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Original article

Induction of periodontal disease via retentive ligature, lipopolysaccharide injection, and their combination in a rat model

H. Mustafa^{1,2}, C.H. Cheng¹, R. Radzi¹, L.S. Fong¹, N.M. Mustapha¹,
H.O. Dyary²

¹Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

²College of Veterinary Medicine, University of Sulaimani, Sulaymaniyah, Kurdistan Region, Iraq

Abstract

Periodontitis is a highly prevalent, chronic immune-inflammatory disease of the periodontium that results in the periodontium and alveolar bone loss's progressive destruction. In this study, the induction of periodontal disease via retentive ligature, lipopolysaccharide, and their combination at three different times were compared in a rat model. Seventy-two Sprague Dawley rats were distributed into four treatment groups: 1) control group with no treatment; 2) application of 4/0 nylon ligature around second maxillary molars; 3) combination of ligature and LPS injection (ligature-LPS); 4) intragingival injection of *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) to the palatal mucosa of the second maxillary molars. Six rats were sacrificed from each group after 7, 14, and 30 days of periodontal disease induction. Alveolar bone loss, attachment loss, number of inflammatory cells, and blood vessels were evaluated histologically. A micro-CT scan was used as a parameter to know the rate of alveolar bone loss. Parametric data were analyzed using two-way ANOVA followed by Bonferroni correction with a significance set at 5%. Non-parametric data were analyzed using Kruskal-Wallis, followed by multiple comparisons with Bonferroni correction. The histological results revealed significant destructive changes in the periodontal tissues and alveolar bone following the ligature and ligature-LPS induction techniques. These changes were evident as early as seven days, maintained until 14 days post-treatment, and declined with time. The ligature technique was effective in inducing acute periodontal disease. The LPS injection technique did not induce alveolar bone loss, and its combination to ligature added insignificant effects.

Keywords: alveolar bone, periodontal disease, *Porphyromonas gingivalis* lipopolysaccharide, retentive ligature

Introduction

Periodontal disease is a significant oral disorder affecting humans, caused by bacterial plaque in the periodontium (Petersen 2004). The disease can vary from mild plaque-induced gingivitis to severe degradation of the periodontal connective tissue, alveolar bone loss, and potential loss of teeth (How et al. 2016). It is a multifactorial disease that occurs due to the host's response to the periodontium's microbial plaque (Cochran 2008). Developing an economic animal model will accelerate the preclinical screening of potential treatment modalities for this disease.

Various species of animals have been used to induce periodontal disease experimentally. Non-human primates, dogs, pigs, ferrets, rabbits, rats, and mice have been used as animal models (Polak et al. 2009). Rats are models of import for experimental periodontal research because they are inexpensive and easy to handle (Oz and Puleo 2011). The dental gingival area structure in rodents is quite similar to that observed in humans (Yamasaki et al. 1979). Rats are preferred for surgical procedures in the oral cavity than mice as the size of the oral cavity in mice is very small, and doing surgical procedures would be a challenging task.

Different experimental methods have been described to induce periodontitis in rats. Retentive ligatures have been reported in other literature. It is a reliable technique but is technically challenging. A combination of the ligature technique with inoculation of *P. gingivalis* has been reported to induce alveolar bone loss in the rat model (Meulman et al. 2011). Taguchi *et al.* reported a transient increase in gingival tumour necrosis factor- α but not interleukin 6, following intragingival injection of lipopolysaccharide (*Pg*-LPS) derived from *P. gingivalis* (Taguchi et al. 2015a). Injection of *Pg*-LPS could induce periodontal inflammation but may need a longer time to induce alveolar bone loss (Taguchi et al. 2015b). Whether a combination of the ligature technique with an intragingival injection of *Pg*-LPS would accelerate the process is not well described.

The variability of the literature models prevents direct comparisons between the results and conclusions of the studies. Comparative assessment of periodontitis progression using different techniques can be helpful to compare the results of previous studies and select the appropriate technique for future experiments. This study aimed to describe and compare the histological changes induced by ligature, injection of *Pg*-LPS, and a combination of both techniques at different times in the Sprague Dawley rat model.

Materials and Methods

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee (IACUC) at Universiti Putra Malaysia Approved this study through the Animal Utilization Protocol (AUP) number UPM/IACUC/AUP-R048/2016.

Animals of the study

A total of 72 male Sprague-Dawley rats, aged 8-9 weeks and weighed 292-360 g, were used in this study. All rats were purchased from the Animal Resource Unit and kept at the Animal Research Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The rats were acclimatized for one week before starting the experiment. They were kept in pairs in plastic cages of 800 cm² floor area and 17 cm in height. The room was well ventilated, and the temperature was regulated at 22±2°C. A 12-hour light-dark system was followed with lights on at 7:00 p.m. All rats were fed with standard laboratory rat chow pellets, and filtered tap water was available *ad libitum*. All rats were healthy based on physical examination. The experimental protocol was approved by the Universiti Putra Malaysia's Animal Care and Use Committee (UPM/IACUC/AUP-R048/2016).

Experimental protocol

Rats were randomly divided into four treatment groups, each containing 18. Group C was the control group with no treatment except for examination under general anesthesia, while group L was treated with the placement of 4/0 nylon ligature on the second upper molar teeth, bilaterally. Group LPS was treated with an intragingival injection of *Pg*-LPS. Group L-LPS was treated with a combination of 4/0 nylon ligature and intragingival injection of *Pg*-LPS.

Ligature-induced periodontal disease

The intraoral procedures in groups L, LPS, and L-LPS, were performed under general anaesthesia. A mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) was administered intraperitoneally to induce anesthesia, while isoflurane was used to maintain anesthesia via a small animal anesthesia machine (Smith Medical, USA). Anesthesia was maintained with 2-3% isoflurane in 100% oxygen (1.5-2.0 L/min) using a small nose cone modified from a circuit adaptor and strips of elastic bandage (Hana et al. 2017). The ligature model was created by placing a sterile 4/0 nylon suture (Ethilon 4/0. W1620, 2018, USA) around the second

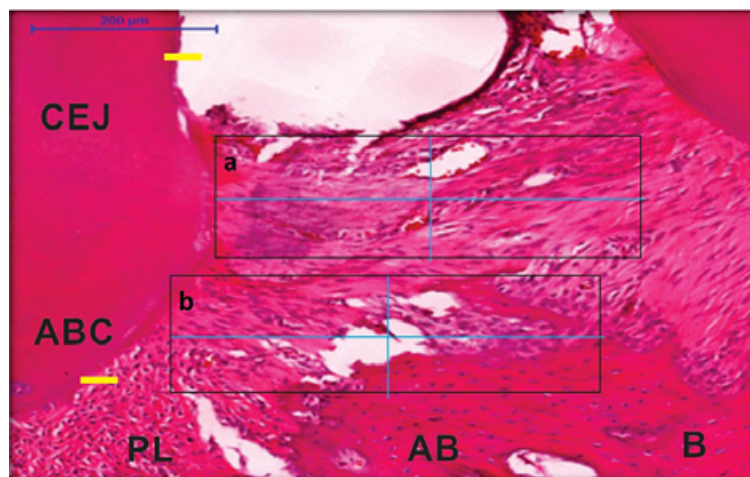


Fig. 1. Photomicrograph of a close-up view of the evaluated structures described in A. a) the subepithelial region, near the gingival sulcus/periodontal pocket. b) the supracrestal region above the ABC. The CEJ-ABC distance (yellow lines) was measured in groups. Hematoxylin and eosin (H&E) stain. CEJ = cemento-enamel junction; ABC = alveolar bone crest; PL = periodontal ligament; AB = alveolar bone.

maxillary molar bilaterally and securing with the surgeon's knots on the lingual surface. The ligatures were examined every 3-4 days throughout the experimental period.

Intragingival injection of *Pg*-LPS

Ten micrograms of a purified preparation of *Pg*-LPS (Invivo Gen Inc., San Diego, CA, USA) in 10 μ L saline (1.0 mg/mL) was injected twice per week with a microsyringe (Hamilton 60330,701RN 10 μ L SYR USA) to the subgingival tissue at the palatal side of the second maxillary molars (Chang et al. 2013).

Animal sacrifice and analyses

Six rats from each group were sacrificed after 7, 14, and 30 days of the experiment by euthanasia with an intraperitoneal dose of pentobarbitone (200 mg/kg). Following euthanasia, skin, muscles, and connective tissues of the maxillary jaws were removed and resected into halves for histological analyses.

Histological analyses

Right jaw segments were fixed in 10% neutral buffered formalin for 72 hours, followed by decalcification in 10% formic acid for 5-7 days. The hemimaxillae were trimmed to obtain the three molars and sectioned horizontally in the middle of a mesiodistal direction. The sections were then washed and dehydrated in graded alcohol concentrations (70%, 90%, 95%, and 100%), followed by clearing in xylene and then embedded in paraffin. After that, serial sections of 5 μ m thickness were cut in the mesiodistal direction with a microtome (Leica Jung Multicut 2045, Leica, Germany), mounted on slides, and stained with hematoxylin and

eosin (H&E). All slides were scanned with a digital slide scanner (Pannoramic Desk® Budapest, Hungary), and panoramic viewer software (Pannoramic viewer 1.15, 3DHISTECK, 2014) was used at X100 or X200 magnification.

The histological analysis included examining the area corresponding to the periodontal tissues in the palatal side at the interproximal junction of the first-second and the second-third molars. This area was closest to the injection site of the *Pg*-LPS.

The presence and intensity of the inflammatory infiltrate were evaluated in two specific regions. The regions were the subepithelial region, near the gingival sulcus/periodontal pocket, and the supra crystal region, above the alveolar bone crest (ABC; Fig. 1). The severity of inflammation was classified in each region using polymorphonuclear leukocyte and mononuclear cell inflammation scoring, as described previously by Molon *et al.* (de Molon et al. 2014a). Severity was ranked as 0 = no inflammatory cells; 1 = mild inflammation (some inflammatory cells); 2 = moderate inflammation with a remarkable number of inflammatory cells scattered throughout the connective tissue above the bone crest; or 3 = severe inflammation (predominance of inflammatory cells). The number of blood vessels was counted according to the method described by de Souza *et al.* (de Souza et al. 2011). The region of interest for the analysis, an area involving the palatal side of the first molar palatal root and the connective tissue subjacent to the gingival sulcus, was examined for the presence of any other tissue morphologic changes.

Micro-CT analyses

The distance from the cemento-enamel junction (CEJ) to the ABC was measured as a parameter to know

Table 1. Effect of Ligature, LPS, and Ligature-LPS on histological parameters of maxillary jaws after 7, 14, and 30 days of disease induction.

Sec.	Parameter	Days after induction	Control	Ligature	Ligature-LPS	LPS
A	CEJ-JE distance (μm)	7	4.3 ^{bx} \pm 4.1	259.1 ^{ax} \pm 11.2	264.9 ^{ax} \pm 17.0	11.6 ^{bx} \pm 4.9
		14	8.4 ^{bx} \pm 2.3	223.1 ^{ax} \pm 11.4	301.0 ^{ax} \pm 25.1	15.5 ^{bx} \pm 7.3
		30	8.0 ^{bx} \pm 3.3	205.7 ^{ax} \pm 24.1	269.5 ^{ax} \pm 43.3	47.3 ^{bx} \pm 9.6
B	CEJ-ABC distance (μm)	7	457.5 ^{bx} \pm 13.0	668.6 ^{ax} \pm 37.9	655.0 ^{ax} \pm 22.0	471.0 ^{bx} \pm 29.7
		14	468.8 ^{bx} \pm 20.6	682.3 ^{ax} \pm 13.2	674.8 ^{ax} \pm 13.7	498.8 ^{bx} \pm 17.4
		30	446.2 ^{bx} \pm 29.3	645.8 ^{ax} \pm 22.8	636.6 ^{ax} \pm 6.4	502.4 ^{bx} \pm 26.4
C	Inflammatory cells (%)	7	15.0 ^{bx} \pm 2.5	60.0 ^{ax} \pm 27.5	59.0 ^{ax} \pm 25.6	18.8 ^{bx} \pm 8.1
		14	16.3 ^{bx} \pm 6.9	48.8 ^{ax} \pm 9.4	55.0 ^{ax} \pm 9.37	20.0 ^{bx} \pm 5.6
		30	17.3 ^{cx} \pm 2.5	33.8 ^{aby} \pm 5.6	40.0 ^{ay} \pm 5.6	23.8 ^{bxc} \pm 4.4
D	Vascularization (%)	7	15.0 ^{ax} \pm 2.5	28.8 ^{ax} \pm 7.5	19.0 ^{ax} \pm 2.5	16.8 ^{ax} \pm 3.8
		14	14.0 ^{bx} \pm 6.1	25.3 ^{axy} \pm 10.9	27.8 ^{ay} \pm 11.4	17.5 ^{bx} \pm 5.6
		30	13.0 ^{bx} \pm 6.4	28.8 ^{ay} \pm 8.3	27.5 ^{ay} \pm 7.0	17.5 ^{bx} \pm 3.1
E	Bone volume (mm^3)	7	5.9 ^{cx} \pm 0.3	4.4 ^{abx} \pm 0.7	4.1 ^{ax} \pm 0.3	5.9 ^{bxc} \pm 0.2
		14	6.9 ^{cx} \pm 0.1	4.6 ^{axy} \pm 0.1	5.9 ^{aby} \pm 1.3	6.2 ^{bxc} \pm 0.5
		30	7.2 ^{cx} \pm 0.2	6.6 ^{aby} \pm 0.8	5.9 ^{ay} \pm 0.3	7.6 ^{bxc} \pm 0.5

Data are presented as mean \pm Standard error (sections A, D, and E) or median \pm interquartile range (sections B and C). Different alphabets denote significant difference, $P < 0.05$. abc indicate differences between treatment groups, and xyz indicate differences over time within treatment groups. Statistical analysis used was Two-way ANOVA, followed by Tukey's post hoc test (sections A, D, and E), or Kruskal-Wallis test, followed by the Bonferroni test (sections B and C). C = Control group, L = Ligature group, LPS = Lipopolysaccharide injection group L-LPS = Ligature with LPS injection group, CEJ = cemento-enamel junction, JE = junctional epithelium.

the rate of alveolar bone loss. The right hemimaxilla samples were fixed in 4% paraformaldehyde and stored at room temperature until they were scanned. The palates were scanned using a high-resolution μ -CT system (Skyscan 1076, Skyscan, Belgium).

The maxillae were scanned with a pixel size of 9.0 μm . The x-ray tube voltage used was 50 kV, and the current was 120 μA , with a 0.5 mm thick aluminum filter. The x-ray projections were obtained at 0.70 degrees with an average frame of 3. Three-dimensional reconstructions were performed using NRecon software (Skyscan 1076, Skyscan, Belgium) using Data-Viewer (Skyscan 1076, Skyscan, Belgium). The sagittal dataset was used to analyze the distance from the CEJ to the ABC of second molars in micrometers. The measurements were done three times for each sample.

The volumetric bone measurements were performed for three rats of each group using CTAnalyser software (SkyScan 1076, Skyscan, Belgium). The volume of interest (VOI), consisting of a stack of regions of interest (ROI), was selected from the alveolar bone at the level of the distal root of the first molar to the distal root of the second molar, which served as the endpoint landmark borders. Within the VOI, ROI was selected with a freehand drawing technique, and

semi-automatic contouring was applied every 4 to 5 slices to segment alveolar bone from the dental root accurately. Mean micro-CT attenuation (arbitrary units) of alveolar bone within the VOI was obtained using one fixed threshold for all scans that were selected visually. Morphological parameters of the segmented alveolar bone, namely, bone volume (BV; mm^3), were calculated using CTAnalyser software. This BV represents the volume of the regions segmented as bone (Bouxsein et al. 2010).

Data analysis

Statistical analysis was performed using Statistical Package for Social Sciences (IBM SPSS Statistics version 19.0, USA) software. Parametric data were tested for treatment and time effect using a two-way analysis of variance (ANOVA), followed by Bonferroni correction. The histological analysis results for inflammatory cells and blood vessel counting were evaluated using the non-parametric Kruskal-Wallis test followed by a post hoc. Differences were considered significant at $p < 0.05$.

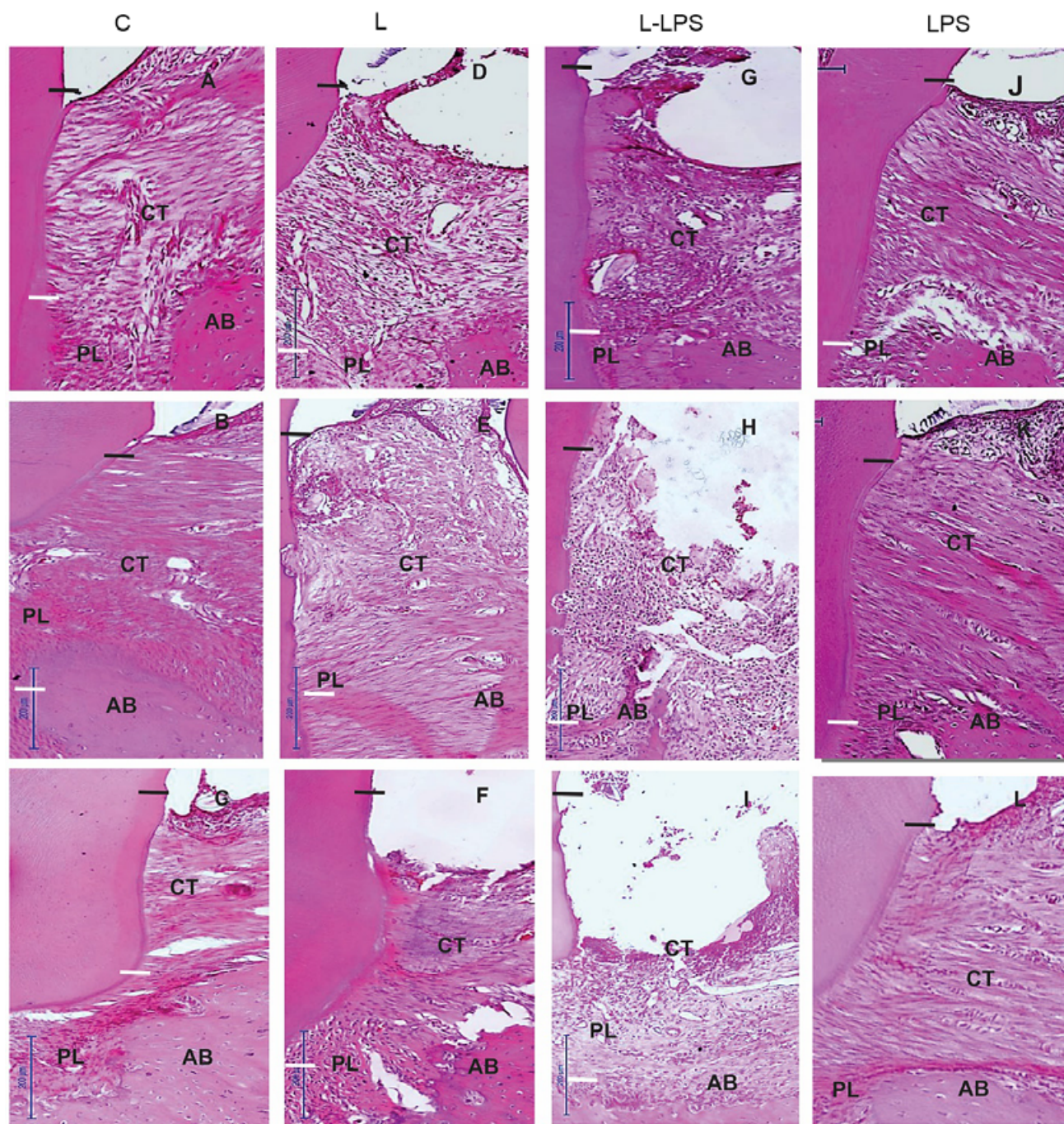


Fig. 2. Histologic views of sections from the labial side frontal aspect of the maxillary second molars in the C = control, L = ligature, L-LPS = ligature with LPS injection, and LPS = LPS injection groups on days 7 (A, D, G, J), 14 (B, E, H, K), and 30 (C, F, I, L). The histomorphologic aspect of the periodontium was conserved. Ulcerations of the gingival tissues, the influx of inflammatory cells, and bone loss were observed in groups treated with ligature and the ligature and LPS injection combination after 7, 14, and 30 days. A comparison of the distance from the cement-enamel junction (CEJ, black line) to the alveolar bone crest (ABC, white line) showed noticeable epithelium apical down growth in the ligature and combination of the ligature with LPS groups at all experimental periods. PL = periodontal ligament; AB = alveolar bone, CT = Connective tissue. (H&E stain, x100).

Results

Histology

The descriptive histological analyses of all groups in the different experimental periods are presented in Fig. 2. Ligature of the maxillary molar tooth induced

periodontitis after 7, 14, and 30 days. The inflammatory changes included intense infiltrations of polymorphonuclear and mononuclear leukocytes, loss of connective tissue attachment, and alveolar resorption. The loss of connective tissue attachment was characterized by an increase in the distance between the CEJ to the

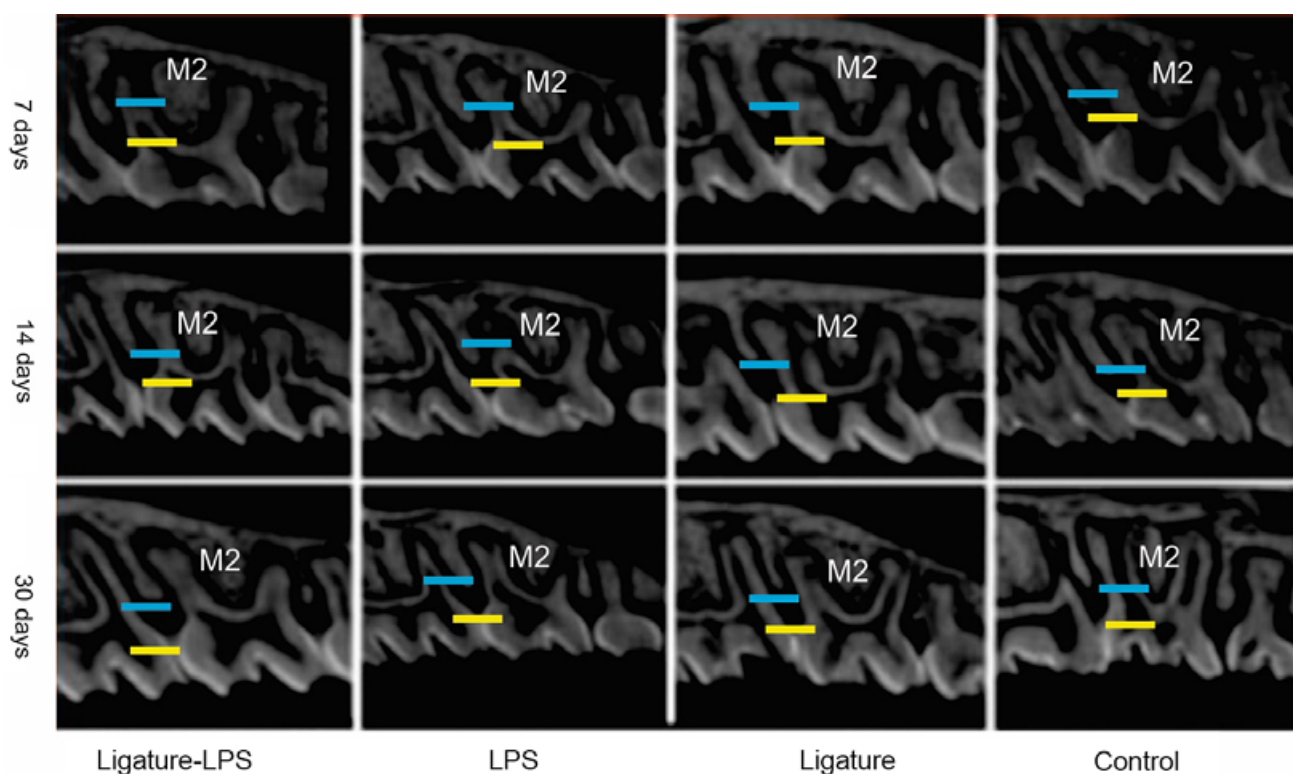


Fig. 3. Two-dimensional sagittal micro-CT views of the maxillary groups of different models from each group at 7, 14, and 30 days. The yellow lines indicate the cemento-enamel junction (CEJ), and the blue line denotes the alveolar bone crest (ABC). M2 = second molar teeth.

junctional epithelium (JE) coronal position attached to the root surface (Table 1A).

Alveolar bone resorption was characterized by an increase in the space between the CEJ and ABC. The distances between CEJ and ABC in the different rat groups are shown in Table 1. Groups treated with ligatures and a combination of ligature-LPS developed larger spaces between the CEJ and ABC ($655.0 \pm 22.0 \mu\text{m}$), compared to the control ($457.5 \pm 13.0 \mu\text{m}$) and LPS ($471.0 \pm 29.7 \mu\text{m}$) groups. There was no difference in the CEJ-ABC distance between the group that underwent ligature ($668.6 \pm 37.9 \mu\text{m}$) and the group treated with ligature-LPS. Furthermore, no differences were observed between the LPS and control groups at any time. The duration of exposure to the different treatment methods did not significantly affect the extent of periodontitis ($p \geq 0.05$). The distance from CEJ to the epithelial cells' apical level was higher in the ligature and ligature-LPS groups than the control and LPS groups.

The degree of infiltration of inflammatory cells was higher in the ligature and ligature-LPS groups than the control and LPS groups after 7 and 14 days of inducing periodontitis (Table 1C). Accumulation of inflammatory cells was the highest in the ligature and ligature-LPS groups after one week and reduced with time. By day 30, infiltration was the highest in the ligature-LPS

group, followed by ligature, LPS, and control. Significant differences were not detected between the LPS and control at any time.

Higher vascularization rates were found by day 14 in both the ligature and ligature-LPS groups, which persisted until day 30 (Table 1D). There was no difference in vascularization rate between the groups of ligature and ligature-LPS ($p \geq 0.05$). No differences were observed between the LPS and control at any time. Significant time-dependent changes were not detected in the LPS group.

Micro-CT

The two-dimensional sagittal micro-CT views of the maxillary molars from each group at days 7, 14, and 30 are presented in Fig. 3. The CEJ-ABC distance was prominent in the ligature and a combination of ligature and LPS injection groups (Fig. 3). The mean distance from the CEJ to the ABC is shown in Table 1B. Both ligature and combination of ligature-LPS groups developed greater CEJ-ABC distance than control and group LPS at all experimental periods. There were no differences between ligature and ligature-LPS and between LPS and control at any time. Within groups, the time effect was not significant.

The BV was used as a volumetric parameter in this study, as presented in Table 1E. Apparent bone loss

through BV was observed in the ligature model on days 7, 14, and 30, with significant difference ($p=0.02$), and the ligature + Pg-LPS group was significantly different ($p=0.03$) in comparison with the control group. However, the Pg-LPS group did not have any effect on bone loss ($p=0.937$).

The bone volume was significantly lower at the region of interest in the ligature and ligature-LPS groups than the control and LPS groups at all experimental periods. There were no differences between ligature and ligature-LPS and between the control and LPS groups. There were significant differences over time in the same group between days 7 and 30 of the experiment in the ligature and the combination of ligatures with LPS injection groups.

Discussion

This study described and compared the periodontal histological changes following the induction of inflammation by ligature, gingival injection of Pg-LPS, and a combination of these techniques in a rat model. Developing a secure and economical animal model to induce periodontitis is essential to further study periodontal changes following different therapeutic approaches. This study revealed that significant degenerative changes in the periodontal tissues and alveolar bone could be induced as early as seven days following the use of ligature and combination of ligature-LPS. However, only mild inflammatory changes were observed after the injection of Pg-LPS into the gingiva compared to the control. This study showed that an acute periodontitis model could be created in Sprague Dawley rats using the ligature method.

In the present study, the ligature technique involved a 4/0 nylon suture around both maxillary second molars. This technique is more difficult than the placement of ligatures on the first molar or the incisor (Ionel et al. 2015), but the advantage was that we did not record any incidences of slipped ligatures. The present study confirmed that the application of ligature alone is an effective method to create acute periodontitis in the Sprague Dawley rat. This method produced significant histopathological changes when compared to control and LPS-treated groups. In this ligature model, the nylon suture caused mechanical trauma to the dentogingival area and acted as a subgingival plaque retentive device.

Histological sections showed increased infiltration of inflammatory cells in the ligature and L-LPS groups as early as seven days post-induction. This local destructive inflammatory response in the periodontium resulted in the loss of connective tissue attachment and alveolar bone resorption (Spolidorio et al. 2014). As a result of the alveolar bone breakdown, periodontal

tissues tend to migrate to an apical position to recover the biologic space. The distance from the CEJ to ABC has been measured to indicate alveolar bone resorption (de Molon et al. 2014b). Furthermore, the distance between the CEJ and the most apical level of the JE's epithelial cells attached to the root surface was measured as an indicator of attachment loss (Nakatsu et al. 2014). The CEJ-ABC and CEJ-JE spaces were calculated by examination of the histological sections. The CEJ-ABC and CEJ-JE distances were more substantial in the ligature and ligature-LPS groups, indicating increased alveolar bone resorption and attachment loss.

In this study, the CEJ-ABC space was measured using both histological sections and micro-CT. Both methods showed that the CEJ-ABC distance was more significant in both the ligature and ligature-LPS groups, showing enhanced alveolar bone reabsorption in these two groups. Results from three-dimensional micro-CT further affirm the findings, as the bone volume in these two groups was significantly less compared to the control and LPS groups at similar periods. The increasing bone volume over time in both control and LPS may be explicated by continuous bone growth as the rats advanced from 10 weeks to 14 weeks old.

The histological changes of tissue inflammation tended to decrease by day 30 in the ligature and ligature-LPS groups. This observation concurred with the results of other ligature models in mice (de Molon et al. 2014b) and rats (de Souza et al. 2011). In the present study, the ligatures were not repositioned in an apical position to maintain contact with the marginal periodontal tissues. As the disease progressed, the periodontal tissues migrated apically, away from the ligature. This outcome may explain why inflammation and bone loss intensities were significant initially but decreased with time. Furthermore, the nylon suture used in our study was monofilament, previously reported to cause minimal tissue reactions (Javed et al. 2012). Therefore, to increase and maintain the disease intensity with time, multifilament suture and ligature repositioning to maintain intimate contact with peripheral tissues should be employed.

The intragingival injection of 10 μ g Pg-LPS twice a week resulted in mild inflammation, which could be observed on days 14 and 30. However, it did not result in alveolar bone loss, even after 30 days. In the study by Cirelli *et al.* (Cirelli et al. 2009), injections of 10 μ g Pg-LPS per site (total of 40 μ g/rat), three times a week, resulted in an observable bone loss by week 4. Park *et al.* (Park et al. 2007) reported that the injection of 10 μ g Pg-LPS, three times a week, causes a significant bone loss by week 8. The frequency of application and the amount of Pg-LPS may not have been adequate

to sustain the study's degenerative inflammatory reactions. Thus, it is advisable to increase the amount and frequency of *Pg*-LPS injections in future studies of animal models.

Asides from increasing the frequency of injection, the use of higher concentrations of LPS may be considered. Dumitrescu *et al.* reported that the gingiva's tightly bound tissue in rats might not expand to accommodate an injection of 10 µg LPS in a volume of 10 µL saline (Dumitrescu *et al.* 2004). Thus, some of the injected LPS may be lost along the needle track, and, hence, more concentrated LPS can be used to adjust for smaller volume.

Conclusions

This study showed that acute periodontitis could be created in Sprague Dawley rats using the ligature method. Gingival injection of *Pg*-LPS induced only mild inflammatory changes and did not have an additive effect when combined with the ligature method. The results from this study provide the basis for future preclinical preventive or treatment modalities for periodontal disease.

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