were also analyzed, and the results indicated a significant difference in the heat-killed *Pg* and ligature models at 15 and 30 days (Figs. 3E and 3F). Figures 3G through 3I illustrate, respectively, what was considered mild, moderate, and severe inflammation.

Micro-CT

The bidimensional and three-dimensional sagittal micro-CT views of the maxillary molars from each group at 30 days are shown in Figures 4A through 4F and 4G through 4L, respectively. Alveolar bone loss was obvious in the ligature and heat-killed *Pg* injection models (Figs. 4E, 4F, 4K and 4L). Graphs of all experimental periods with the results of BV and linear measurements during a 30-day period are shown in Figures 5A through 5D. 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 during the 30-day period ($P < 0.05$). The heat-killed *Pg* injection model displayed continuous bone loss starting from day 15 ($P < 0.05$) compared to the oral gavage models and the control group.

Quantitative Analyses of mRNA Expression

At 7 days, the mRNA expression of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and the cytokines IL-1β and IL-6 was more pronounced in the ligature model, with a consequent reduction by day 15. In the heat-killed *Pg* model, there was a tendency for increased expression in all of the cytokines analyzed, RANKL, OPG, IL-1β, and IL-6, during the 15-day period, but there was no significant difference among the groups (Figs. 5 and 6).

DISCUSSION

Experimental models of periodontal disease are used to obtain a better understanding of physiology,

**** GraphPad Software, San Diego, CA.
†††† TaqMan Gene Expression Assays, Applied Biosystems.

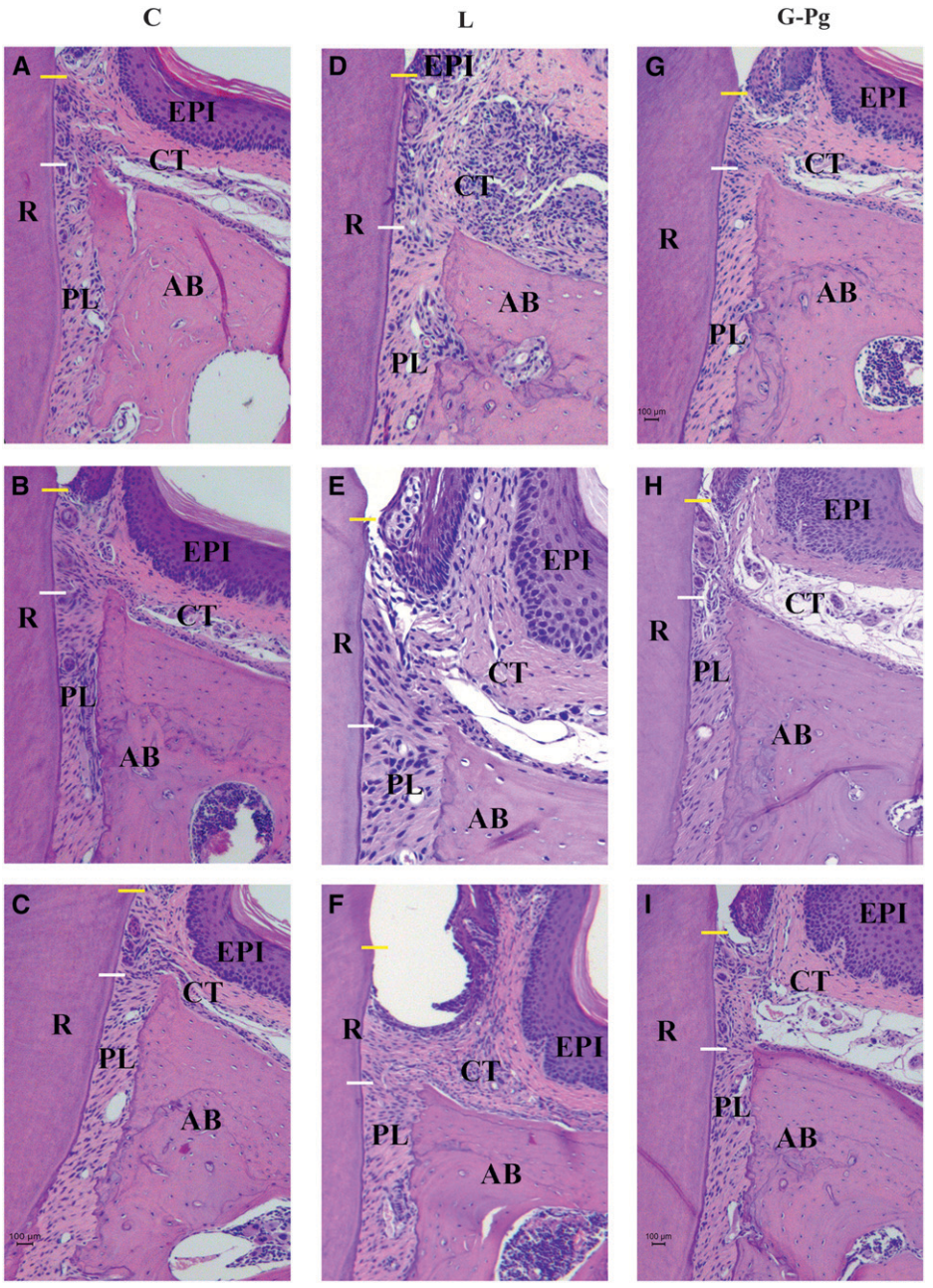

Figure 1.
Histologic views (H&E stain, magnification ×100) of the sections of the palatal root frontal aspect of the first maxillary molars in the C, L, and G-Pg groups at 7 ***(A, D,*** *and* ***G)****, 15* ***(B, E,*** *and* ***H)*** *and 30* ***(C, F,*** *and* ***I)*** *days. The histomorphologic aspect of the periodontium was conserved, and evident ulcerations of the gingival tissues, influx of inflammatory cells, and bone loss from the ligature model were observed by days 7, 15, and 30 (D through F). The distances from the CEJ (yellow line) to the ABC (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 D through F compared to the C group and the G-Pg model. R = root; EPI = oral epithelium; PL = periodontal ligament; AB = alveolar bone.*

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, efforts were focused on characterizing and evaluating the host response caused by three different models of experimental periodontitis in mice at histologic, molecular, and radiographic levels, aiding in the selection of the model to be used in preclinical experiments according to their purpose.

In the present study, similar to other published reports,[20,26,33] an oral gavage model is used to induce periodontal disease. The present results indicate that monomicrobial (*Pg*) or polymicrobial (*Pg* + *Fn*) oral infection was not effective at eliciting significant periodontal bone breakdown compared to the control group after a period of 30 days. The present results are in agreement with another report[34] in which the animals infected with *Pg* had no significant alveolar bone loss compared to the control group. However, the present results are not consistent with other studies that have shown significant bone breakdown after oral inoculation with *Pg* 42 days after the last infection.[20,26,33] A previous study[12] using polymicrobial infection with *Pg/Fn* in BALB/c mice displayed more destructive alveolar bone loss and a stronger inflammatory response compared to the monomicrobial infection. However, Kesavalu et al.,[13] using this same model, noted that although the polymicrobial infection with *Pg*, *Tannerella forsythia*, *Treponema denticola*, and *Fn* results in considerable alveolar bone loss compared to monoinfections, when *Fn* was excluded

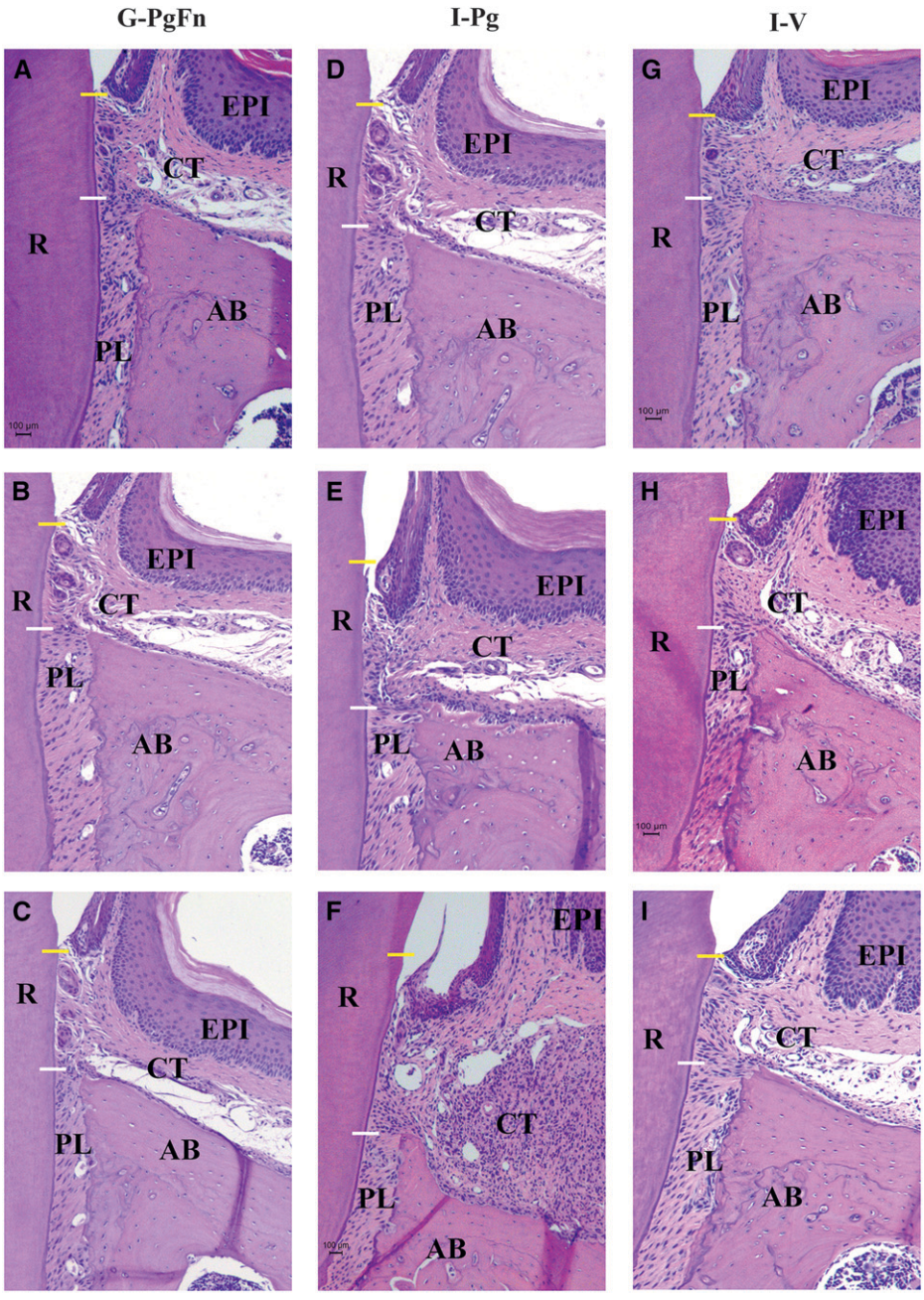

Figure 2.
Histologic views (H&E stain, magnification ×100) of the sections of the palatal root frontal aspect of the first maxillary molars in the G-PgFn, I-Pg, and I-V groups at 7 ***(A, D,*** *and* ***G)****, 15* ***(B, E,*** *and* ***H)****, and 30* ***(C, F,*** *and* ***I)*** *days. The I-Pg model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

from the experimental group, there was no significant difference in bone breakdown. The present findings were consistent with the above work because the association of *Fn* with *Pg* did not aggravate the alveolar bone loss during 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 *Pg* has been shown to induce periodontal disease in experimental models, previous studies[26,27] have suggested that the strains ATCC 52977, 381, and W50 are more aggressive than those used in the present study (ATCC 33277). Furthermore, the same research groups[26,27] used different bacterial concentrations that were higher than the concentrations used in the present study. According to Baker et al.,[33] C57BL/6 mice are more resistant to experimental disease induction than the BALB/c mice used in previous studies.[12] Baker et al.[33] assessed the genetic susceptibility among different strains of mice to induce experimental periodontal disease. An oral gavage model with human strains of *Pg* 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. The present findings are similar to the above-mentioned findings because periodontal disease induction by oral gavage in C57BL/6 mice resulted in no significant difference in alveolar bone loss compared to the control group.

The ligature model developed here was effectively used to induce alveolar bone resorption in C57BL/6 mice.

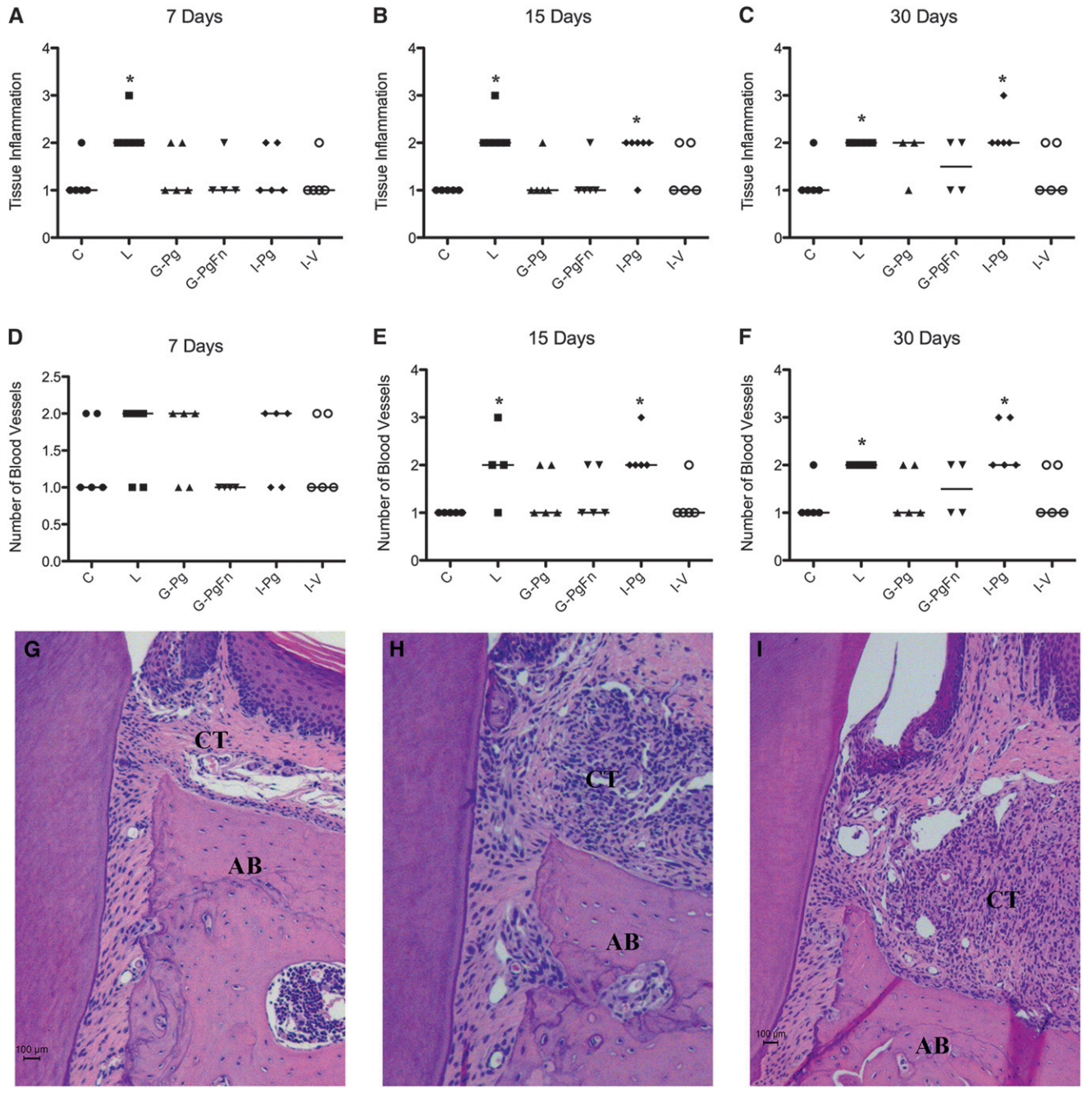

Figure 3.
The graphs show the tissue inflammation scores and blood vessels in the 7-, 15-, and 30-day experimental periods. Note the increased tissue inflammation in all experimental periods in the ligature group ***(A through C)****. Significant differences were also observed at days 15 and 30 in the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

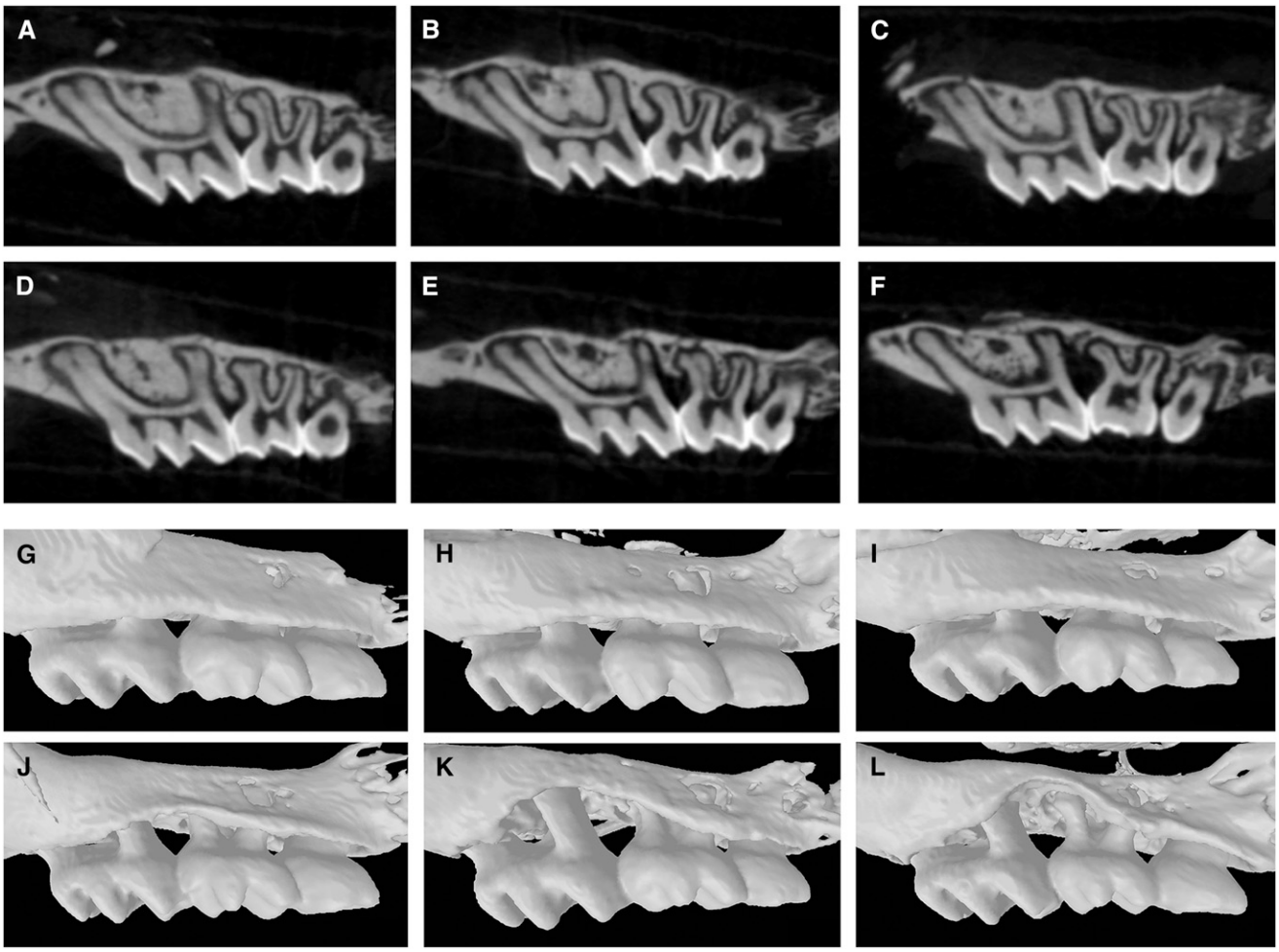

Figure 4.
*Comparison of BV percentage measured in a selected ROI of the maxillary first molars using micro-CT in all experimental groups in a 30-day period. Bidimensional **(A through F)** and three-dimensional **(G through L)** sagittal micro-CT views of the maxillary molars of different animals from each group at 30 days. 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.*

The present results revealed in the histologic sections the apical migration of the junctional epithelium, CT AL, and bone breakdown, especially in the interproximal area between the first and second molars, which is consistent with several studies in the literature that have used the same method using rats[17,19,35] or C57BL/6 mice.[34,36] 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.[37]

Some authors[31] 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 with time. As a result of alveolar bone breakdown, periodontal tissues tend to migrate to an apical position in an attempt to recover the biologic space, as corroborated by the present authors' research group.[31,38,39] For maintenance of the disease intensity with time, some authors[34-36] have used ligature incubated with *Pg* and repositioned the thread daily in an apical position to maintain the ligature in intimate contact with the marginal tissues. In the present 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.[34-36] Ligature-induced bone loss is a model of acute periodontal disease in which bone loss occurs primarily during the first 7 to 15 days.[19] The present authors are in

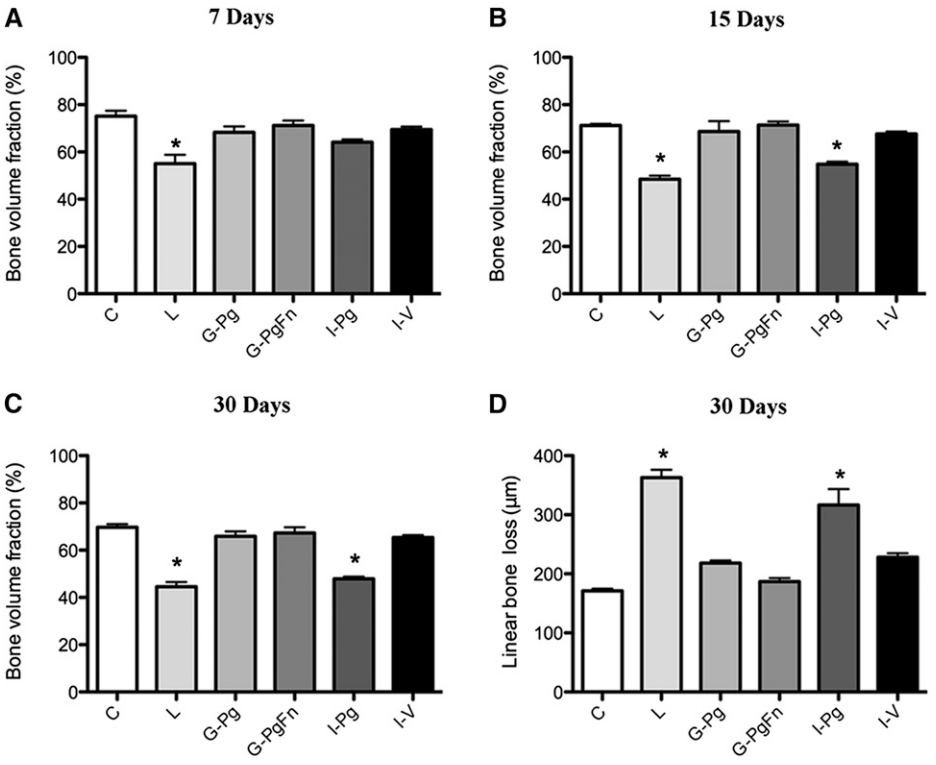

Figure 5.
*The graphs show the BV fraction in all experimental periods **(A through C)** and linear bone loss measured from the CEJ to ABC in a 30-day period **(D)**. All results are expressed as the mean ± SD. *P <0.05, significant difference compared to all the other groups (one-way ANOVA and Tukey post hoc tests).*

agreement with this statement because their own 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 breakdown, 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 used widely to evaluate gene expression and the induction of bone resorption in animal models.[31,40-43] 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 histologic and micro-CT analyses demonstrate that this model

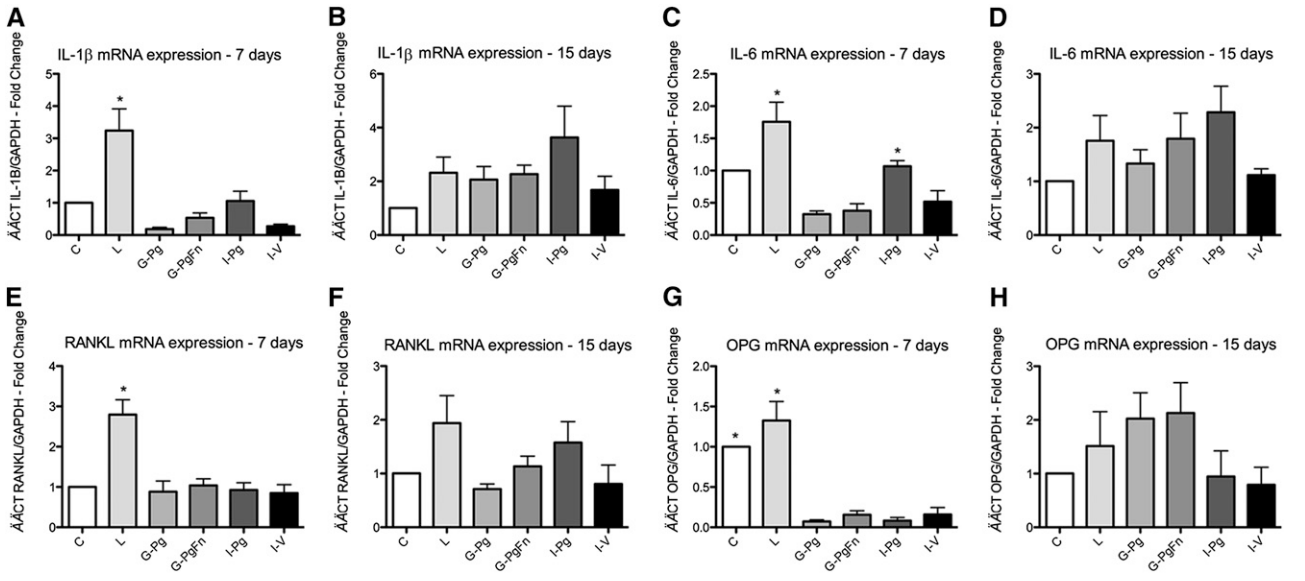

Figure 6.
*mRNA expression of the proinflammatory cytokines IL-1β **(A** and **B)** and IL-6 **(C** and **D)** and the proteins involved in osteoclastogenesis RANKL **(E** and **F)** and OPG **(G** and **H)** during the course of the experimental periodontal disease in all experimental groups (7- and 15-day time points) accomplished by RT-qPCR. Cytokine expression levels were normalized to the expression of the housekeeping gene GAPDH. *P <0.05, significant differences compared to the other groups (one-way ANOVA and Tukey's post hoc tests).*

displays some characteristics of human periodontitis, especially in the area in which the injections were made, including the CT AL and bone breakdown observed at 15 and 30 days and that increased with time.

In the present study, the mRNA of different proinflammatory 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 breakdown. Several biologic mediators released during the tissue breakdown can be induced by increased levels of cytokines, especially RANKL, IL-1β, and IL-6.[22,44] 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 OPG, which in turn inhibits bone resorption by preventing the interaction of RANK and RANKL.[45,46] Changes in the balance between protein expression of RANKL and OPG define the pathogenesis of various bone diseases, including periodontitis.[47] During the progress of periodontal disease, high levels of RANKL can be found in periodontal tissues, explaining alveolar bone resorption.[48] 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.

Conversely, 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 CT degradation and, ultimately, bone resorption.[49,50] Also, IL-1β can induce regulation of adhesion molecules on leukocytes and endothelial cells and stimulate the production of chemokines 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 proinflammatory mediators, and bone loss.

In the present 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[31,38,39] 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 with time. The reason for decrease of the inflammation severity at the 15-day 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 biologic 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. Conversely, in the heat-killed *Pg* 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 three times per week for the entire duration of the experiment. Similarly, both oral gavage models displayed a tendency to increase the expression of proinflammatory mediators with time.

CONCLUSIONS

The results obtained in this study suggest that the ligature and heat-killed *Pg* 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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