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Micro-CT analysis of LPS-induced Alveolar Bone Loss in Diabetic Mice

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Periodontal disease induces an increased incidence of tooth loss, particularly in cases with an associated loss of alveolar bone and periodontal ligaments. In this study, alveolar bone loss was detected by micro-computed tomography (CT) following exposure to *E. coli* lipopolysaccharide (LPS) in a streptozotocin (STZ)-induced diabetic mouse model. A 10 mg/ml dosage of *E. coli* LPS was applied between the first, second and third molars of the mice three times a week for 10 weeks. The loss of periodontal ligaments and alveolar processes was then evaluated by micro-CT using two and three dimensional microstructure morphometric parameters. In the diabetic mice, *E. coli* LPS induced the destruction of periodontal ligaments and loss of alveolar process spaces. The distances between periodontal ligaments were significantly widened in the STZ-LPS group compared with the untreated STZ group. The 10 mg/ml exposure to *E. coli* LPS in the STZ mice also resulted in a significant decrease in the alveolar bone volume fraction. The results of our study suggest that alveolar bone loss can be readily detected by volumetric micro-CT analysis as an increase in the distance between periodontal ligaments and in the alveolar process length.

Keywords : lipopolysaccharide (LPS), diabetes, micro-CT , alveolar bone loss

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Introduction

Periodontal diseases were common diseases and public health burden world wide. They involved the chronic and progressive destruction of the tooth supporting tissues, mainly the alveolar bone and periodontal ligament. These diseases often lead to tooth loss. Periodontal diseases were gives rise to gingival inflammation and tooth loss by a bacterial biofilm initiated inflammatory reaction [1]. Periodontal disease and other oral pathologies were frequent complications of diabetes. Diabetes were an important risk factor for more severe and progressive periodontitis, infection or lesions resulting in the destruction of tissues and supporting bone that form the attachment around the tooth [2]. Destruction of the periodontal tissues was caused loss of alveolar bone with ultimate exfoliation of teeth [3]. Periodontal diseases are inflammatory lesion caused by bacteria [4]. This inflammation induces disattachment of the periodontal ligament, with loosening of the connection between the gingiva and alveolar bone, allowing the extension of bacterial infection such that with *Fusobacterium nuleartum, Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis* [5,6]. *Porphyromonas gingivalis,* one of the major periodontal pathogens were initial stages of periodontal inflammation are accompanied by vascular hyperpermeability [7]. Lipopolysaccharide (LPS) stimulated an up-regulation of adhesion molecules- and cytokines via- a endotoxemia in the cardiovascular tissue of the diabetic compared with normal mice [8]. Topical application of LPS was widely used for the study of periodontitis in rats. LPS from *Escherichia coli* induces

keratinocyte proliferation in vitro [9] and in vivo, a one-time application of *E. coli* LPS were increase cell proliferative activity in the rat junctional epithelium [10].

Micro-computed tomography (micro-CT) offers significant potential for identifying mineralized structures [11]. Micro-CT for preclinical application provides higher spatial resolution images than medical or dental CT for clinical assessment. We could not find the report about the effects of *E. coli* LPS on alveolar bone loss in diabetic mice using the micro-CT. This study investigated the effects of *E. coli* LPS application in diabetic mice. The effects of *E. coli* LPS on alveolar bone loss were assessed by micro-CT.

Materials & Methods

Animals prepared with diabetes animal models

BALB/c 8-week-old female mice were randomly divided into 2 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 groups received high-dose streptozotocin (STZ, 70 mg/kg body weight Sigma, MO, USA) to induce diabetes. The experimental protocols were approved by the Chonnam National University Institutional Animal Care and Use Committee.

Induction of diabetes and determination of fasting blood glucose levels

Diabetes mellitus was induced with the diabetogenic agent, STZ diluted in 0.3 ml of 1 M citrate buffer, pH 4.4 [12]. The drug was injected intraperitoneally once a week for 3 weeks. Glycosuria was measured with a Glucotest strip (Roche, Mannheim, Germany), and blood glucose concentrations were measured withthe Accu-Chek Active monitoring system (Roche, Mannheim, Germany), according to the manufacturer's instructions. Animals with overnight fasting serum glucose levels 125 mg/ml less excluded. The body weight and amounts of food intake were checkedfor weekly interval.

Murine model of LPS-induced periodontitis

LPS-induced periodontitis was generated as previously described [13]. Briefly, Oral applications of 10 mg/ml of *E. coli* LPS were repeated three times per week over a 10 weeks period. The mice (20 g per each) showed periodontal inflammation. The mice were divided into two groups : STZ-LPS group versus control STZ group. The numbers of animals studied were 10 in each group.

Tissue Specimen Preparation for Micro-CT

After euthanasia, the molar with intact surrounding tissue from each animal was dissected and fixed in freshly prepared 4% paraformaldehyde (pH 7.2) 4°C overnight, and transferred to a 70% ethanol solution, and stored at 4°C. Specimens were scanned by micro-CT, and results were analyzed with GE microview software (Microview Software Technologies, USA) for quantification of bone volume fraction (BVF). 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).

Micro-CT and image capture

Micro-CT image capture was done using the Skyscan 1172 Desk Top micro-CT X-ray scanner (SKYSCAN, Kontich, Belgium). The x-ray generator was operated at an accelerated potential of source voltage (kV) of 50 with a beam current of 198 μA, and filter of Al 0.5 mm. The x-ray source combines with a 2D detector operating with a shutter speed of 1,100 ms, which produces images with a vertical position of 0.4 mm, pixel size of 2 μm and rotation degree 10. System were scanner of Skyscan1172, Instrument S/N=054, hardware version of A, software of Version 1. 5. Image reconstruction changed dynamic image were minimal value of 0.047351 and maximal value of 0.24852. The 3D volume viewer program (CTAn) and 2D analyzer software (dataviewer) was used for the visualization and quantification. Post processing images were colorized with a software program (Adobe Photoshop CS4, Adobe Systems, San Jose, CA).Given a series of projection images, a stack of 2D sections was reconstructed for each specimen (the number of sections depending of the desired height) and stored in the bmp format condition.

Statistics

Data are expressed as mean±SD from two independent 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

Intraperitonial injection of high-dose STZ (70 mg/kg) produced the STZ-induceddiabetic mice. The blood glucose levels were highly elevated in STZ-induced diabetic mice. Fasting blood glucose levels in the STZ-induced diabetic mice were ≥200 mg/ml (Fig. 1). The STZ-induced diabetic mice denoted STZ group. The STZ-induced diabetic mice were oral injection with 10 mg/ml of *E. coli* LPS denoted STZ-LPS group. Effects of oral applications of *E. coli* LPS on food intake and body weight was assessed. A 10 mg/ml of *E. coli* LPS treatment had no effect on food intake and body weight checked by time dependently (Fig. 2). Micro-CT technique for evaluation of alveolar bone loss in mice is relatively simple and accurate, and due to its high sensitivity, enables the investigator to reduce the number of animals needed in each experimental group. Two-dimensional serial sections display a right mandible included all three molars. Alveolar bone loss was estimated by measuring length of periodontal ligament and alveolar process space in 2D reconstruction images. Transversal serial sections (0.5 mm) images with the corresponding micro-CT marked of first molar (M1), second molar (M2) and third molar (M3). Lengthof alveolar process space in the STZ-LPS group showed increased distance of alveolar process space compared with STZ group (Fig. 3A). Quantification of alveolar process space distance reveals statistically significant increased in STZ- LPS group compared with STZ group (Fig. 3B). The STZ-LPS group showed more increased distance of periodontal ligament compared with STZ group (Fig. 4A). The results of this study suggest that distance in interdental periodontal ligament status were changes may predispose loss of alveolar bone. Quantification of periodontal ligament distance reveals statistically significant increased in STZ-LPS group compared with STZ group (Fig. 4B).

Volumetric micro-CT measurement shows three dimensional structures in periodontal ligament and alveolar process space. 3D reconstruction of a right mandibleshows the lingual surface at alveolar bone. Analysis of alveolar bone including all three molars can be done by using the method for creating 3D ROIs. STZ-LPS group showed reduced the alveolar bone process. The areas displaying yellow color were crown- and root of tooth- and white color were al

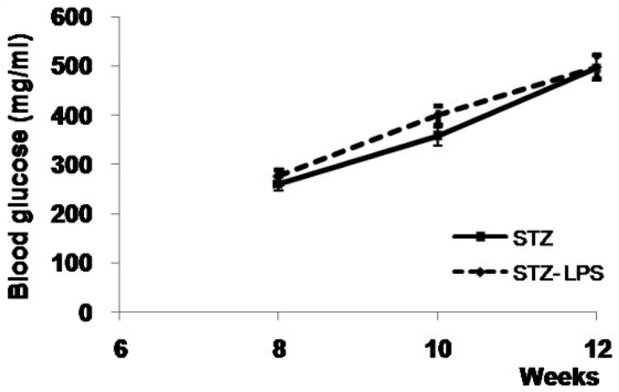

Fig. 1. Fasting blood glucose level in streptozotocin induced diabetic mice. Highly elevated blood glucose levels on high-dose streptozotocin (STZ; 70 mg/kg) induced STZ diabetic mice. Fasting blood glucose levels (mg/ml) in the STZ-induced diabetic mice were ≥200 mg/ml for time dependently. Experimental mice were selected ≥125 mg/ml fasting blood glucose levels. Numbers of animals studied were 10 in each group.

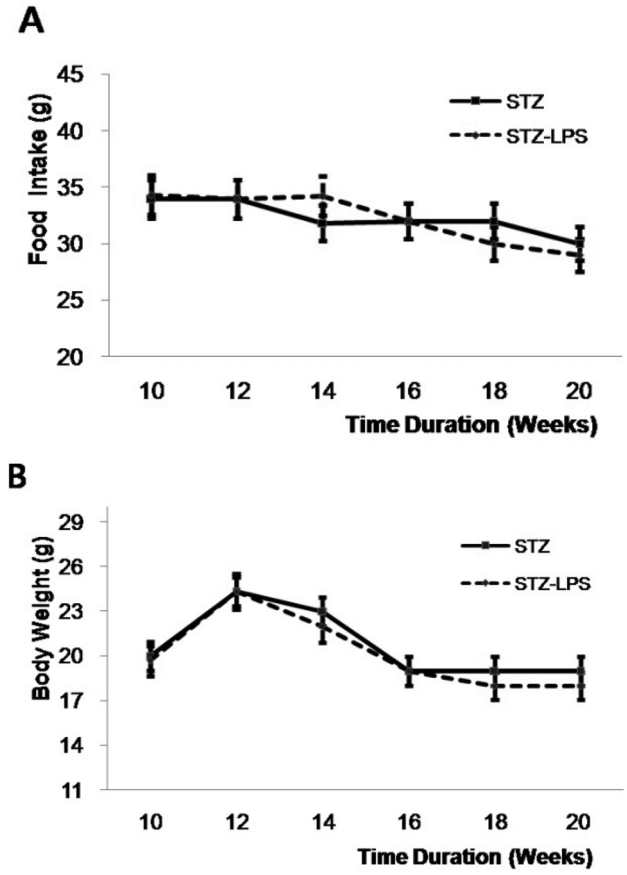

Fig. 2. The daily food uptake and body weight of mice. A daily food intake (g/day) was checked in STZ- and STZ-LPS group (A). Body weight changes were monitored in STZ- and STZ-LPS group until 20 weeks (B). Numbers of animals studied were 10 in each group.

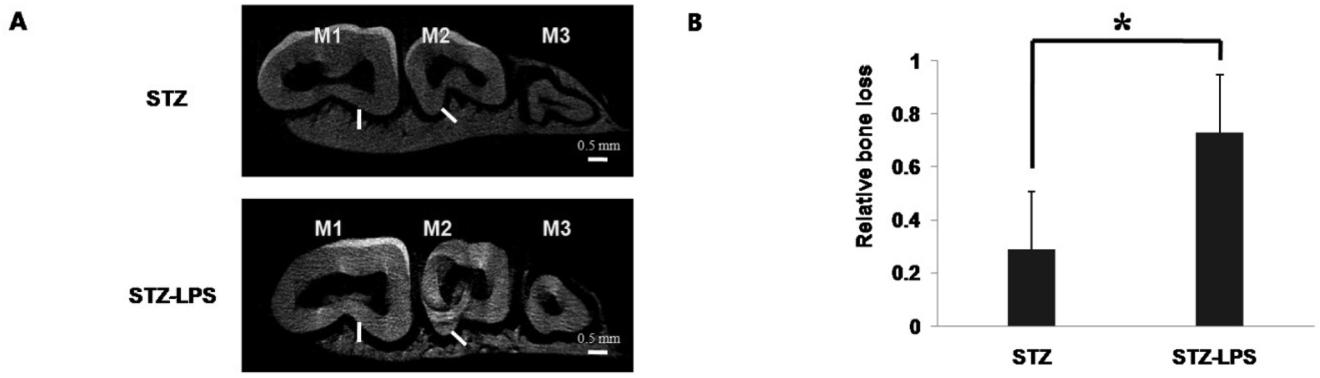

Fig. 3. The 2D representative specimens displayed periodontal ligament. The 2D displayed periodontal ligament length at STZ group and STZ-LPS group (A). Comparison of relative bone loss indicated that periodontal ligament length in STZ-LPS group was significantly greater than that in STZ group (B). The indicated white bar was 0.5 mm. White bar was distance of periodontal ligament length. * indicates a significant difference, $p < 0.05$.

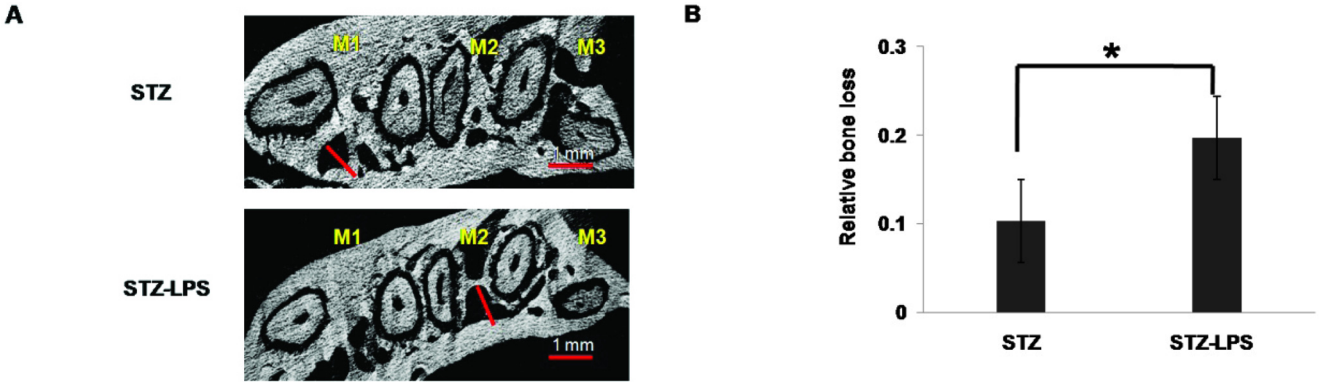

Fig. 4. The 2D representative specimens displayed interdental alveolar process space. The 2D displayed in length of alveolar process space loss at STZ group and STZ-LPS group (A). Comparison of relative bone loss indicated that alveolar process space length in STZ-LPS group was significantly greater than that in STZ group (B). The indicated red bar was 1 mm. Red bar was distance of alveolar process space. * indicates a significant difference, $p < 0.05$.

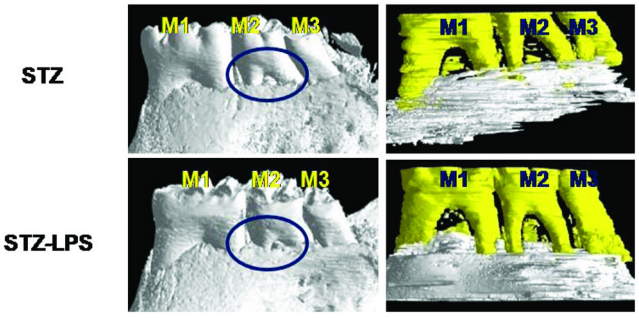

Fig. 5. The 3D representative specimens displayed periodontal ligament and alveolar process space. STZ-LPS group demonstrated decreased alveolar bone volume fraction (BVF) in compared with STZ group. The volume of interest (VOI) was established micro-CTreconstruction. VOI was established three dimensional reconstruction displayed alveolar bone loss in STZ- and STZ-LPS group . Yellow color = crown and tooth root, white color = alveolar bone process. M1; first molar, M2; second molar and M3; third molar.

veolar bone process. STZ-LPS group represented reduced the alveolar bone process (Fig. 5). 3D micro-CT measurements showed the alveolar bone loss in STZ-LPS group compared with STZ group.

Discussion

Periodontal disease is the most frequent cause of tooth loss in humans and is the most prevalent disease associated with bone loss, including osteoporosis [14]. In association with the infection, bacterial antigen and LPS weaken connective-tissue attachments in the gingiva and induce an immune response in the subgingival crevicular fluid [15]. Periodontal patients with type 1 diabetes produce an immune inflammatory response to LPS compared with non-diabetics [16,17]. Diabetic patients tend to suffer from periodontitis with severe alveolar bone loss caused by lowered

immune reaction and delayed tissue recovering. Periodontal pathogens such as LPSand several cytokines stimulate osteoclast differentiation in gingival connective tissue. Then, alveolar bone resorption progresses and the resultant tooth loss happens [16,17].

In this study, LPS-inducing alveolar bone loss enhances inflamed alveolar loss in STZ-induced diabetic mice. Intragingival injection of LPS has been accepted as useful experimental models of periodontitis with alveolar bone loss. LPS injection resulted in a significant gingival and periodontal inflammation with inflammatory infiltrate interdental bone loss. [18]. Micro-CT is a recent technology available in bone laboratories. Micro-CT has been widely used in the study of bone metabolism. Previous reports showed that micro-CT is an effective method for histomorphometrical analysis of long bone in ovariectomized rats and gene-deficient mice [19-21]. A 3D finite element model of a tooth with different levels of bone height was constructed to estimate the reduction [22]. These approaches for quantitative assessment of periodontal osseous structures demonstrated the reliability and reproducibility of 3D micro-CT measurements of alveolar bone loss. The selected landmarks for quantification of alveolar bone provided critical criteria and reproducibility to measure volumetric osseous parameters. Future studies should focus on 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.

There is a report about LPS induced inflammatory bone resorption in a mouse calvarian osteolysis model using a micro-CT [23]. But we could not find the report about the effects of *E. coli* LPS on alveolar bone loss in diabetic miceusing the micro-CT. In this study it was tested the roles of *E. coli* LPS on alveolar bone loss in STZ-induced diabetic mice using a micro-CT. Alveolar bone loss distances were represented as length of periodontal ligament and alveolar process. The periodontal ligament and alveolar process space was affected by oral applications with 10 mg/ml of *E. coli* LPS. Significantdecrease in bone volume fraction was observed by volumetric micro-CT analysis. Especially, periodontal ligaments and alveolar process space were more effect of STZ-LPS group compared with STZ group. Even though there is diabetes, still alveolar bone loss is quite small. But if there is diabetes and periodontitis happened together, alveolar bone loss is quite a lot. So management of diabetes and periodontitis can be separated.

In conclusion, *E. coli* LPS can initiate periodontitis and inflammatory responses, then induce the loss of periodontal ligaments and alveolar process space length leading to alveolar bone loss.

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