Imaging Science in Dentistry 2021
https://doi.org/10.5624/isd.20210058

A 2-plane micro-computed tomographic alveolar bone measurement approach in mice

Raisa Queiroz Catunda[1], Karen Ka-Yan Ho[1], Srushti Patel[1], Maria Febbraio[1,*]

[1]*Department of Dentistry, Faculty of Medicine and Dentistry, School of Dentistry, University of Alberta, Edmonton, AB, Canada*

ABSTRACT

Purpose: This study introduces a standardized 2-plane approach using 8 landmarks to assess alveolar bone levels in mice using micro-computed tomography.

Materials and Methods: Bone level differences were described as distance from the cemento-enamel junction (CEJ) to alveolar bone crest (ABC) and as percentages of vertical bone height and vertical bone loss, comparing mice infected with *Porphyromonas gingivalis* (Pg) to controls. Eight measurements were obtained per tooth: 2 in the sagittal plane (mesial and distal) and 6 in the coronal plane (mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual).

Results: Significant differences in the CEJ-to-ABC distance between Pg-infected mice and controls were found in the coronal plane (middle-lingual, mesiobuccal, and distolingual for the first molar; and mesiobuccal, middle-buccal, and distolingual for the second molar). In the sagittal plane, the distal measurement of the second molar was different. The middle-buccal, mesiobuccal, and distolingual sites of the first and second molars showed vertical bone loss relative to controls; the second molar middle-lingual site was also different. In the sagittal plane, the mesial sites of the first and second molars and the distal site of the second molar showed loss. Significantly different vertical bone height percentages were found for the mesial and distal sites of the second molar (sagittal plane) and the middle-lingual and distolingual sites of the first molar (coronal plane).

Conclusion: A reliable, standardized technique for linear periodontal assessments in mice is described. Alveolar bone loss occurred mostly on the lingual surface of the coronal plane, which is often omitted in studies. ***(Imaging Sci Dent 20210058)***

KEY WORDS: X-Ray Microtomography; Alveolar Bone Loss; Periodontitis; Porphyromonas Gingivalis

Introduction

Periodontitis is a multifactorial inflammatory disease that can lead to an irreversible destruction of the soft and hard tissue structures in the oral cavity, ending in tooth loss. It occurs as a result of complex interactions between the dental plaque biofilm, which contains bacteria, and a susceptible host's efforts to fight the infection, with contributions from environmental and epigenetic factors.[1] Although the subgingival environment is characterized by a large variety of bacteria, with more than 300 species identified from different individuals and ~40 at a single site, only a few species are associated with periodontitis, and they are mainly Gram-negative and anaerobic. One key pathogenic bacterium in periodontitis is *Porphyromonas gingivalis* (Pg).[2]

Studies of the etiology and progression of periodontal disease, as well as pharmacological and surgical interventions, have been widely explored in animal models.[3-8] There remain, however, unanswered questions, given the ethical barriers associated with non-intervention in humans. The benefit of animal models is that they enable a longitudinal assessment of the disease, with similar onset and progression characteristics in several animals, in addition to allowing analysis of the cellular and molecular

We acknowledge the Fund for Dentistry, the Dental Hygiene Research and Master's Stipend Funds (all from the University of Alberta, Department of Dentistry), Frederick Banting and Charles Best Canada Graduate Scholarship Doctoral and Master's Awards (CIHR).
Received March 11, 2021; Revised May 27, 2021; Accepted June 11, 2021
Published online Aug 11, 2021
*Correspondence to : Prof. Maria Febbraio
Department of Dentistry, Faculty of Medicine and Dentistry, University of Alberta, 11361 87 Avenue, Edmonton, AB T6G 2E1, Canada
Tel) 1-780-492-3066, E-mail) febbraio@ualberta.ca

composition of the tissues involved.[4,9,10] The selection of an experimental model is determined by the objective of the scientific research. Mice are a good model for studying periodontitis because they show susceptibility to experimental induction of periodontal disease, are relatively low-cost and easy to handle, have been extensively characterized in terms of their inflammatory and immunological processes, and can be readily manipulated genetically.[11]

Clinical attachment level and, consequently, alveolar bone level, are factors accounted for in the most recent classification of periodontal diseases in humans.[1] With the help of a periodontal probe, the measurements are assessed at 6 standardized sites per tooth: mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual. In mice, the alveolar bone level is a tangible 2-dimensional (2D) measurement that has been used over the years to assess periodontal disease, various treatment modalities, and pathology progression.[3,7,12,13] However, there is a clear lack of standardization of linear measurements in mice, even after the introduction of micro-computed tomography (micro-CT). Regardless of the technology used for assessment, in some studies, sites are randomly assigned, while in others, the sites are established, but standardization of the measurement remains unclear. The most commonly used landmarks comprise the interproximal region (mesial and distal), either within the sagittal plane or in the coronal plane.[3,7,12,13]

There are already multiple variables that can make it difficult to compare data collected from animals from study to study, such as strain, method of periodontal disease induction, age, environmental factors, and diet.[9,10,14] Therefore, a standardized method for alveolar bone level assessment is essential for reproducibility and in order to compare results among studies. The goal of this paper is to introduce a standardized 2-plane approach with 8 linear landmarks for the assessment of alveolar bone levels in mice utilizing micro-CT. Furthermore, the utility of this method is demonstrated in a study of mice orally infected with Pg.

Materials and Methods

Animals

In this study, 10- to 12-week-old C57Bl/6j male mice were used. The animals were maintained and bred in a specific pathogen-free facility at the University of Alberta. The mice received water and were fed a standard rodent chow diet (4% total fat) *ad libitum*. They were kept in the same environmental conditions concerning temperature, humidity, and light/dark cycles. Ethics approval from the University of Alberta Animal Care and Use Committee was granted for this research project prior to study commencement (AUP 2935).

Bacteria

Pg bacteria (ATCC strain 33277) were grown for 72 hours on a blood agar plate under anaerobic conditions and then inoculated into Schaedler broth containing vitamin K and hemin for 24-48 hours, until the optical density of the culture reached 1.3 at 660 nm (approximately 10^9 colony-forming units (CFU)/mL). Cultured Pg were resuspended in saline containing 2% carboxymethyl cellulose (as a thickener to promote adherence) before oral inoculation of the mice.

Experimental design

A periodontal disease induction protocol was used as an experimental model to determine whether this standardized measurement method would adequately and reliably discriminate bone loss. In order to determine the sample size, a power calculation was performed. A 3% difference in bone loss relative to control and a 15-20% difference between groups was considered to be statistically and clinically significant, as per the current literature.[7,12,15] This yielded 20 teeth/group (left and right first molars and left and right second molars ($=4$ teeth $\times 5$ animals/group). Since significant variability in measurements of the third molar was noted in previous studies (unpublished data), only the first and second molars were included in the present study.

Pg infection

Ten mice were divided into 2 groups, Pg-infected and control. Infection was carried out by oral lavage with 200 μL of carboxymethyl cellulose containing ~10^{10} CFU/mL Pg, using a micro-brush to apply the suspension at the gingival margin throughout the mouth on alternate days for 2 weeks (7 applications total). The mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylene (intraperitoneal injection) for the inoculation. This procedure, a modification of the classical technique of Lalla et al.,[16] was performed in a level 2 biocontainment facility. Two weeks after the last inoculation, the mice were euthanized by pentobarbital overdose (200 mg/kg, intraperitoneal injection) followed by bilateral thoracotomy, according to Canadian Council on Animal Care guidelines.[17]

The heads were collected and fixed in 4% formaldehyde for 48 hours.

Histological assessment of inflammation

The micro-CT findings were compared to the histological assessment of inflammation for validation. Following micro-CT scanning, the jaws were decalcified, embedded in paraffin, and sectioned (7-µm thickness). After de-paraffinization, the slides were stained with hematoxylin and eosin and with a pan-leukocyte marker (CD45) to identify inflammatory cells (catalog #53665, Santa Cruz Biotechnology Inc., Dallas, TX, USA), according to the supplier's instructions. CD45 positive cell counts were performed by 3 independent examiners and the results were calculated as the ratio of the difference between the Pg-infected group divided by the control group (fold change formula). A total of 50 images (5 per animal) of the masticatory gingiva were analyzed.

Scanning of the mandibles

The mouse heads were 3-dimensonally (3D) micro-CT scanned at a 25-µm voxel size resolution using the following settings: 360°; exposure, 75 ms; voltage, 50 kV; current, 0.24 mA (MILabs U-CT, Utrecht, Netherlands). Each head was placed separately and scanned and saved as a Neuroimaging Informatics Technology Initiative (NIftI) file, a format commonly used in imaging informatics. In this format, the first 3 dimensions define the 3 dimensions in space (x, y, and z). The scanned image was then reconstructed using MILabs Software (MILabs, Utrecht, Netherlands) aligning the sagittal, coronal, and axial planes, using the nasal septum and the occlusal plane of the first molars as references. The reconstructed scan was saved as a "parcel" file (a tool that bundles all the parts of the files into a way they can be compacted and exported).

Quantification of alveolar bone level

Three-dimensional reconstructions were performed using Avizo software (version 2019.1, Berlin, Germany). To assess the alveolar bone levels of mice, a measurement protocol with 3 landmarks was defined: the cemento-enamel junction (CEJ), alveolar bone crest (ABC), and root apex. The distance from the CEJ to ABC was measured in millimeters (up to 2 decimal points). These measurements were completed for the first and second molars of the mandible on both sides, since mandibular molars are most often used for measurements in the literature and are birradicular (anatomical consistency).[3,13,18-20]

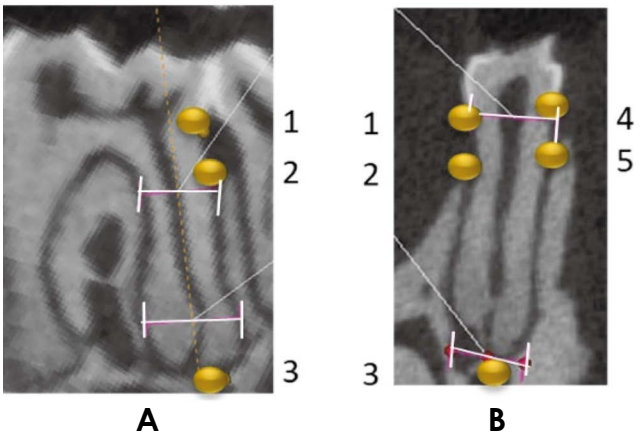

Fig. 1. A. Sagittal landmarks (dots). 1. cemento-enamel junction. 2. alveolar bone crest. 3. root apex. B. Landmarks of the coronal plane (the line defines the ruler used to define the middle of the tooth). 1. cemento-enamel junction (lingual). 2. alveolar bone crest (lingual). 3. root apex. 4. cemento-enamel junction (buccal). 2. alveolar bone crest (buccal).

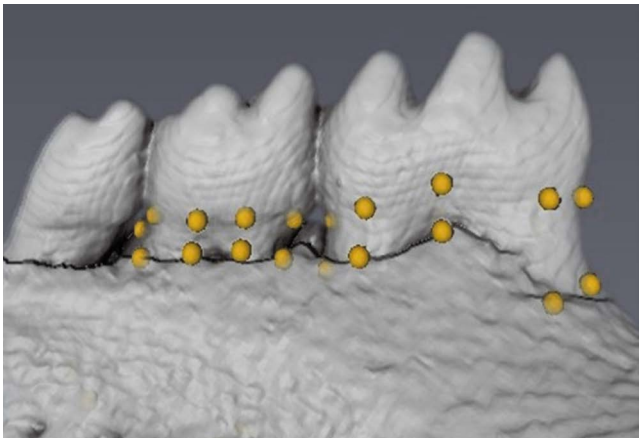

Fig. 2. Three-dimensional lingual view of the landmarks (dots) used for linear measurements.

Similar to human examinations, a total of 8 measurements were obtained: 2 in the sagittal plane (mesial and distal, Fig. 1A) and 6 in the coronal plane (mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual, Fig. 1B), totaling 32 measurements per animal (first and second mandibular molars). Figure 2 shows a 3D view of measurements from the buccal perspective.

Landmark standardization according to planes

In order to achieve standardized sagittal measurements, the coronal plane of the first molar was initially oriented. Sagittal plane measurements were taken, using the soft-

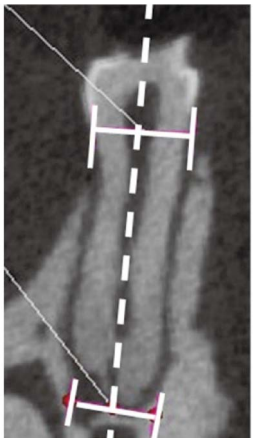

Fig. 3. Definition of the sagittal plane in the coronal plane (the same for both roots). In white: ruler tool; white line + dashed red line represents the middle point.

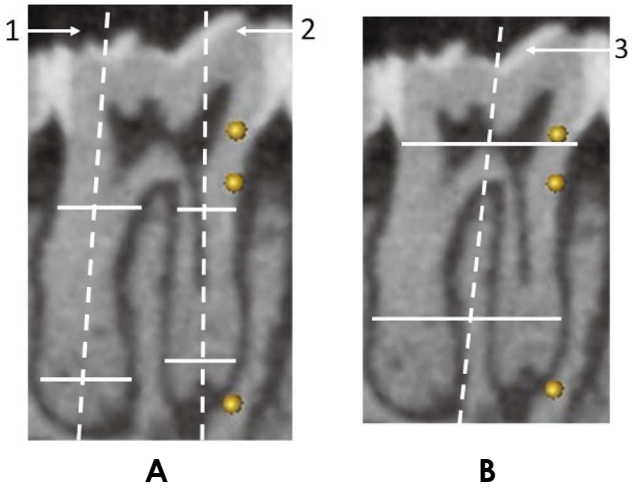

Fig. 4. A and B. Definition of the coronal plane in the sagittal plane for mesial, distal, and middle measurements. 1. Orientation plane for lingual and buccal coronal distal measurements. 2. Orientation plane for lingual and buccal coronal mesial measurements. 3. Orientation plane for lingual and buccal coronal middle measurements (dots demarcate landmarks; dashed lines represent the mid-points of the root/pulp chamber).

ware's measurement line and ruler tools to precisely ensure the center of the middle pulp chamber and root apex (Fig. 3). This can be achieved by drawing a line that passes through the middle of the CEJ, ABC, and root apex.

For coronal measurements, the sagittal plane was used for orientation of the buccal and lingual sides (Fig. 4). The coronal plane measurement line was precisely aimed at the middle of the floor of the pulp chamber in the designated root and the middle of the last third of the root. Again, the ruler tool with millimeter markings was used to ensure that the center of the root was established (the root was divided in thirds and the ruler was used from each root external wall–buccal and lingual–to establish the middle point).

To obtain the middle-buccal and middle-lingual measurements, the plane was defined as the center of the pulp chamber and the middle distance between the last third of the 2 roots (Fig. 4C). After the planes were established, measurements could then be performed.

Results were presented as the distance in 3 different ways: 1) millimeters from the CEJ to the ABC; 2) as a percentage of vertical bone height (utilizing the formula CEJ-ABC / total root length [TRL: distance from the root apex to the CEJ] × 100); in humans, vertical bone height is classified as the remaining vertical bone height and the vertical bone height that has been lost, whereas in mice, there is no standardization regarding what would be "clinically" significant; therefore, the calculation of how much vertical height was lost within the same animal was called "vertical bone height" to avoid any confusion with actual bone loss, which is described in #3 below; and 3) bone loss relative to the animals that were not exposed to Pg, calculated using the formula below:

$$\text{Vertical bone height } \% = \frac{(\text{CEJ to ABC})}{\text{TRL}} \times 100$$

$$\text{Bone loss } (\%) = (\text{Vertical bone height of PG-infected group}) - (\text{Vertical bone height of control group})$$

All alveolar bone measurements were performed by calibrated and blinded examiners. Using the Euclidean distance formula below (calculated from the Cartesian coordinates of the points using the Pythagorean theorem), data were collected for statistical interpretation.

$$d(p,q) = \sqrt{\sum_{i=1}^{n} (q_i - p_i)^2}$$

p, $q = 2$ points in Euclidean n-space, q_i, p_I = Euclidean vectors, n = n-space

Statistical analysis

Prism software was employed to perform statistical analyses (GraphPad, La Jolla, CA, USA). Data were analyzed for normal distribution using the Shapiro-Wilk test. If normally distributed, data were subjected to the parametric t-test; if not, the non-parametric Mann Whitney-U test was used. The mean and standard error of the mean (SEM) were calculated for both groups. Statistical signifi-

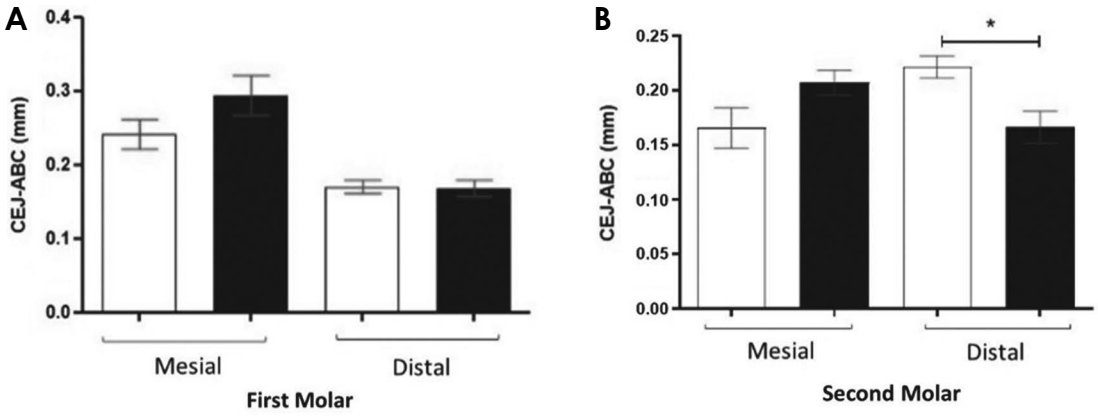

Fig. 5. Distance measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) in millimeters for each group (Pg-infected versus control) in the sagittal plane for mesial and distal sites. A. First molar. B. Second molar. *$P<0.05$, n = 10 first and 10 second molars/group. Pg: *Porphyromonas gingivalis*.

cance was set at $P<0.05$.

To calculate the error of the method, 3 blinded examiners were calibrated, and inter- and intra-examiner correlations were evaluated using the Cohen kappa for all measurements. Five animals were randomly selected, and a total of 160 measurements were compared (first and second molar, right and left side of the mandible; 4 teeth/mouse for a total of 20 teeth). The analysis was performed by each examiner with a 1-week interval between each measurement. The average error accepted was $\leq 3\%$ or 0.02 mm.

Results

The inter-examiner Cohen kappa scores for measurements were determined to be 0.80, 0.85, and 0.90 for each measurement cycle, and the intra-examiner Cohen kappa was 0.61, 0.70, and 0.80 for each examiner (all showing "substantial" to "strong" agreement).

The Pg-infected group presented some degree of alveolar bone loss in both planes, as expected. However, the differences in the percentage of vertical bone loss and the ABC-to-CEJ (mm) distance depended upon which planes were analyzed.

In the sagittal plane (Fig. 5), only 1 site (the distal site of the second molar) showed a statistically significant difference in the CEJ-to-ABC distance ($P<0.05$) (Fig. 5B). In the control group, the distance varied from 0.16 mm to 0.24 mm; in the Pg-infected group, the distance varied from 0.16 mm to 0.29 mm. The average ± SEM values for the control group versus the Pg-infected group per site were as follows: mesial first molar: 0.24 ± 0.02 mm versus 0.29 ± 0.02 mm; distal first molar: 0.16 ± 0.008 mm versus 0.16 ± 0.01 mm; mesial second molar: 0.16 ± 0.01 mm versus 0.20 ± 0.01 mm; distal second molar: 0.16 ± 0.01 mm versus 0.22 ± 0.01 mm.

The average values of the combined coronal measurements of the CEJ-to-ABC distance (control versus Pg-infected group) were as follows: 0.15 ± 0.01 mm versus 0.19 ± 0.01 mm for the first molar ($P<0.05$) and 0.12 ± 0.006 mm versus 0.15 ± 0.005 mm for the second molar ($P<0.05$) (Figs. 6A and B). When analyzing all CEJ-to-ABC distance measurements in the coronal plane separately, the distolingual and middle-lingual measurements of the first molar and the distolingual measurement of the second molar showed statistically significant differences compared to the control group ($P<0.05$). On the buccal surface, the mesial measurement of the first molar and second molar, as well as the middle-buccal measurement for the second molar showed differences that were statistically significant ($P<0.05$) (Figs. 6C-F).

In terms of vertical bone loss, of the 6 locations in the coronal plane for each molar, 4 sites showed loss relative to control on the buccal side: the middle and mesial sites of the first molar (1.71% and 1.14%, respectively) and the middle and mesial sites of the second molar (1.05% and 3.32%). On the lingual side, 3 locations showed loss: the distal site of the first molar (2.08%) and the middle and distal sites of the second molar (4.43% and 1.15%) (Figs. 7A and B). In the sagittal plane, the sites that showed loss were the mesial sites of the first molar and second molar (3.11% and 4.82%, respectively) and the distal site of the second molar (5.18%) (Fig. 7C).

In terms of percentage of vertical bone height, for the sagittal plane, the control group showed variation from 12.50% to 16.19%, whereas the Pg-infected group ranged

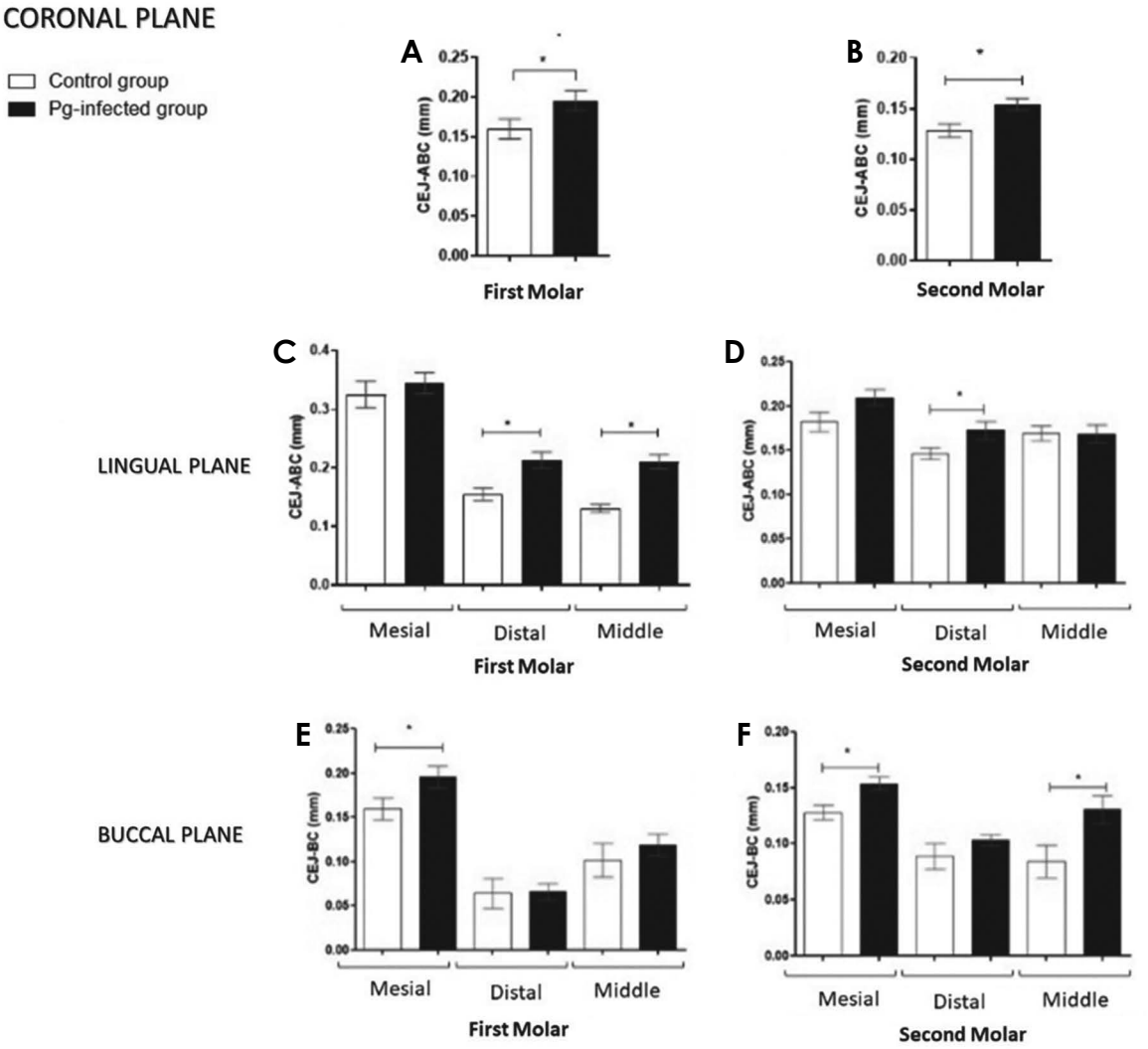

Fig. 6. Distance measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) in millimeters for each group (Pg-infected versus control) combined in the coronal plane. A. First molar. B. Second molar; Individual measurements of the lingual surface. C. First molar. D. Second molar; Individual measurements of the buccal surface. E. First molar. F. Second molar. *$P < 0.05$, n = 10 first and 10 second molars/group. Pg: *Porphyromonas gingivalis*.

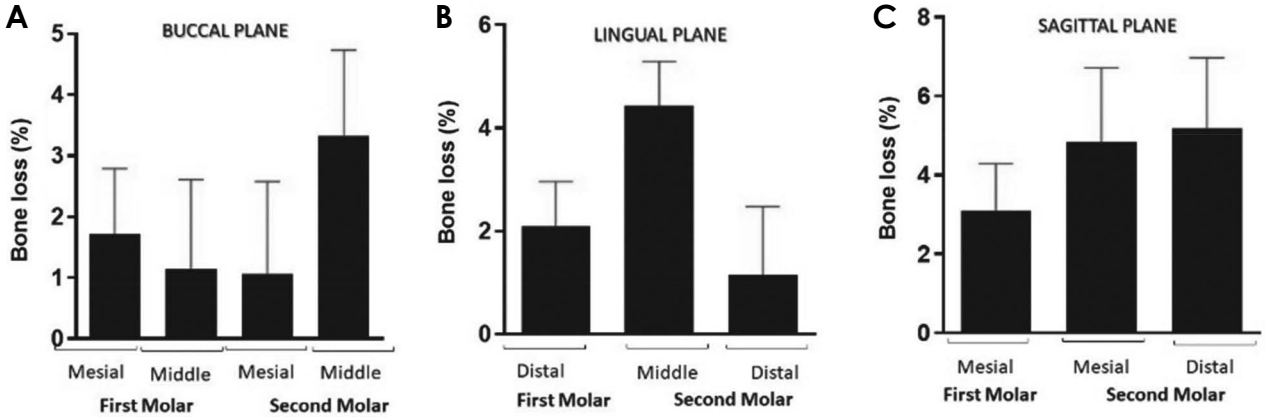

Fig. 7. Percentage of bone loss in the Pg-infected group relative to control. A. Buccal surface. B. Lingual surface. C. Sagittal plane. Only sites that presented bone loss are shown. Pg: *Porphyromonas gingivalis*.

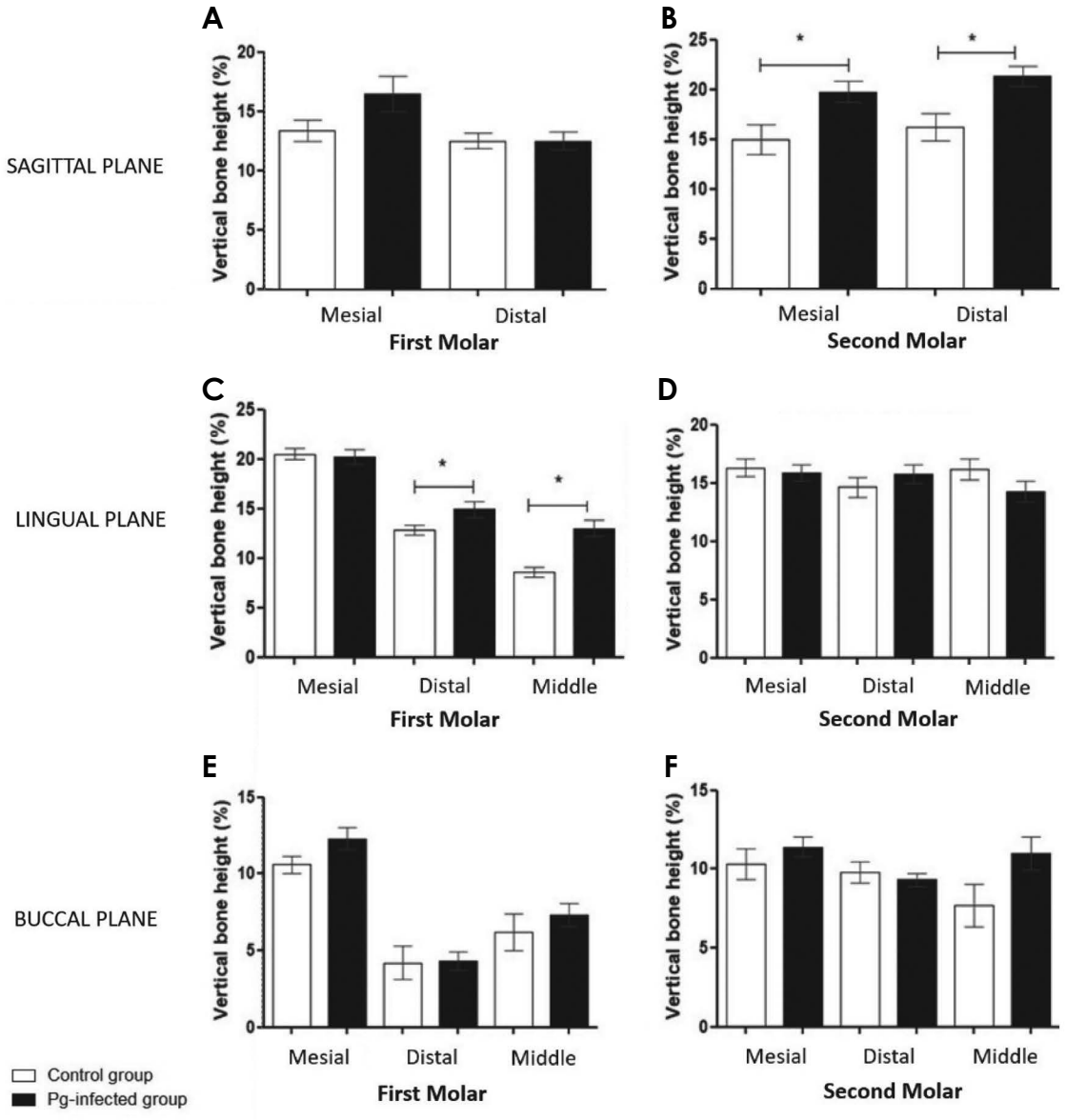

Fig. 8. Alveolar bone height shown as the percentage of total root length for Pg-infected and control groups. Combined measurements in the sagittal plane. A. First molar. B. Second molar; individual measurements of the lingual surface. C. First molar. D. Second molar; individual measurements of the buccal surface. E. First molar. F. Second molar. *$P<0.05$. Pg: *Porphyromonas gingivalis*.

from 12.49% to 21.37% (Figs. 8A and B). Statistically significant differences were observed at the mesial and distal sites of the second molar ($P<0.05$). In the coronal plane, the percentage of vertical bone height varied from 4.21% to 20.5% in the control group and from 4.34% to 20.15% in the Pg-infected group, with the lowest percentages observed on the buccal surface and the highest percentages on the lingual surface (Figs. 8C-F). Out of the 6 locations in the coronal plane, 2 sites showed a significant difference relative to control: the middle-lingual and distolingual sites of the first molar ($P<0.05$).

A classic method of assessing oral inflammation is via histology; hematoxylin and eosin staining and immunocytochemistry with a specific inflammation marker (CD45) were utilized to show that oral infection with Pg induced inflammation concomitant with bone loss (Fig. 9). The Pg-infected group presented more CD45-positive cells than the control group (2.53-fold increase; $P<0.05$).

Discussion

This manuscript describes a standardized micro-CT approach to perform linear periodontal measurements of 8 different sites per mandibular molar in a murine model.

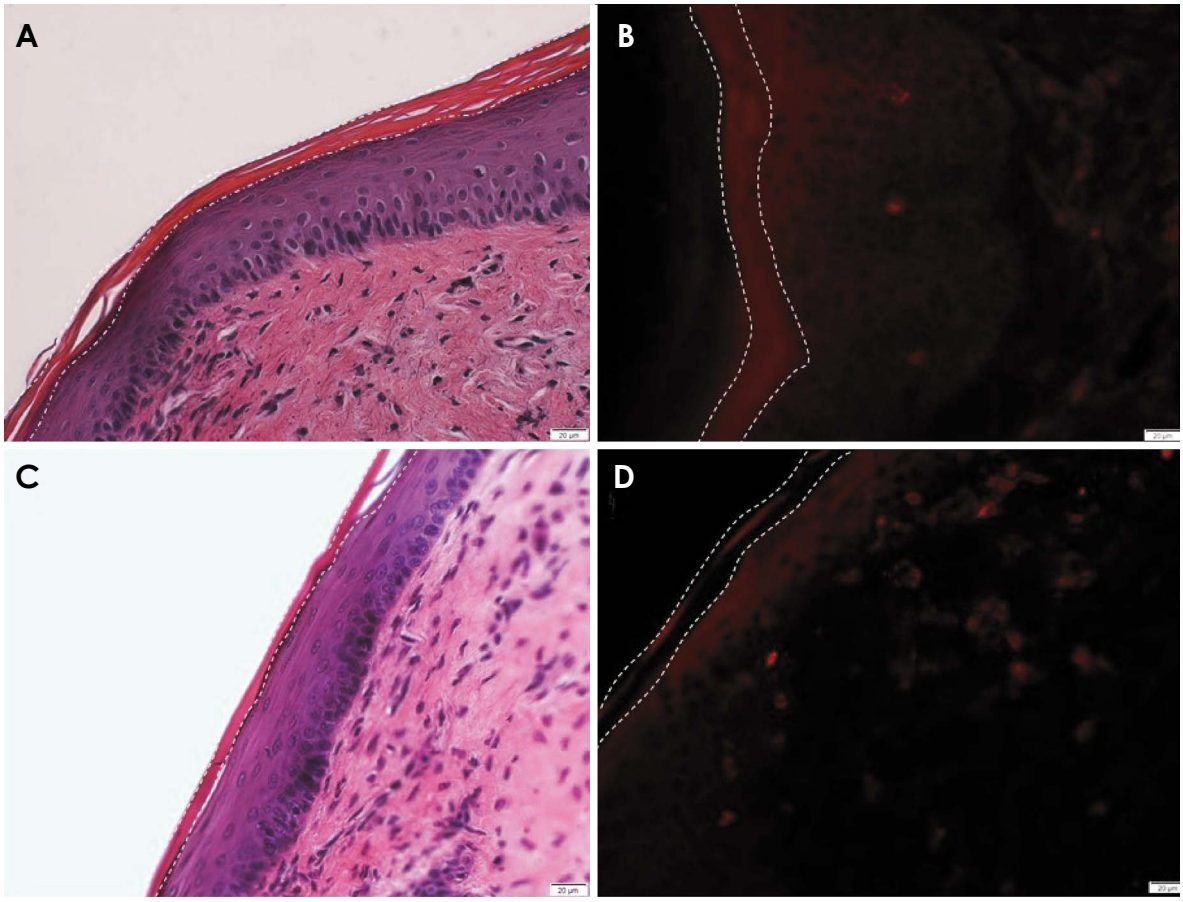

Fig. 9. Hematoxylin and eosin-stained oral cavity tissue from masticatory gingiva. A. Control and C. Pg-infected mice; CD45-positive cells in the oral cavity tissue from masticatory gingiva. B. Control and D. Pg-infected mice. Dashed lines show the keratinized oral tissue border for localization purposes; CD45-positive cells were visualized by red fluorescence (magnification: ×400). Pg: *Porphyromonas gingivalis*.

There is a great variety of linear measurements described in the literature,[3,7,13,18,20,21] but the lack of consensus regarding the sites chosen and the variability in the assessed teeth and single-plane measurements remain concerns. In Figure 10, alveolar bone loss is shown in a 3D view of the lingual sites (middle and mesiobuccal). The results clearly show that if only the sagittal or buccal surfaces, for example, were measured, there could be an underestimation of the disease extent.

When performing research using an animal model of periodontal disease, many factors play a role in the degree and severity of the disease. Some of these factors include the species of animal, the method utilized to promote bone resorption (e.g., bacteria or ligation), the specific bacteria used for infection, the period of study following infection or ligation, and the methods associated with disease evaluation. The methodology used to perform measurements should not be a source of bias introduced into the evaluation.[5,8-10,14,15,21,22] The importance of a consistent set of sites and landmarks reflects the fact that linear measurements are technique-sensitive and minor changes in angulations/positions of the sample might impact the results.[7]

The most commonly used measurement to determine alveolar bone loss in mice is the distance from the CEJ to the ABC.[3,6,7,18,21,23-26] The results of the present study showed that alveolar bone loss occurred mostly on the lingual surface of the coronal plane, a surface not often included in studies, and in the sagittal plane.[7,18,21] Studies found in the literature that did include the coronal plane (buccal and lingual/palatal), combined their results as an average; therefore, it is not possible to confirm whether those findings are similar to the findings of the present study.[3,6,15,23-27] The studies that analyzed bone loss utilizing a percentage method had findings that varied considerably, with reported values of 6-8%,[12] 10-30%,[13] 15-29%,[20] and 5-28%.[28] Those studies included vertical bone loss, bone loss compared to control, or the bone volume fraction. There was substantial variability (5-30%) due to the sites measured or methodology applied; howev-

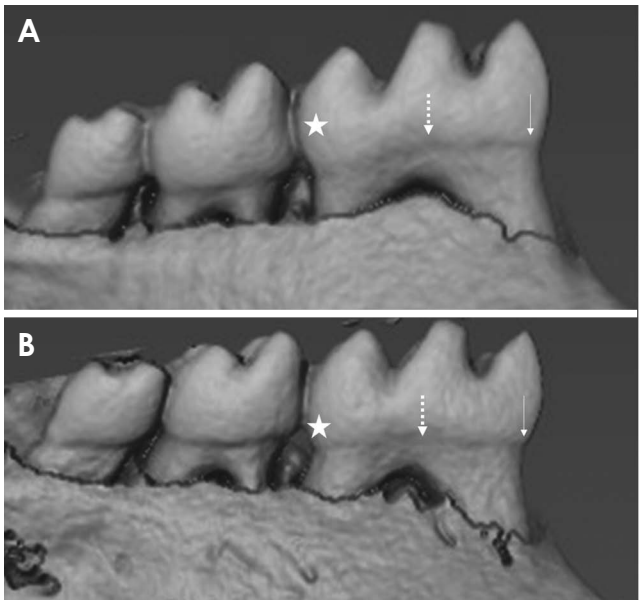

Fig. 10. Lingual view of 3 mandibular molars. A. Control mouse. B. Pg-infected mouse. Stars: distolingual, dashed arrows: middle-lingual, arrows: mesiolingual.

er, some of the reported values are broadly similar to the variability found in the present study (4.34-21.37%).

It is fundamental to note that the goals of an individual study often dictate the method of infection and the type of bacteria used for infection (or a combination), and that different methods will generate different patterns of bone resorption. Oral lavage with Pg was used in this study as a test method of infection. It is paramount to have a measurement strategy that examines the tooth completely, similarly to what is done in human periodontal probing to assess bone loss. This ensures that no instances of isolated bone loss will be excluded from the analysis.[1,29-32]

Vertical bone height characterizes the length of the part of a tooth that appears above the ABC (measured based on the CEJ) versus the part that lies below (CEJ up to the root apex). This number can also be expressed as a ratio or a percentage.[33] After Pg infection, all planes presented some degree of bone loss. When assessing alveolar bone loss, the vertical bone height percentage was compared by subtracting the value found in the Pg-infected group from that found in the control group to assess how much loss occurred at each site. This is important because animals and their root length may vary in size. Expressing the measurement in this manner eliminates any root size bias; therefore, this method is often used in the literature to assess both bone loss and bone gain.[18] The present study showed that lingual sites had a higher percentage than buccal sites, but similar percentages when compared to sagittal, mesial, and distal sites. In the literature, the studies that included vertical bone height, unfortunately, only included buccal sites.[12,34] This could be a very significant omission, given that lingual sites showed percentages of vertical bone height of up to 20% and buccal sites had a maximum of 12% in the present study; the latter value is similar to what has been published in the literature.[12,34] When determining any type of loss, it is important not to underestimate or overestimate the disease. Since lingual sites showed double the percentage of vertical bone height of buccal sites, this dramatic difference should be considered when conducting assessments.

Some of the limitations of the present study are that only 1 type of software was used (Avizo); therefore, the landmarks and planes might not be applicable to other types of software that do not allow the user to move the sample freely. The linear measurements accounted for isolated bone loss but not intra-bone loss; therefore, volume and density would need to be measured to assess alveolar bone internally. This method was utilized to measure alveolar bone in C57Bl/6j male mice molars; therefore, the measurements may be applicable to animals with similar dento-alveolar anatomy such as hamsters and rats, but not larger mammals, given the variability across species in the number and shape of teeth.

Acknowledgments

The authors gratefully acknowledge Dr. Manuel Lagravere-Vich for his support with Avizo software and for his advice in developing landmarks for this protocol. We also acknowledge Dr. Maria Alexiou and Pranidhi Baddam for support in scanning and reconstructing the animal heads.

Conflicts of Interest: None

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