International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 11, Special Issue 4, 2019

Full Proceeding Paper

EFFECT OF LIPOPOLYSACCHARIDE INDUCTION OF *PORPHYROMONAS GINGIVALIS* ON OSTEOCLAST AND OSTEOBLAST CELL NUMBER OF WISTAR RATS' (RATTUS NORVEGICUS) ALVEOLAR BONE

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Received: 20 Jan 2019, Revised and Accepted: 25 May 2019

ABSTRACT

Objective: This experimental laboratory research too serves the effect of LPS *P. gingivalis* induction on the osteoclast and osteoblast cell number of Wistar rats' alveolar bone.

Methods: *in vivo* laboratory, experimental research was conducted using posttest only control group design. The samples were 20 male Wistar rats divided into 4 groups. Groups I and II were groups injected using LPS *P. gingivalis* for 6 w and were decapited on the day 3 and day 7, groups III and IV were the control groups (not injected using LPS *P. gingivalis*) and decapited on the day 3 and day 7. Subsequently, conducting tissue preparation, staining using haematoxilin eosin, and calculating the number of osteoclasts and osteoblasts cells using a microscope (Optilab) with 400x magnification. The results of osteoblast and osteoclast cell calculation were analyzed using ANOVA and LSD one-way test.

Results: Induction of LPS *P. gingivalis* affected the number of osteoclasts and osteoblasts cell number of Wistar rats' alveolar bone.

Conclusion: Induction of LPS *P. gingivalis* in Wistar rats (Rattusnorvegicus) increases osteoclast cell number and decreases osteoblast cell number.

Keywords: Lipopolysaccharide, Osteoblast, Osteoclasts, Periodontitis, *Porphyromonas gingivalis*

DOI: http://dx.doi.org/10.22159/ijap.2019.v11s4.35294

INTRODUCTION

Riset Kesehatan Dasar (Riskesdas) described that prevalence of dental and oral disease increase, included periodontal diseases, from 23.2% in 2007 to 25.9% in 2013 [1]. According to the oral health services program in 2012, periodontal diseases was the second of oral diseases in Indonesia, particularly periodontitis [2, 3]. Periodontitis is inflammation disease in periodontal tissue or tooth-supporting tissue which there is loss attachment between tooth, root surface, cementum and alveolar bone [4]. Periodontitis is usually related with presence or enhancement specific pathogen bacteria, such as *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Bacteroides forsytus* [5]. *Porphyromonas gingivalis (P. gingivalis)* is the dominant of oral microorganism which is able to greatly colonize in the oral cavity [6]. These bacteria are able to express several virulence factors, as well as fimbriae; lipopolysaccharide (LPS); proteinase; organic metabolite, such as butyric acid; and enzymes, such as arginine, gingipain, Collagenase, gelatinase and hyaluronidase [7].

LPS is Gram negative-endotoxin that is one of the pathogenic factors and identified as the main cause of sepsis [8]. LPS of *P. gingivalis* has the ability to activate host response and disturb alveolar bone remodeling. LPS stimulates macrophage producing pro-inflammatory cytokines, such as interleukin-1α (IL-1α), *tumor necrosis factor-α* (TNF-α) and prostaglandin E2 (PGE2), which cause alveolar bone resorption [9]. Alveolar bone resorption is a dynamic process resulting in an imbalance of osteoclast and osteoblast number, whereas the number of osteoclasts are higher than osteoblast. LPS is played a role in bone remodeling disturbance started as LPS penetrates into periradicular tissue and causes inflammation and bone resorption [9, 10].

Alveolar bone resorption studies were performed using rats as the animal model because the genome was as similar or homolog as human [11]. Likewise, in the periodontal tissue of molar area, the anatomy and histology resemble with the human. With that reasons, this recent study used rats as the animal model of the study [11, 12]. The objective of this study was to know the effect of the lipopolysaccharide of *P. gingivalis* to the number of osteoclast and osteoblast in alveolar bone of Wistar rats (*Rattusnorvegicus*).

MATERIALS AND METHODS

Material

Ethical clearance

This study was experimental laboratories (in vivo study) with the posttest only control group design. All of the procedures were approved by Research Ethic Commission of Dentistry Faculty of Gadjah Mada University, Yogyakarta No. 0748/KKEP/FKG-UGM/EC/2016.

Model periodontitis

Animal models (rats) were acclimatized about a week and divided into 4 groups (I=rats were injected LPS of *P. gingivalis* for 6 w and euthanized on the 3rd day; II= rats were injected LPS of *P. gingivalis* for 6 w and euthanized on the 7th day; III= rats were a control group without LPS of *P. gingivalis* injection and euthanized on the 3rd day; IV rats were a control group without LPS of P. gingivalis injection and euthanized on the 7th day). Every group was consisted five rats. 10 µl of 0.5 mg/ml LPS of *P. gingivalis* was injected in the gingival sulcus of the proximal area of the mandible first and the second molar using 30G tuberculin syringe. The injections were three times a week for 6 w [13]. After that, on the 3rd and 7th day the rats were euthanized using the overdose of ether per inhalation.

Micro-computed tomography (Micro-CT)-based investigation

The sample was positioned on a horizontally rotating holder of the Micro-CT scanning device (Bruker Micro-CT SkyScan 1173, High Energy Micro-CT, FMIPA ITB). The specimens were scanned using a source X-Ray voltage of 40 kV. The current of the source is 130 mA with exposure time of 500 ms and using a 1.0-mm aluminium filter. The sample was scanned through a 180° rotation in a rotation step of 0.2 °. During image acquisition, the 10 frames were averaged; and the scanning process took about 2 h per sample. By using the camera binning of 1×1, the produced projection images have a dimension of 2240×2240 [14].

Hematoxylin and eosin staining

Then, the mandibles were removed and transversally (buccal-lingual direction) sectioned in the first and second molar area. The

specimens were fixed using 10% buffer formalin. Multiple, 5-to 6-µm sections were sectioned from each sample, and representative sections were stained with hematoxylin and eosin (H and E). The sections were observed under the microscope with 400 magnifications. The number of osteoclast and osteoblast were counted on three field areas by three observers. The areas were the top third, middle third and bottom third of sections. All of the calculations were summed up and counted on the average.

Statistical analysis

The results of osteoblast and osteoclast cell calculation were analyzed using ANOVA and LSD one-way test.

RESULTS AND DISCUSSION

Fig. 1a shows a cross-section of a slice of a sample of rats induced by LPS *P. gingivalis* for 6 w. The image is generated from projection techniques with maximum intensity projection. Fig. 1b shows a 3D cross-section of a rat's teeth that were not treated (control) for 6 w. Images are created using 3D volume rendering mode that produces a 3D display by displaying the buccal side. The arrow indicates the longest distance between Cemento enamel junction and peak bone.

The osteoclast size was larger than osteoblast and the nucleolus number of osteoclast, multinucleated, was more than osteoblast. Moreover, osteoblast was cuboids or trapezoidal and single nuclei; and the osteoblast was tent to lie on the bone tissue surface fig. 2. According to the calculation of cells number, The II group had the highest of osteoclast and the III group had the lowest osteoclast. However, the highest of osteoblast number was in IV group, and the I group had the lowest of osteoblast number (fig. 3). Based on the statistic test, there were significant differences of the osteoclast number between the groups ($p<0.05$), except between the I to the II group and the III to the IV group. The osteoblast number among and between the groups had significantly different ($p<0.05$).

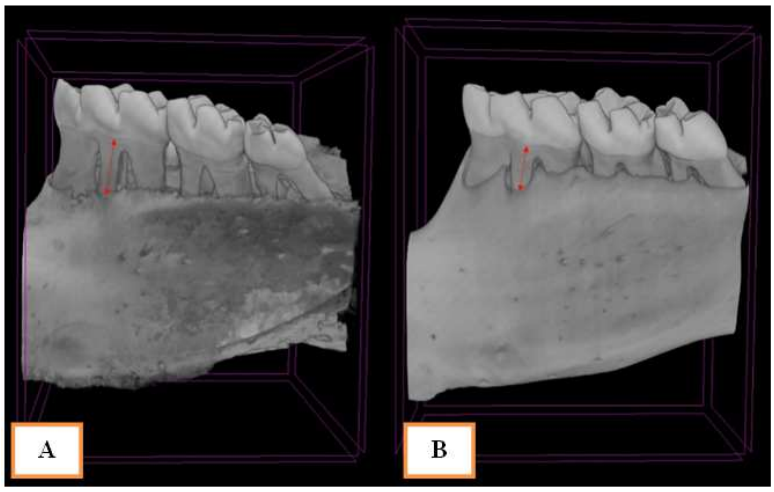

Fig. 1: a. 3D Image of µ-CT are shown rat induced by LPS *P. gingivalis* for 6 w, b. 3D image of µ-CT are shown untreated rats for 6 w. The red arrow indicates the distance between the CEJ and the longest peak of the bone

The MCT description shows a significant difference in the treatment and control groups, where the treatment group was given injection LPS *P. gingivalis* greater alveolar bone resorption in molar teeth (data not shown)

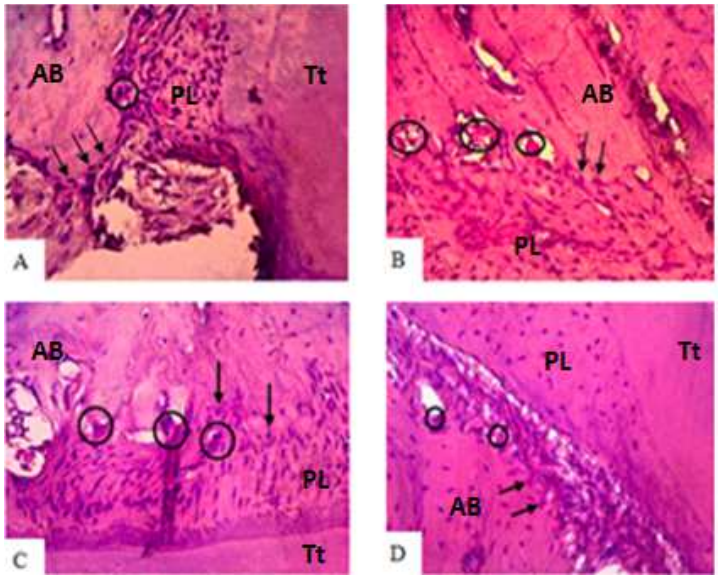

Fig. 2: Histology analysis of osteoclast and osteoblast in alveolar bone with 400x magnification. AB, Alveolar Bone; PL, Periodontal Ligament; Tt, Tooth

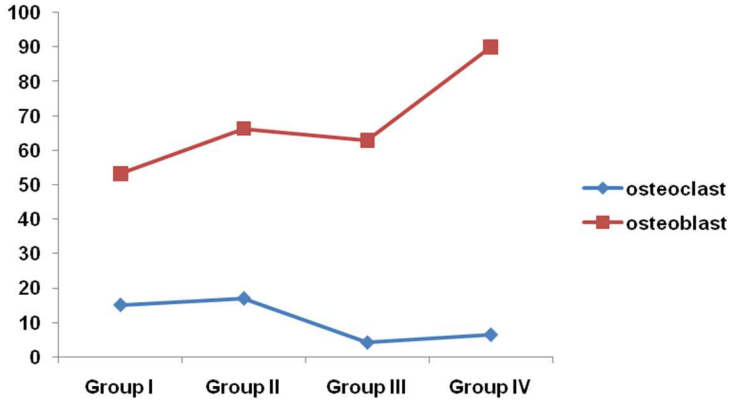

Fig. 3: Histogram of the number osteoclast and osteoblast in alveolar bone of rats

Bone remodeling process is the physiological process which osteoblast and osteoblast are the main factors played a role in that process. Osteoblast is as bone formation cells, and osteoclat is as bone resorption cells. However, this process can be disturbed in some conditions which the osteoclast is more than osteoblast or there is the imbalance in osteoclast and osteoblast [10]. The recent study described that LPS injection enhanced osteoclast in alveolar bone of rats. LPS might be mainly played the role as inflammation stimulator in order to stimulate macrophage to produce pro-inflammatory cytokines which could increase osteoclast genesis activity (new osteoclast cells forming) [9]. The LPS affects inflammation responds since LPS is released and bound with LBP (*Lipopolysaccharide Binding Protein*) which is a protein in plasma as LPS reactant. The bond of those components will config. a molecule complex which is subsequently recognized by macrophage through *Toll-Like Receptor-4* (TLR4), the receptor activating macrophage as adaptive immune respond inducing pro-inflammatory cytokines production, such as interleukin-1α (IL-1α), *tumor necrosis factor α* (TNF-α) and prostaglandin E2 (PGE2) [10].

Statistically, there were significant differences ($p<0.05$), except between the I to II group. This study suggested that there was bone remodeling process in the II group which proliferation of osteoclast enhanced on the 3rd day and declined on the 7th day. Kusumastuti *et al.* described that LPS induction caused inflammation response through immune system resistance, that host immune responses was going to play a main role to control inflammation response due to minimizing the inflammation severity. The healing process is consisted of inflammation phase on the 1st to 3rd day, proliferation phase on 3rd to 7th day, and remodeling phase on 7th to 14th day after injuries [15]. While there was no significant difference between the III and IV group. There might be absence pro-inflammatory cytokines stimulation so it did not affect in osteoclastogenesis. Osteoclastogenesis will occur when bacteria products, such as lipopolysaccharide, induce pro-inflammatory cytokines production [16].

Furthermore, this study showed that LPS significantly decreased the number of osteoblast in the alveolar bone of rats. It might be caused by LPS induced pro-inflammatory cytokines, which stimulated osteoblast apoptosis and decreased *osteoprotegerin* (OPG) production. OPG reduction conduce RANKL configuration which induces inhibition of osteoblast differentiation and production which result in the decline of osteoblast [17]. Moreover, there was the significant difference in the average of osteoblast in the III and IV group which was caused there was no inflammation in the IV group, so osteoblast was still survived and physical activity was presence till 7th day. Physical activity, body movement, directly stimulate *growth hormone* (GH) secretion by way of *insulin-like growth factor-1* (IGF-1), whereas GH will promote stromal cell proliferation and differentiation to be osteoblast in bone marrow [17]. Bone is continuously formed by osteoblast and resorbed by osteoclast. This is the balance process in order to constantly form new bone [18].

The recent study revealed that there were no significant differences between the I, II and III groups. Those were suggested that the animal models in the I group had a good response, so not all of the osteoblast was apoptosis. Osteoblast apoptosis is part of physiology integrity, especially in bone repair and regeneration process. The balance in osteoblast proliferation, differentiation, and apoptosis process assigns osteoblast population in certain time and condition which apoptosis determines the number of osteoblasts. If there is osteoblast apoptosis alteration prevalence, bone formation rate will be affected [16]. Furthermore, osteoblast in the II group was not significantly different with control groups (III and IV). This study suggested that there was bone remodeling in the II group. Osteoclast enhancement and osteoblast depression in this study illustrated that LPS injection could induce inflammation response which disturbed alveolar bone remodeling and caused bone resorption [19].

CONCLUSION

This study concluded that LPS of *P. gingivalis* injection in Wistar rats (Rattusnorvegicus) escalated osteoclast and decreased osteoblast. This study needed further study about LPS of *P. gingivalis* effect to the number of osteoclast and osteoblast after 7th day exposure and about comparison alveolar bone resorption rate induced by LPS of *P. gingivalis* injection.

ACKNOWLEDGMENT

All authors would also like to thank the Micro CT Laboratory (Bruker Micro-CT SkyScan 1173) of the Faculty of Mathematics and Natural Science, Institut Teknologi Bandung.

AUTHORS CONTRIBUTIONS

All authors contributed to this research. Desi Sandra Sari: drafting this conceptual research, doing research in the laboratory, collecting data, doing the discussion and compiling the articles. Desy Futri Intan Gandini Abdi Nagari: performs data analysis and data interpretation, evaluation of laboratory results. Depi praharani: drafting conceptual research, drafting and revising the article. Tantin Ermawati: designing conceptual research, compilation and revision of article submission and the preparation of articles.

CONFLICT OF INTERESTS

All the authors hereby declare that there is no conflict of interest.

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