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The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A_2 inhibitor on alveolar bone loss in an *in vivo* mouse model of periodontitis

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Background and Objective: Live-animal micro-computed tomography is a new and promising technique that can be used to quantify changes in bone volume for periodontal disease models. The major aim of this study was to develop the methodology of live-animal micro-computed tomography and to determine the effect of a novel secretory phospholipase A_2 inhibitor on alveolar bone loss.

Material and Methods: Periodontitis was induced in mice by oral infection with *Porphyromonas gingivalis* over a period of 13 wk, and live-animal micro-computed tomography scans were taken at different time-points to determine bone volume changes with disease progression. This enabled conclusions to be made as to when treatment was most likely to be effective. In addition, the model was used to investigate a novel drug, the secretory phospholipase A_2 inhibitor, KHO64, and its potential ability to inhibit osteoclast bone resorption and treat periodontitis.

Results: The results from live-animal micro-computed tomography scans revealed greater, statistically significant, bone volume loss in diseased mice compared with normal mice ($p < 0.05$). This corresponded to a larger area from the cemento–enamel junction to the alveolar bone crest, as assessed by stereo imaging ($p < 0.001$). These techniques can therefore detect and quantify alveolar bone loss. Both methods revealed that KHO64 had no significant effect on the volume of bone resorption.

Conclusion: Live-animal micro-computed tomography is a robust, reproducible technique that clearly demonstrates significant time-dependent changes in alveolar bone volume in a small-animal model of periodontitis.

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Periodontitis is a destructive inflammatory disorder of the periodontium that is characterized by enhanced osteoclast development and activity, resulting in pathological alveolar bone resorption (1–3). This disease is one of the most common forms of pathological bone loss that occurs in humans and negatively impacts a substantial proportion of the population. Up to 60% of the population suffers from chronic periodontitis and it is a significant cause of tooth loss in adults (4). Despite this, there are few effective therapies to treat the destructive bone resorption.

Mouse models of periodontitis are commonly used to investigate the effects of novel drugs on periodontitis-induced alveolar bone resorption (5). Determining the extent of disease induced in these models requires analysis of the alveolar bone resorption. Many of the current methods used (histomorphometry and two-dimensional radiography) are not effective at giving a true indication of disease severity and can also be error-prone (6). One of the most effective methods for determining the extent of periodontitis is micro-computed tomography which has many distinct advantages as it enables detailed three-dimensional microarchitecture of the alveolar bone to be produced with data that can be analyzed in different planes (7). Many studies have investigated the use of micro-computed tomography in animals, but these have not used live animals scanned at different time-points. Wilensky's group demonstrated the high sensitivity for detection of alveolar bone loss that can be achieved using micro-computed tomography and suggested that it could be used effectively to study the role of periodontal bacteria in mouse models of periodontitis (8). A subsequent study by Park and colleagues investigated three-dimensional micro-computed tomography imaging of alveolar bone in experimental bone loss (6). In this study, significant differences were recorded in the area measured from the cemento–enamel junction to the alveolar bone crest between the healthy group and the infected group. The authors also noted that there was no significant difference between the baseline and the 8 wk measurement in the animals without disease, indicating the reproducibility of the method (6).

Although these studies demonstrated the reproducibility of micro-computed tomography, they involved the need to kill large numbers of animals at various time-points for scanning to occur. New technology has been developed which allows quantitative live-animal micro-computed tomography analyses to be performed over the duration of a time-course experiment. This allows investigators to follow live animals throughout the disease process and to monitor changes in bone loss before and after drug administration. Mice can be repeatedly scanned throughout a study and so can also form their own controls.

The novel drug, KHO64, is an inhibitor of the enzyme secretory phospholipase A_2, specifically the group IIa isoform which is involved in the osteoclastogenesis process (9,10). By decreasing osteoclast bone resorption, this drug may potentially provide an effective treatment adjunct for periodontitis. Secretory phospholipase A_2 enzymes increase arachidonic acid release from membrane phospholipids, eventually resulting in the production of prostaglandin E_2 that has been shown, under some circumstances, to stimulate osteoclast formation and modulate bone resorption (11,12). KHO64 has been shown to be an effective inhibitor of bone loss in osteoporotic rats (13). Therefore, secretory phospholipase A_2 inhibitors, such as KHO64, may also convey benefits by reducing arachidonic acid metabolite levels (such as prostaglandins, thromboxanes and leukotrienes) in the body (14). Accordingly, it is suggested that secretory phospholipase A_2 inhibitors may have potential value in inhibiting osteoclast bone resorption and could ultimately provide an effective adjunct treatment for periodontitis.

This study had two main aims: first, to develop novel methods of accurately assessing bone loss in a mouse model of periodontitis using recently developed live-animal micro-computed tomography; and, second, to use these methods to determine the effect of a secretory phospholipase A_2 inhibitor, KHO64, on alveolar bone resorption in a mouse model of periodontitis.

Material and methods

Mouse model of periodontitis

Experimental periodontitis was induced in 8-wk-old female BALB/c mice ($n = 10$). All mice were housed in the Institute of Medical and Veterinary Science Animal House and special bedding was used to ensure that there were no antibacterial products in the surrounding environment. Kanaomycin (1 mg/mL in water) was administered to all mice for 1 wk before beginning the experiment to ensure eradication of all endogenous bacteria. At the completion of the study, all mice were killed by CO_2 inhalation and the heads were kept for bone analysis. Animal ethics approval for this study was obtained from the Institute of Medical and Veterinary Science/Central Northern Adelaide Health Service, Animal Ethics Committee.

Porphyromonas gingivalis inoculations

Porphyromonas gingivalis (strain W50) was seeded onto anerobic blood agar plates and incubated under anerobic conditions at 37°C. After 3–4 d of culture, bacterial cells were suspended in 2 mL of carboxymethyl-cellulose (2% carboxymethyl-cellulose in phosphate-buffered saline) for oral inoculation of mice. The viable count of the bacteria was determined to be 24.3×10^{10} colony-forming units/mL and the dry weight was 12.5 mg/mL. All mice receiving the disease were inoculated with *P. gingivalis* bacteria throughout the entire study. The first inoculation sequence (four inoculations over 8 d) began following antibiotic administration and continued for 1 wk with 0.1 mL of bacteria (in 2% carboxymethyl-cellulose) being orally administered to each mouse. Following each inoculation, mice were kept without food and water for 1 h to ensure the effectiveness of the inoculation. Following the first inoculation

sequence, all mice were inoculated twice a week for 2 wk. This was followed by a second inoculation sequence (four inoculations over 8 d). All mice continued to be inoculated twice a week throughout the remainder of the experimental period.

Drug treatment by oral gavage

To investigate its effect on alveolar bone resorption, KHO64 was administered to mice following the second inoculation sequence when bone loss was found to be greatest. Mice (*P. gingivalis* and drug-treated mice, $n = 5$) were treated daily for a period of 5 wk by oral gavage. The drug-treated mice received 0.1 mL of KHO64 (5 mg/kg/d) suspended in olive oil (10).

Live-animal computed tomography treatment protocol

Mice were scanned using the live-animal computed tomography scanner (Skyscan 1076 High Resolution *In Vivo* Scanner; Skyscan, Kontich, Belgium) to determine changes in bone volume. This machine has a 10 megapixel camera with a tungsten 100 kV X-ray source and a spot size of 5 μM. For scanning, the X-ray source was operated at 75 kV and 120 mA with a 1-mm tungsten filter. The scanning width was set to 35 mm with a pixel size of 9 μm and spatial resolution of 9-μm pixels. At time 0, mice were scanned to obtain a baseline measurement before disease was induced as described above. Mice were subsequently scanned at weeks 7 and 13 to allow time-dependent analysis of bone volume. Five control mice (no disease and not treated with drug) were scanned at the same time-points to give an indication of the increase in bone volume that occurs with normal mouse growth.

Live-animal computed tomography methods

For scanning, mice were anesthetized, positioned in a polystyrene foam holder and placed in the 3-cm (mouse) carbon fibre bed. Each scan took approximately 25 min to complete. Scans were then reconstructed using the volumetric reconstruction software 'NRECON' (Skyscan). This program uses a modified Feldkamp algorithm with automatic adaptation to reconstruct the three-dimensional images with 8.7 voxels. The vertical boundaries of the area of interest were selected from the beginning of the first molar (maxilla) until no teeth remained in the slices (this normally occurred after 490 slices). For the first slice, the entire quarter of the image was outlined to ensure that the image was resliced in the correct orientation. The histogram setting was adjusted to a threshold of 72–175 to distinguish between hard tissues (i.e. alveolar bone and enamel from the soft tissues). The white areas indicate the areas included in the analysis, whereas the black regions were not included. The range selected was 60–2708.1 Hounsfield unit (HU), as enamel has a threshold value of 200, which equates to 3095 HU, and a soft tissue value of 60, equating to 50 HU (15).

To create this model, a density window was set from 0 to 190 to ensure that only bone would be reconstructed. A plane (1024×1024 pixels) was aligned on the model along the cemento–enamel junction of the three molars and the model was resliced to obtain a bone volume analysis. The plane was aligned identically for all three scans to allow comparisons to be made. Reslices were selected from the cemento–enamel junction down to the alveolar bone. A rectangle (5×2.01 mm) was selected ensuring that all bone (both alveolar bone and other dental hard tissues) was included and its size was kept constant for all scans. The histogram was set from 0–180 (previously determined to be normal alveolar bone density). The bone volume was calculated and used for comparisons in individual mice at the three different time-points (time 0, and at weeks 7 and 13).

Stereo imaging of heads

After the mice were killed, their heads were skinned and placed in 1% NaOH until all the flesh had disappeared (approximately 48 h). The skulls were stained with methylene blue (0.003%) and imaged using a stereomicroscope (MZ16FA Leica Stereomicroscope; Leica Microsystems GmbH, Wetzler, Germany). Right-hand and left-hand sides, buccal and lingual surfaces were imaged using a ×1.0 objective lens. The area from the cemento–enamel junction to the alveolar bone crest was measured for each molar (molar 1 to molar 3) using IMAGE J (http://www.rsbweb.nih.gov/ij/download.html).

Statistical analysis

Results were presented as mean ± standard error of the mean. Statistical differences between groups were analyzed using the Student's *t*-test. A one-way analysis of variance was used to compare two or more groups, followed by a Tukey's *post hoc* test. Statistical significance was accepted when the *p*-value was less than 0.05 ($p < 0.05$).

Results

Induction of periodontitis in the mouse model

Periodontitis was initially induced in mice over a period of 13 wk to determine when bone loss occurred and therefore to determine the appropriate time for drug treatment. Oral inoculation with *P. gingivalis* bacteria in these mice appeared to be a good model of human periodontitis, with development of chronic inflammation (increased tissue volume and redness) and associated alveolar bone loss (decreased bone volume). Analysis of bone volume using computed tomography revealed a significantly smaller increase in bone volume from time 0 to week 13 in diseased mice compared with the controls ($p = 0.0037$), as shown in Fig. 1. Computer three-dimensional models revealing the change in alveolar bone from day 0 to week 13 are shown in Fig. 2A,B, respectively. The increase in bone volume in controls indicated that mice of the age used in this study were still growing. From week 7 to week 13 there was a slight decrease (0.43%) in bone volume in diseased mice, whereas bone volume in the control mice increased by an average of 12.8% ($p < 0.05$). This indicated that bone loss occurred in weeks 9–13 in

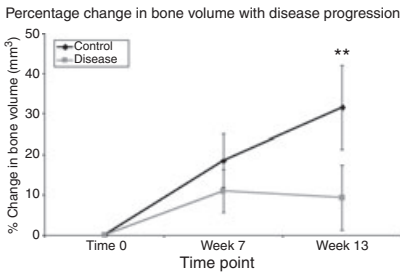

Fig. 1. Average percentage change in bone volume (mm^3) for diseased mice (*Porphyromonas gingivalis* inoculated; $n = 5$) compared with controls (no disease or treatment; $n = 5$) for the following three time-points: time 0, week 7 and week 13. Percentage changes are shown from time 0 to week 7 and from time 0 to week 13. Bars represent mean ± SEM. The Student's *t*-test was used to determine significance. $^{**}p < 0.01$, statistically significant compared with the control at week 13.

this model and hence this was the most appropriate time for drug treatment.

Stereo imaging (Fig. 3) of mouse jaw bones retrieved at week 13 confirmed this result as it revealed a significantly ($p < 0.001$) larger area from the cemento–enamel junction to alveolar bone crest for diseased mice compared with the controls, confirming that periodontitis had been induced. Figure 3 shows a stereo image of a control and a diseased mouse jaw bone in which bone loss is evident. This model of periodontal disease also resulted in an average loss in body weight of 8.5% compared with an increase of 4% observed in control mice. However, this was not statistically significant ($p > 0.05$). The weight loss is probably a systemic effect of the inflammatory periodontitis.

Effect of a secretory phospholipase A_2 inhibitor on bone resorption

Following the initial study, the effect of the secretory phospholipase A_2 inhibitor KHO64 was investigated to determine its effectiveness as a treatment for periodontitis. This drug, however, was not found to reduce bone resorption associated with periodontitis. Surprisingly, we observed an increase in the amount of bone compared with control mice, but this was not statistically significant ($p > 0.05$; see Fig. 4). From weeks 7 to 13 there was a 3.7% decrease in bone volume in treated mice compared with the 12.8% increase in bone volume in control mice ($p < 0.001$), as shown in Fig. 4. This bone loss in treated mice was also evident in the three-dimensional model (Fig. 2D), which can be compared with the control at 13 wk (Fig. 2C).

Stereo imaging supported the bone volume analysis using computed tomography as it revealed no significant reduction in the area from the cemento–enamel junction to the alveolar bone crest for the drug-treated group in comparison to the untreated mice. The total area (from the cemento–enamel junction to the alveolar bone crest) for the three molars from the diseased mice was also found to be larger than in the treated group, as shown in Fig. 5, but this was not statistically significant.

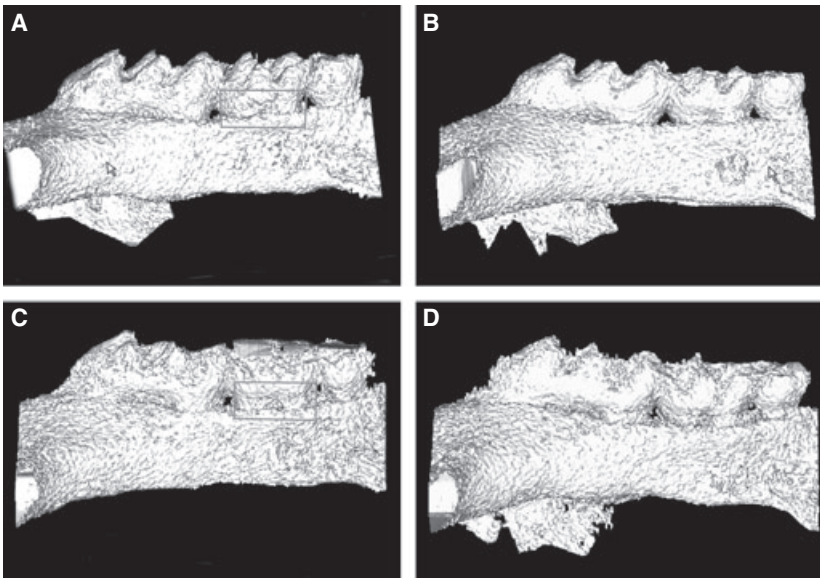

Fig. 2. Three-dimensional models (8.7 voxels) of the three molars of the maxilla (created using the Skyscan analysis program ANT) demonstrating the bone resorption in diseased mice and drug-treated mice compared with the control. The box in panels A and B shows the same area around molar no. 2 in which bone resorption became evident as the disease progressed. (A) Three-dimensional model of a diseased mouse at time 0. (B) Three-dimensional model of a diseased mouse at week 13. (C) Three-dimensional model of a week 13 control mouse. (D) Three-dimensional model of a week 13 drug-treated mouse.

Discussion

The mouse model used in this study was found to be a reliable method for inducing bone loss similar to that found in human periodontitis. This was demonstrated by the observation of a significant increase in bone loss in diseased mice measured using both micro-computed tomography and stereo imaging. One of the important findings of this study was that live-animal micro-computed tomography was found to be an accurate method for determining bone volume changes in the periodontitis model. This technique enabled a three-dimensional model of the alveolar bone to be produced and allowed comparisons of bone volume to be made within individual animals at various time-points throughout the disease process.

Many studies (6–8,16) have demonstrated the advantages of micro-computed tomography analysis over other methods to determine bone changes. Micro-computed tomography allows areas of interest to be easily selected, permitting analysis of only the affected areas. The three-dimensional images of scans allowed accurate measurements of bone volume to be made as it was possible to align images from each scan, thus reducing errors that can be encountered using other methods with different animals. Previous studies

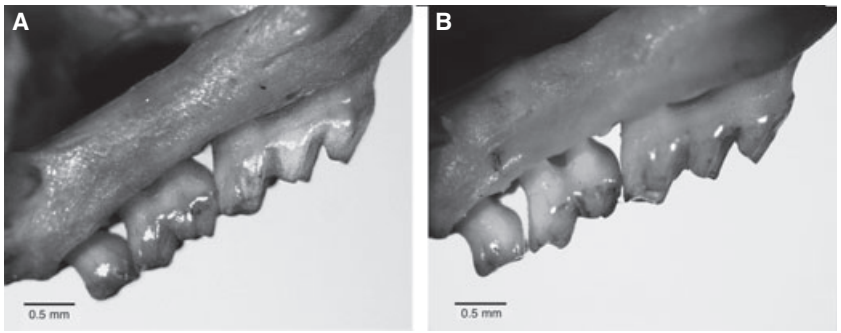

Fig. 3. Stereo images of mouse heads showing the three molars for one diseased mouse (*Porphyromonas gingivalis* inoculated) compared with a control mouse (no disease or treatment). Images were taken using a stereomicroscope (MZ16FA) with a ×1.0 objective lens. Scale: 280 pixels/mm. (A) Control mouse right-hand side lingual view. (B) Diseased mouse (*P. gingivalis* inoculated) right-hand side lingual view.

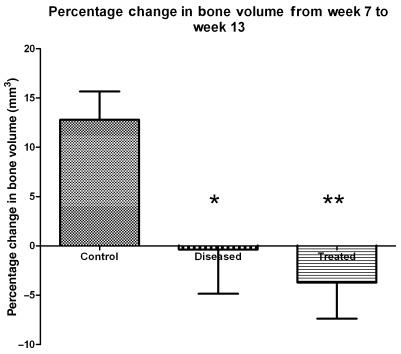

Fig. 4. Average percentage change in bone volume (mm^3) for diseased mice (*Porphyromonas gingivalis* inoculated) and drug-treated mice (*P. gingivalis* inoculated and treated with 5 mg/kg/d KHO64; $n = 5$) compared with the controls (no disease or treatment) from week 7 until week 13 for both right-hand and left-hand sides combined. Bars represent mean ± SEM. The data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. $*p < 0.05$, $**p < 0.01$, statistically significant compared with the control.

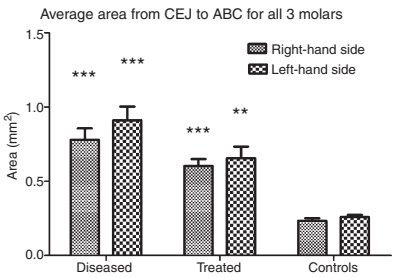

Fig. 5. Comparison of the average area (mm^2) from the cemento–enamel junction to the alveolar bone crest for all three molars combined for diseased mice (*Porphyromonas gingivalis* inoculated; $n = 5$) and treated mice (*P. gingivalis* inoculated and treated with 5 mg/kg/d KHO64; $n = 5$) with control mice (no disease or treatment; $n = 5$). The area from the cemento–enamel junction to the alveolar bone crest was determined from stereo imaging (MZ16FA Stereomicroscope) using IMAGE J. Bars represent the mean ± SEM. The data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. $**p < 0.001$, $***p < 0.01$, statistically significant compared with controls. ABC, alveolar bone crest; CEJ, cemento–enamel junction.

(6–8,16) have investigated the use of micro-computed tomography in periodontitis, but this is the first study to use live-animal computed tomography. This method has many distinct advantages over the use of normal micro-computed tomography in which mice are killed for scanning to be conducted. As live-animal computed tomography is nondestructive and can be repeatedly carried out on the same animal, it reduces the number of animals required for each study. For instance, in a recent study, 100 mice were required as they had to be killed at various time-points for scanning using micro-computed tomography (17). In live-animal computed tomography, individual animals can be scanned at different time-points throughout the study, permitting for a time-dependent bone analysis to be made on each mouse. This reduces sources of error that can be encountered as a result of structural variations of bone in the region of interest in individual animals (18). Statistically, it means that data can be paired for statistical analysis as initial scans of each animal can act as the control. While errors may occur during the alignment of scans to calculate bone volume changes, these errors were found to be small (< 5%). In addition, the use of three-dimensional images means that more detailed effects of the disease on bone structure can also be studied that cannot be undertaken with the use of two-dimensional X-ray methods (8).

Live-animal computed tomography also has advantages over stereo imaging methods that were carried out in this study. There are many sources of error with stereo imaging, including subjective user error in alignment, and loosening of teeth as a result of the defleshing process. This can provide misleading results when measuring the area from the cemento–enamel junction to the alveolar bone crest. Moreover, stereo imaging does not take into account the normal increase in alveolar bone volume that occurs with mouse growth. Overall, the errors made when calculating bone volumes using micro-computed tomography are relatively small, consistent with a previous study which found that three-dimensional measurements provide better alveolar bone analysis than other methods (6).

The secretory phospholipase A_2 inhibitor, KHO64, was found to be ineffective at inhibiting this bone resorption. Stereo imaging and live-animal computed tomography revealed that bone loss in the treated animals compared with the untreated animals was not statistically significant. Secretory phospholipase A_2 inhibitors are known to reduce the formation of the inflammatory mediator prostaglandin E_2 as well as other arachidonate metabolites by reducing arachidonic acid levels. However, conflicting evidence exists over whether prostaglandin E_2 is actually a stimulatory factor of osteoclasts because both stimulatory and inhibitory effects have been observed (11,19–23). It is likely that the responses may depend on the stage of osteoclast development and the involvement of other arachidonate

metabolites. In addition, the species of subject may influence responses to prostaglandin E_2 (22). Our findings are consistent with a recent study which showed that locally applied prostaglandin E_2 could enhance the rate of bone formation in a rat mandible (24). Another possibility is that while this drug suppresses the inflammation, it may concurrently stimulate bone resorption in a manner similar to that proposed for cyclooxygenase-2 inhibitors in rheumatoid arthritis (25).

In conclusion, this study demonstrated the advantages of live-animal-computed tomography to assess changes in bone volume after inducing an inflammatory disease. This method could therefore be used in future studies to determine the effects of drugs on periodontitis and other bone-loss diseases.

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