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Quantification of Microstructures in Mice Alveolar Bone using Micro-computed tomography (μCT)

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Periodontal inflammation increases the risk of tooth loss, particularly in cases where there is an associated loss of alveolar bone and periodontal ligament (PDL). Histological and morphometric evaluation of periodontal inflammation is difficult. Especially, the lengths of the periodontal ligament and interdental alveolar bone space have not been quantified. A quantitative imaging procedure applicable to an animal model would be an important clinical study. The purpose of this study was to quantify the loss of alveolar bone and periodontal ligament by evaluation with micro-computed tomography (micro-CT). Another purpose was to investigate differences in infections with systemic *E. coli* LPS and TNF-α on *E. coli* lipopolysaccharide (LPS) in loss of alveolar bone and periodontal ligament model on mice. This study showed that linear measurements of alveolar bone loss were represented with an increasing trend of the periodontal ligament length and interdental alveolar process space. The effects of systemic *E. coli* LPS and TNF-α on an *E. coli* LPS-induced periodontitis mice model were investigated in this research. Loss of periodontal ligament and alveolar bone were evaluated by micro-computed tomography (micro-CT) and calculated by the two- and three dimensional microstructure morphometric parameters. Also, there was a significantly increasing trend of the interdental alveolar process space in *E. coli* LPS and TNF-α on *E. coli* LPS compared to PBS. And *E. coli* LPS and TNF-α on *E. coli* LPS had a slightly increasing trend of the periodontal ligament length. The increasing trend of TNF-α on the LPS-induced mice model in this experiment supports the previous studies on the contribution of periodontal diseases in the pathogenesis of systemic diseases. Also, our findings offer a unique model for the study of the role of LPS-induced TNF-α in systemic and chronic local inflammatory processes and inflammatory diseases. In this study, we performed rapidly quantification of the periodontal inflammatory processes and periodontal bone loss using micro-computed tomography (micro-CT) in mice.

Key words: Micro-CT, alveolar bone loss, periodontal ligament, TNF-α, *E. coli* LPS

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Introduction

A common inflammatory oral disease involves periodontal disease and periodontal tissue linked with a systemic disorder. Bacteria are important causes of bone pathology in common conditions such as periodontitis and dental cysts [1]. Periodontal disease increases the risk of tooth loss, particularly in cases with an associated loss of alveolar bone

and periodontal ligament (PDL) [2]. Periodontal inflammation is represented by the loss of supporting connective tissue and alveolar bone around the teeth [3]. Bacterial infections produce destruction of the periodontal tissue caused by the inflammatory response to the pathogenic bacteria. Immune mediators such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and IL-6 have been found to be abundantly expressed in periodontal disease [4]. Periodontal diseases are a group of infections that lead to inflammation of the gingiva, destruction of the periodontal tissues, and, in severe cases, loss of alveolar bone with eventual exfoliation of the teeth [5]. The inflammation induces detachment of the periodontal ligament, with loosening of the connection between the gingiva and alveolar bone, allowing the extension of the oral bacterial infection [6]. Periodontal disease has been suspected as a trigger of systemic disorders. Penetration of bacterial products, such as lipopolysaccharide (LPS) may reach into deeper periodontal tissues. This may affect the systemic blood circulation and cytokine production [7]. Interleukin-1β (IL-1β) shows a significantly increased production of low dose LPS groups but TNF-α does not show a significant increase in production [8]. The cytokine dysregulation associated with prolonged TNF-α expression represents a mechanism through which bacteria may induce a more damaging inflammatory disease [9]. Lipopolysaccharides (LPS) of gram-negative bacteria were potent inducers of pro-inflammatory mediators and could initiate numerous host-mediated destructive processes [10,11].

Micro-computed tomograpy (Micro-CT) offers significant potential for identifying microstructures [12,13]. Micro-CT for preclinical applications provides higher spatial resolution images than medical or dental CT for clinical assessment.

To achieve three-dimensional evaluation of alveolar bone loss in periodontitis animal models, micro-computed tomography (micro-CT) has been used in recent studies [14,15]. This study was investigated the effects of alveolar bone loss in *E. coli* LPS and TNF-α on *E. coli* LPS-induced mice evaluated by micro-CT. And also this method suggested that the periodontal ligament and interdental alveolar process space length could be quantified using the micro-CT. Our findings offer a TNF-α on LPS-induced mice model to study the role of inflammatory processes and the degree as quantified by micro-CT. This study was done to assess periodontal tissue breakdown after *E. coli* LPS and TNF-α on LPS-induced experimental periodontitis in mice and the use of micro-CT as a rapid quantification method for alveolar bone and PDL loss assessment.

Materials & Methods

Animals model preparation

BALB/c 8-week-old female mice were randomly divided into 3 groups (n = 10 per each groups). All animals were housed at standard temperature and humidity with a 12-h light/dark cycle, and their body weights were recorded before the test procedures. The mice were fed with standard rodent chow and water *ad libitum*. The experimental protocols were approved by the Chonnam National University Institutional Animal Care and Use Committee. The mice (20 g per each) had experimental periodontitis induced in the interdental region between the mandible first (M1), second (M2) and third molars (M3).

Inflammatory Bone Loss Model

The bone loss model was generated as previously described [16]. To initiate alveolar bone loss, micro-injections of 10 mg/kg *E. coli* LPS (20 μg *per* injection in 2 μl PBS total volume, Sigma, MO, USA) were made directly into the palatal interproximal gingiva between the first and second molars on the left side. The same volume of PBS was injected into the same region on the right side [17]. *E. coli* LPS and PBS injections were repeated 3 times each week over 10 weeks (denoted by LPS and PBS). The *E. coli* LPS treated side was injected with TNF-α (PeproTech, seoul, Korea) (denoted by TNF/LPS). Oral injections of 100 μg/kg of TNF-α were repeated two times per week over a 10 week period.

Micro-CT and Bone Volume Fraction (BVF) Analysis

Micro-CT analysis was performed as previously described [18]. Briefly, after euthanasia, the molar with intact surrounding tissue from each animal was dissected and fixed in freshly prepared 4% paraformaldehyde (pH 7.2) at 4°C overnight for scanning by Micro-CT. Specimens were scanned by Skyscan 1172 Desk Top micro-CT. The system used was Scanner of Skyscan1172, Instrument S/N=054, Hardware version of A, software of Version 1. 5. Analysis with GE microview software (Microview Software Technologies, USA) was done for quantification of the bone volume fraction (BVF). Loss of bone volume was assessed by means of 3-D displays as

previously described [19]. Briefly, given a series of projection images, a stack of 2D sections was reconstructed for each specimen (the number of sections depended on the desired height) and stored in the bmp format condition. In each scan, a standardized tridimensional region of interest (ROI) was defined by the following landmarks: first molar (M1), second molar (M2) and third molar (M3).

Statistics

Data were expressed as the mean±SD from three independent triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test. For all analyses, $p<0.05$ was considered statistically significant.

Results

The effects of oral administration of *E. coli* LPS and TNF-α on food intake and body weight was assessed. The mice were given an oral injection with 10 mg/kg of *E. coli* LPS repeated 3 times each week over 10 weeks. The mice were orally injected with 10 mg/kg of *E. coli* LPS with the addition of 10 μg/kg of TNF-α repeated two times per week over a 10 week period. The control mice were treated with PBS over the same period. The effects of *E. coli* LPS and TNF-α/*E. coli* LPS on food intake and body weight were checked time dependently for 20 weeks. The food intake of the TNF/LPS mice was decreased compared with that of the PBS and LPS mice in a time dependent manner (Fig. 1A). The body weight of the LPS and TNF/LPS mice was decreased more

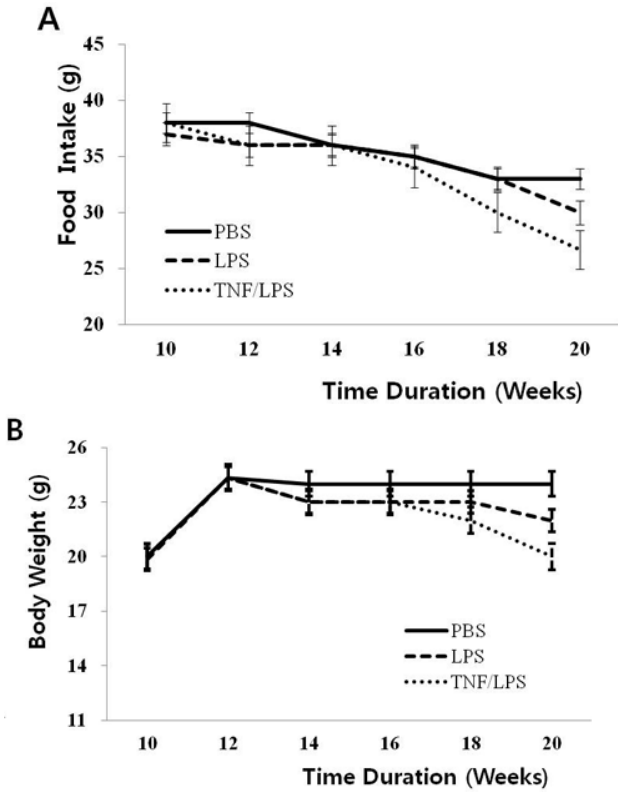

Fig. 1. The daily food uptake and body weight of mice. A daily food intake (g/day) was checked in PBS, LPS and TNF/ LPS mice (A). Body weight changes were monitored in PBS, LPS and TNF/LPS mice until 20 weeks (B). Numbers of animals studied were 10 in each group.

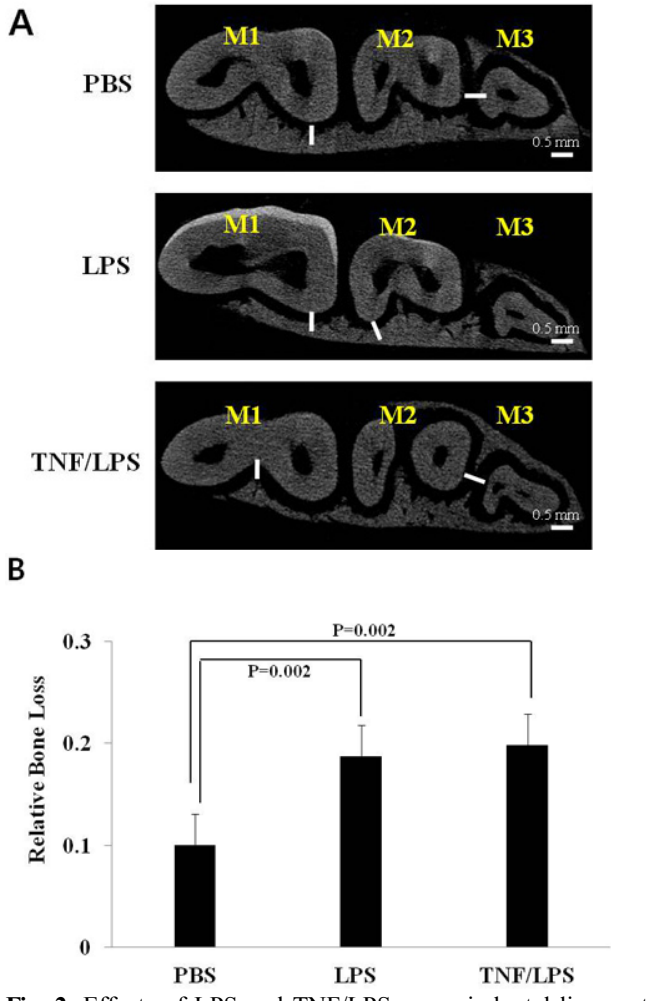

Fig. 2. Effects of LPS and TNF/LPS on periodontal ligament length. The 2D displayed periodontal ligament length at PBS, LPS and TNF/LPS mice (A). Comparison of the relative bone loss indicated that the periodontal ligament length in LPS and TNF/LPS mice was significantly greater than that in PBS mice (B). The indicated white bar was 0.5 mm. The white bar was the distance of the periodontal ligament length. *indicates a significant difference, $p < 0.05$.

than the body weight of the PBS mice (Fig. 1B).

Two-dimensional (2D) serial sections showed all the mandibular molar teeth extracted from the mice. Alveolar bone loss was estimated by measuring the length of the periodontal ligament and interdental alveolar process space in the 2D reconstruction images. Transverse serial sections (0.5 mm) images with the corresponding micro-CT marked off the first molar (M1), second molar (M2) and third molar (M3). The length of the periodontal ligament in the LPS and TNF/LPS showed an increase compared with PBS (Fig. 2A). Quantification of the periodontal ligament length revealed statistically significant increases in LPS and TNF/LPS compared with PBS (Fig. 2B). The interdental alveolar process space length in the LPS and TNF/LPS was greater than in PBS (Fig. 3A). Quantification of the alveolar process space length revealed statistically significant increases in LPS and TNF/LPS compared with PBS (Fig. 3B). There was no difference between LPS and TNF/LPS in the length of the periodontal ligament and interdental alveolar process space. There was no difference in alveolar bone loss between TNF-α and TNF-α in the *E. coli* LPS mice. The alveolar bone loss was represented as the length of the periodontal ligament and interdental alveolar process space using 2D- and 3D measurements. The treatment with 10 mg/kg of *E. coli* LPS and 100 μg/kg of TNF-α caused destruction of the periodontal ligament and alveolar process space. The lengths of the periodontal ligament and alveolar process space were more affected by 100 μg/kg of TNF-α (TNF/ LPS) in 10 mg/kg of *E. coli* LPS-induced on mice.

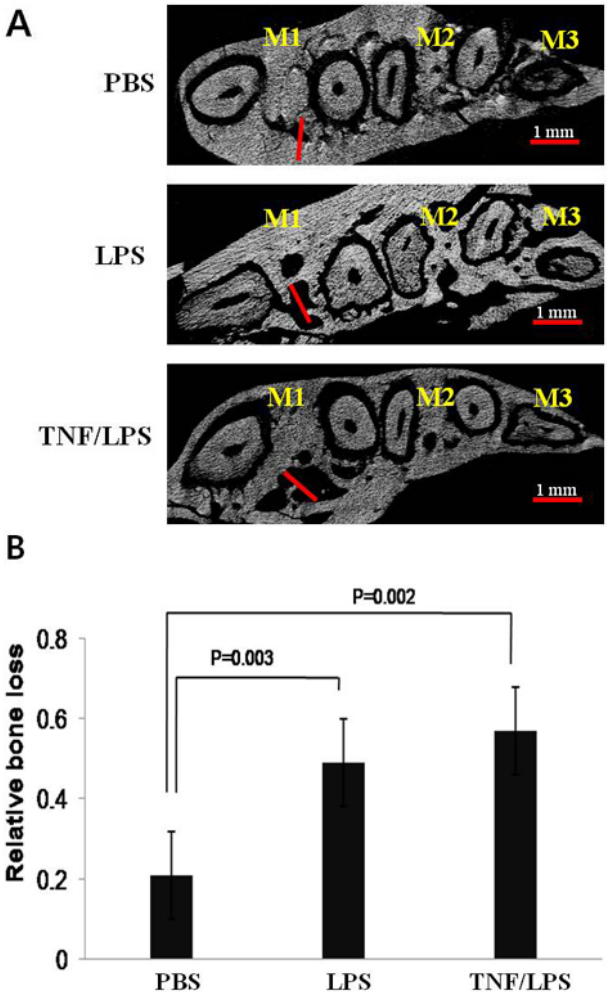

Fig. 3. Effects of LPS and TNF/LPS on the interdental alveolar process space. The 2D displayed the length of the alveolar process space loss at PBS, LPS and TNF/LPS mice (A). Comparison of the relative bone loss indicated that the alveolar process space length in LPS and TNF/LPS mice was significantly greater than that in PBS mice (B). The indicated red bar was 1 mm. The red bar was the distance of the alveolar process space. *indicates a significant difference, $p < 0.05$.

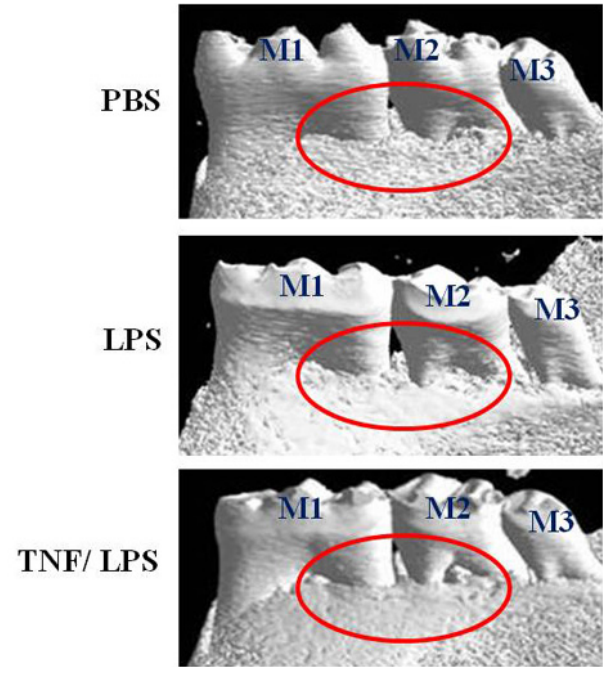

Fig. 4. LPS and TNF/LPS treated mice demonstrated significantly decreased alveolar bone volume fraction (BVF) in comparison with PBS. The 3D representative specimens displayed the periodontal ligament and alveolar process space. TNF or TNF/LPS mice demonstrated a decreased alveolar bone volume fraction (BVF) in comparirson with PBS mice. The volume of interest (VOI) was established in the micro-CT reconstruction. VOI was established with a three dimensional reconstruction displaying the periodontal ligament and alveolar bone loss in PBS, LPS and TNF/LPS mice. M1; first molar, M2; second molar and M3; third molar.

Volumetric micro-CT measurements were three dimensional (3D) structures in the periodontal ligament and alveolar process space. 3D reconstruction of the mandibular bone showed the lingual surface at the alveolar bone. Analysis of the alveolar bone including all three molars can be done by using the method for creating 3D ROIs. TNF/ LPS showed reduction of the alveolar bone process (Fig. 4). 3D micro-CT measurements showed the alveolar bone loss in LPS and TNF/ LPS compared with PBS.

Discussion

In this study, *E. coli* LPS or TNF-α on E. coli LPS induced inflammation was successfully used to induce experimental periodontitis in mice. TNF-α can be induced by bacterial products and is considered as an early event in the cascade leading to inflammation [20]. Intragingival injection of 10 mg/kg of *E. coli* LPS has been accepted as a useful experimental model of periodontitis with alveolar bone loss. 10 weeks after, LPS injection resulted in significant gingival and periodontal inflammation with inflammatory infiltrate interdental bone loss. It indicated that intragingival injection of LPS in the rat provides an easily induced reproducible experimental model of periodontal inflammation [21]. Our results showed site-specific, alveolar bone loss, and inflammation in this model. Quantification of the periodontal ligament length and interdental alveolar process space was done using micro-CT methods. We found that the methods yielded comparable results for detecting alveolar bone loss. Moreover, the osteoclast activity observed in this study was consistent with the progression of inflammation in a monkey model [22]. Periodontal disease is the most frequent cause of tooth loss, including osteoporosis [23]. This finding was similar to the research done by Fokkema, *et al*. [24]. Micro-CT is a recent technology available in bone laboratories. Micro-CT has been widly used in the study of bone metabolism. Previous reports showed that micro-CT is an effective method for the histomorphometrical analysis of long bone inovariectomized rats and gene-deficient mice [25]. A 3D finite element model of a tooth with different levels of bone height was constructed to estimate the reduction [26]. The selected landmarks for quantification of alveolar bone (ROF, the most distal end of molar roots, and tangent line between roots on the coronal plane for landmark detection of ROI) provided critical criteria and reproducibility to measure volumetric osseous parameters. These approaches for the quantitative assessment of periodontal osseous structures demonstrated the reliability and reproducibility of 3-D micro-CT measurements of alveolar bone loss.

In this study, the roles of *E. coli* LPS or TNF-α in *E. coli* LPS-induced alveolar bone loss mice model were studied. But no difference between *E. coli* LPS and TNF-α on *E. coli* LPS-induced alveolar bone loss were found using micro-CT. The destroyed periodontal ligaments and alveolar process space showed the same results of alveolar bone loss. This experiment, once again, proved the contribution of periodontal disease in the pathogenesis of systemic diseases.

Further study needs to be done to investigate the close relationship between periodontal disease and systemic disease using *in vivo* micro-CT imaging approaches for real-time assessments of alveolar bone changes that could lead to dynamic, rather than static, measures of osseous change.

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