Advances in Health Sciences Research, volume 4
11th International Dentistry Scientific Meeting (IDSM 2017)

Virulence of periodontopathogens Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis: micro-computed tomography and inflammatory cytokine analysis

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Abstract. The objective of this study is to determine the difference between A. actinomycetemcomitans and P. gingivalis virulence as the cause of periodontal disease. Thirty male Wistar rats were divided into six groups. Ten rats were induced by 10 ug/mL P. gingivalis lipopolysaccharides (LPS), and the other ten rats were induced by 10 ug/mL crude proteins of A. actinomycetemcomitans. The injection was performed three times for six weeks in the interdental area between the first and the second mandibular right molar by using a 30gauge needle. On the third and seventh days, five rats from each group were decapitated and observed with micro-computed tomography (μ-CT) to determine the severity of alveolar bone resorption. The immunohistochemical method was used to find the expression of macrophage inflammatory cytokines (IL-1β and TNF-α). The ANOVA test was performed to determine the difference among each group. The μ-CT image observation of each group revealed that the severity of alveolar bone resorption was different in each group. In the group that was induced by the crude proteins of A. actinomycetemcomitans, resorption was more advanced than those in the LPS and the control group. A significant increase ($p<0.05$) was found in IL-1β and TNF-α expressions after induction in both groups on the third and seventh days, with the highest cytokine expression observed on the seventh day. The expressions of IL-1β and TNF-α in the group that was induced by the crude proteins of A. actinomycetemcomitans were significantly higher than those in the LPS group. The crude proteins of A. actinomycetemcomitans showed a greater level of virulence than the P. gingivalis LPS in either alveolar bone resorption or inflammatory cytokine expression.

Keywords: Periodontal disease, crude protein of A. actinomycetemcomitans, P. gingivalis lipopolysaccharides, inflammatory cytokine, μ-CT

1 Introduction

Periodontal disease is a chronic infectious disease with a multi-bacterial factor, and it is characterized by the destruction of periodontal ligaments and alveolar bone. The severity of periodontal disease has been established to be dependent on the

interactions between the microbial challenge and the host immune inflammatory responses. Some gram-negative bacteria, such as Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis, have been consistently detected as normal constituents of the microbial flora in periodontal disease. P. gingivalis and A. actinomycetemcomitans are important periodontal pathogens [1,2]. All of the periodontopathogenic bacteria have virulence factors, such as lipopolysaccharides (LPS) and crude proteins, which can stimulate inflammatory cytokine expression [3]. LPS have been primarily shown to stimulate monocytes to produce cytokines, such as TNFα and IL-1. The balance between the local levels of cytokines stimulated in response to periodontopathogenic bacteria and their products is important in determining the outcome of an immune response to a given pathogen [4]. IL-1 is a highly pro-inflammatory cytokine. IL-1 is a multifactorial cytokine that can activate many cell types with strong inflammatory features. The Il-1 release is typically observed to occur shortly after LPS stimulation. IL-1 affects nearly all cell types and usually in concert with other cytokines or mediator molecules. The broad biological effects of IL-1 result from its central role in the regulation of many different genes that regulate cytokines, cytokine receptors, acute phase reactants, growth factors, extracellular matrix components, and adhesion molecules [5.6]. Clinical studies found that the amount of IL-1ß is much higher in the gingival crevicular fluid (GCF) in periodontal pockets or in the underlying inflamed gingival tissue than in the healthy sites. Furthermore, IL-1ß levels are higher in active periodontitis sites than in stable sites [7].

TNF-α is released by monocytes and macrophages in a rapid burst after LPS stimulation. TNF-α induces bone and extracellular matrix resorption by activating the osteoclasts. The local cellular effects of TNF-α include the adhesion of polymorph nuclear leukocytes (PMNs) to endothelial cells, degranulation of PMNs, activation of phagocytosis, and intercellular adhesion molecule (ICAM) -1 expression. The amount of TNF-α was demonstrated to be at a high level in the gingival crevicular fluid and diseased periodontal tissues. Furthermore, experimental studies have shown a central role for TNF-α in alveolar bone resorption [6,8]. The current study aims to determine the difference between A. actinomycetemcomitans and P. gingivalis virulence as periodontopathogenic bacteria in periodontal disease.

2 Material and Methods

This experiment was conducted at the Microbiology and Biomedical Laboratory of the Faculty of Dentistry at University of Jember and the Faculty of Medicine at Brawijaya University, the Bioscience Laboratory of Oral and Dental Hospital at University of Jember, and the Faculty of Physics of Earth and Complex Systems of the Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology.

2.1 Preparation of P. gingivalis LPS

One milligram of P. gingivalis LPS (Invivo Gen, San Diego, USA) was dissolved in 2 mL Dulbecco's phosphate-buffered saline, homogenized, and stored at 4 °C before usage.

2.1 Culture and Preparation of Crude Proteins of A.actinomycetemcomitans Toolbars

A actinomycetemcomitans Y4 (serotype b) strains were grown in brain-heart infusion media with the addition of 1% yeast extract at 37 °C with 10% level of CO2 using a CO2. generating GasPack sachet for 7-10 days [9]. A actinomycetemcomitans of the local isolate/serotype b was isolated using the method of Westphal and Jann. The crude proteins were isolated using the phenol method. About 500 ml of bacterial suspension was centrifuged at a rotation of 15,000 g (6000 rpm) for 1 h. The pellets obtained were resuspended in 10 ml deionized water, which was heated at 72 ºC-75 ºC. The suspension was then vortexed for 20 s and placed in a water bath at a temperature of 72 ºC-75 ºC with the mouth of test tube opened. Subsequently, 10 ml of the 88% phenol solution (previously heated at 72 ºC-75 ºC) was added, vortexed, and incubated for 15 min in a water bath [10].

3 Experimental Model of Periodontitis

Thirty male Rattus norvegicus of Wistar strains aged 12-14 weeks and weighing 200-250 g were used in this study. This research was approved by the Ethics and Advocacy Unit of the Faculty of Dentistry, Gadjah Mada University (No.00748 / KKEP / FKG-UGM / EC / 2016). Anesthesia was performed before induction using a combination of intra-muscular ketamine and xylazine. Chronic periodontitis conditioning was conducted by the induction of the P. gingivalis LPS and the crude proteins of A. actinomycetemcomitans. These substances were injected at the interproximal gingiva between the first and second molars of the buccal aspect of a mandible. Each experimental animal was given P. gingivalis LPS and crude proteins of A. actinomycetemcomitans, with a 10 µl volume and 0.5 mg/ml concentration, with a 1 cc/ml tuberculin syringe (Terumo) with a 30 G (BD) needle administered three times a week for six weeks 11,12. The rats were divided into six groups, in which five rats were induced by the crude proteins of A. actinomycetemcomitans and decapitated on the third day post-injection and another five rats on the seventh day post-injection. The other five rats were induced by LPS from P. gingivalis and decapitated on the third day following the injection, and another five were decapitated on the seventh day post-injection. The control group, which consisted of five rats, was decapitated on the third and seventh days after six weeks.

3.1. *Micro-Computed Tomography* (µ-CT)

The microstructural analysis of the mandibles was conducted using the X-ray µ-CT imaging technique. Three samples were scanned: six weeks of the control sample, six weeks of the induced crude proteins of the A. actinomycetemcomitans sample, and six weeks of the induced P. gingivalis LPS sample. The samples were scanned using the Bruker SkyScan 1173 High-energy X-ray µ-CT (Bruker Micro-CT, Kostich, Belgium) using the primary parameters listed in Table 1. The samples were scanned using different settings. However, the adjustment of the parameters was made on the basis of the primary acquisition standard that the intensity of the detected X-ray should be in the min-max range of 30%-90%.

Table 1. Scanning parameters for the three scanned samples

Parameters	Control	Crude protein of A. *Actinomycetemcomitans*	*P. gingivalis* LPS
Source Voltage (kV)	60	60	80
Source Current (uA)	100	50	50
Filter	Al 1.0 mm	Al 1.0 mm	-
Exposure (ms)	700	125	125
Rotation Step (deg)	0.2	0.2	0.2
Image Pixel Size (μm)	12.114213	24.231851	24.231851
Camera Binning	1x1 (2240 x 2240 pix)	4x4 (560 x 560 pix)	4x4 (560 x 560)

Following the scanning process, reconstruction of the projection images was conducted using the NRecon software (using the GPUReconServer kernel). Subsequently, 3D visual analyses were conducted using the CTVox software. Evaluation of the resorption was performed by measuring the distance between the cementoenamel junction (CEJ) and the processus alveolaris. The measurement can be taken using DataViewer or CTAn software [13].

3.2. Decapitation and Immunohistochemical (IHC) Assay

The animal subjects were asphyxiated using either inhalation on the third and seventh days postinjection. Subsequently, the right mandibular bone of the first and second molars was soaked in a 10% buffered formalin fixation solution for at least 8 h before decalcification. The specimen was immersed in an EDTA solution. After the decalcification process was completed, the sample for IHC analysis was examined to investigate the expression of IL-1 and TNF-α. The expression of TNF-α was disclosed in the rodent mandibular tissue with the TNF-α monoclonal antibody (LS Bio, USA). The method of TNF-α expression painting was performed according to the UltraTek HRP Anti-Polyvalent (DAB) Staining System (Scytek, USA). The IL-β expression was detected in the rodent mandibular tissue using the TNF-α monoclonal antibody (LS Bio, USA). The method of IL1β expression painting was based on the UltraTek HRP Anti-Polyvalent (DAB) Staining System (Scytek, USA). The expressions of TNF-α and IL1β were observed under an Olympus CX-31 light microscope at 400x magnification [11,14].

3.3. Statistical analysis

Results are presented as the mean and the standard deviation (SD). The ANOVA test was performed to determine the significance of the results between the selected groups at a significance level of 95%. The significant difference was analyzed by T-test.

4 Results

Reconstruction of the projection images produced a set of 8-bit grayscale images, which are 2D images of the structure in the transaxial slices. The grayscale images contain information on the intensity level (grey level) of the structure of the sample, which can be commonly related to the density of the sample's structure. These images can be visualized in 3D using CTVox (see Fig. 1). For the sample that was induced by the crude proteins of A. actinomycetemcomitans for six weeks, the resorption was so severe (see the bottom inset of Figure 1.B) that the measurement of the CEJ-processus alveolaris distance was difficult to conduct using the grey scale images. Thus, we used the maximum intensity projection (MIP) image of the sample to produce the one shown in Figure 1.B. The result of the measurement of the CEJ-processus alveolaris distance is presented in Table 2.

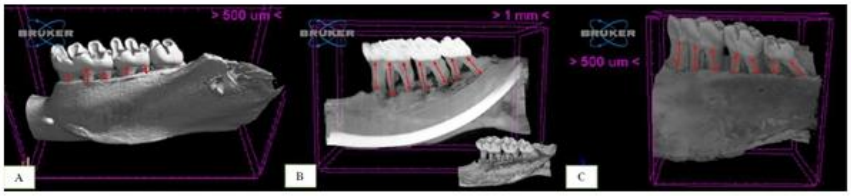

Fig 1. (A) Description of the µ-C T of a six-week control rat. The arrows indicate the CEJ and the processus alveolaris. (B) Description of the µ-CT of a rat that was induced by the crude proteins of A. actinomycetemcomitans for six weeks. (C) Description of the µ-CT of a rat that was induced by P. gingivalis LPS for six weeks

Table 2. Results of the resorption measurement of the alveolar bone of the Wistar rats. The sequence of the measurements of the samples in Figure 1 begins from left to right.

Measurement point	1	2	3	4	5	6	7
CEJ-processus alveolaris distance of the control rat (µm)	420	576	370	525	447	343	293
CEJ-processus alveolaris distance of the crude proteins of A. actinomycetemcomitans (µm)	2400	2472	2160	2040	2088	1440	1560
CEJ-processus alveolaris length of P. gingivalis LPS (µm)	1503	1285	1042	1163	1018	800	969

The µ-CT analysis showed that alveolar bone resorption occurred in the rats that were induced by P. gingivalis LPS and the crude protein of A. actinomycetemcomitans for six weeks. The resorption indicated a decrease in the apical margin of the gingiva in the molar rat tooth area compared with the control sample.

4.1. TNF-α Expression Analysis Using IHC Methods

Fig 2 shows that TNF-α expression in tissue with crude proteins of A. actinomycetemcomitans and P. gingivalis LPS did not affect the TNF-α expression in the control group on either the third day or the seventh day. By contrast, for the treatment group on the 7th day, the injection of the crude proteins of A. actinomycetemcomitans and P. gingivalis LPS increased the TNF-α expression unlike on the third

day. The result of the statistical analysis concluded a significant increase in the TNF-α expression on the seventh day post-injection of the crude proteins of A. actinomycetemcomitans. P. gingivalis LPS and the control group exhibited no significant differences. The analysis was using IHC Methods (Fig 3-5)

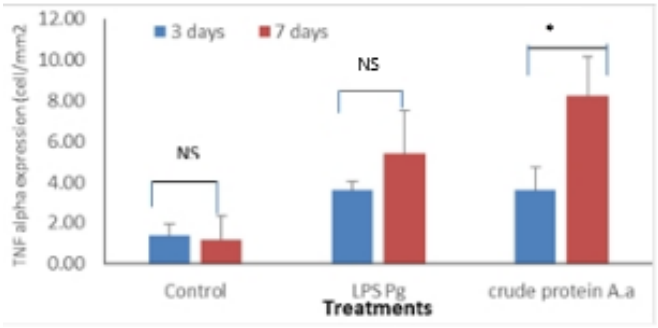

Fig. 2. Bar chart analysis showing a significant increase in the TNF-α expression on the seventh day compared with that on the third day and post-injection control (p=0.02) six weeks after the induction by the crude proteins of A. actinomycetemcomitans. (*: Significant, NS: not significant)

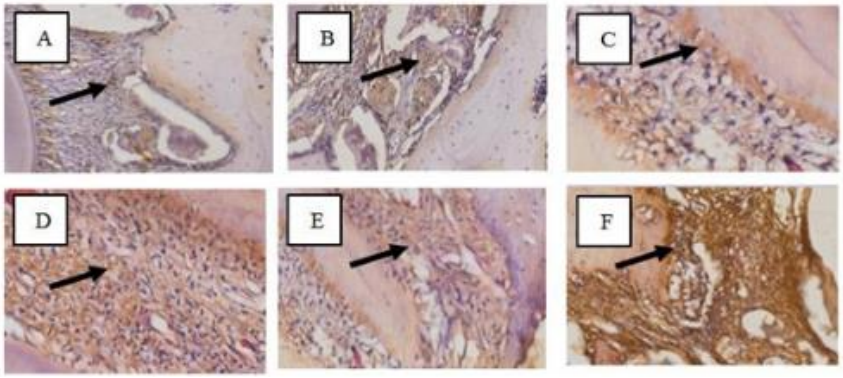

Fig. 3. TNF-α expression on macrophages in the periodontal ligament tissues using IHC methods in the control group and the induction group of crude proteins of A. actinomycetemcomitans. (A) Control group on the third day post-injection. (B) Control group on the seventh day post-injection. (C) Group treated with the crude proteins of A. actinomycetemcomitans on the third day post-injection. (D) Group treated with the crude proteins of A. actinomycetemcomitans on the seventh day post-injection. (E) Group treated with P. gingivalis LPS on the third day post-injection. (F) Group treated with P. gingivalis LPS on the seventh day post-injection (400x magnification).

4.2. IL-1 β analysis using IHC methods

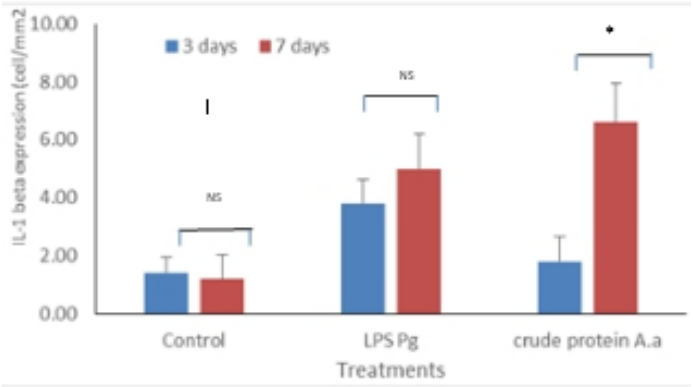

Fig. 4. Bar chart analysis showing a significant increase in the IL-1 β expression on the seventh day compared with that on the third day and post-injection control (p= 0.01) six weeks after the induction by the crude proteins of A. actinomycetemcomitans. (*: Significant, NS: non-significant)

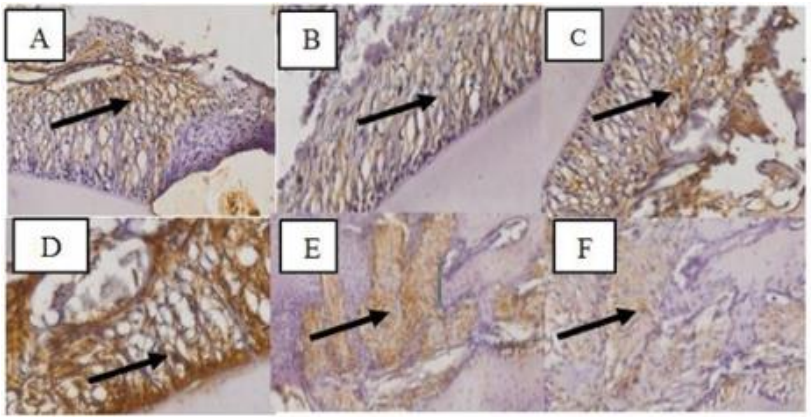

Fig. 5. IL-1β expression on macrophages in periodontal ligament tissues observed using IHC methods in the control group and the group treated with the crude proteins of A. actinomycetemcomitans. (A) Control group on the third day post-injection. (B) Control group on the seventh day post-injection. (C) Group treated with the crude proteins of A. actinomycetemcomitans on the third day post-injection. (D) Group treated with the crude proteins of A. actinomycetemcomitans on the seventh day post-injection. (E) Group treated with P. gingivalis LPS on the third day post-injection. (F) Group treated with P. gingivalis LPS on the seventh day post-injection (400x magnification).

4 Discussion

In the present study, we established a new model of periodontitis in rats through the induction of the crude proteins of A. actinomycetemcomitans and P. gingivalis LPS. In six weeks, evidence of periodontal disease, including increased inflammatory cell infiltration, enhanced inflammatory cytokine expression, and stimulated osteoclastogenesis, indicated a significant alveolar bone loss. Based on the results, the virulence level of A. actinomycetemcomitans in the expression of increased inflammatory cytokines was higher than that of P. gingivalis. A. actinomycetemcomitans produces collagenase that can destroy type 1 collagen, thus causing the collagen degradation in periodontal tissue. These bacteria also have several other virulence factors that accelerate periodontal disease progression. Virulence affects the intensity and potential of the pathogen in host destruction as well as their defense mechanisms toward the host immune system [11,15].

The crude proteins from A. actinomycetemcomitans are a type of protein that can be found inside the bacteria cell, both in endotoxin and in exotoxin form. A. actinomycetemcomitans produces two exotoxins, namely, leukotoxin and cytolethal distending toxin. These toxins can cause cell death, inhibit growth, and stimulate an inflammatory response such as bone resorption. Target cells with receptors involved in leukotoxin induction experience cell lysis, which is related to the function of receptor-related lymphocytes 1 (LFA-1) [16,17].

P. gingivalis LPS, which are important pathogenic bacteria, are closely associated with the inflammatory destruction of periodontal tissues. They induce the release of cytokines and local factors from inflammatory cells, stimulate osteoclastic cell differentiation, and cause alveolar bone resorption. P. gingivalis LPS stimulate the expression of inflammatory cytokines, as inflammatory cytokines such IL-1 and IL-6 are involved in the pathogenesis of periodontal disease [18].

A study Roger et al, on a rat periodontitis model with Escherichia coli endotoxin or using a wire ligature-induced method showed 50% more severe bone destruction on the 7-14 days after induction. Conversely, eight weeks after the induction of the LPS of A. actinomycetemcomitans, the alveolar bone destruction was even more severe than that of the induction of the LPS of E. coli induction. Proinflammatory cytokine production occurs in the pathway of toll-like receptors (TLRs), which have been categorized as a family of innate immune recognition receptors that detect conserved microbial patterns and endogenous ligands. These receptors, along with CD14-expressing immune cells, play a fundamental role in innate immune signaling and lead to inflammatory responses by activating several transcription factors, including the nuclear factor-κB (NF-κB) [11].

LPS, proinflammatory glycolipids and a main component of the cell wall of all Gram-negative bacteria, are shown to be capable of inducing bone resorption in vitro. LPS initiate signal transduction through the toll-like receptor (TLR)-4, which causes the activation of macrophages, lymphocytes, and endothelial cells to release proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. These proinflammatory cytokines can induce osteoclast formation and activation. Moreover, LPS can promote osteoclast differentiation, fusion, survival, and activation independence of IL-1, TNF-α, and RANKL. In these cytokines, TNF-α has been reported to be upstream of other cytokines and plays a central role in inflammatory reaction. TNF-α

exerts its biological effects by binding to the TNF type 1 receptor (TNFR1) and type 2 receptor. These receptors are expressed in the lineages of both osteoclasts and osteoblasts. TNFR1 has already been shown to be critical for LPS-induced osteoclastogenesis in vivo.19,20 LPS promote the survival of differentiated osteoclasts in vitro in the presence of OPG or IL-1 receptor antagonists, or in cells derived from TNF-α receptor-deficient mice, through the activation ofboth the NF-KB and P13K pathways. Therefore, LPS can promote osteoclast suvival both indirectly through the stimulation of cytokines and directly through the activation of survival pathways [21].

TNF-α and/or IL-1β and/or IL-6 levels in blood were found to be high in rats with ligature-induced periodontitis. In the LPS rat periodontitis model, the increase in the TNF-α levels in blood was also reported, although this model could be difficult to interpret because LPS from different Gram-negative bacteria could elicit different host responses. Proinflammatory cytokines expression, primarily T helper 1-type TNF-α, was significantly elevated on the first 30 days post-infection, but the level decreased in the last 30 days post-infection [22].

6 Conclusions

Crude proteins of A. actinomycetemcomitans showed a higher level of virulence than P. gingivalis LPS in either alveolar bone resorption or inflammatory cytokine expression.

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