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Research Article

Osteoblast And Osteoclast Ratio Analysis Based On Bacterial Turbidity Grades On Periapical Bone Resorption induced by*Fusobacterium nucleatum*

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Abstract: Bacterial infection that accumulated in the pulp of the tooth generally provokes periapical lesions and bone resorption. These periapical lesions as results of necrotic pulp mostly inducted by anaerobic bacterial infection. One of the most prevalent anaerobic bacteria in the root canal systems is *Fusobacterium nucleatum* (*Fn*). Bone resorption is influenced by osteoclast activating factor such as prostaglandin, bacterial endotoxins, as well as activator complement products that consist of cytokines including as IL-1β, IL-1α, TNF-α, TNF-β, and IL-6. The main purpose of this study is to determine the difference between ratio numbers of osteoblast and osteoclast in the periapical tissue that induced by *Fn* with various grades of turbidity. This study design used an experimental laboratory. A total 16 male Wistar rats were divided into 3 groups. Group A: the teeth were induced by *Fn* with $10x10^8$ turbidity; Group B: the teeth were induced by *Fn* with $10x10^{10}$ turbidity; and Group C: a control group without any bacterial induction. After euthanasia procedure was applied to the all samples 3 weeks after induction, a histological examination of their periradicular tissues was conducted. The analysis reveals that number of osteoclast cells is significantly higher than osteoblast cells in the treated groups with $10x10^8$ and $10x10^{10}$ turbidities. Meanwhile, the study also shows that number of osteoclast cells is significantly higher in group with $10x10^8$ turbidity than ingroup with $10x10^{10}$ turbidity. An increased number of osteoclast cells is also detected in the area with periapical bone resorption after being inducted by *Fn*.
Keywords: *Fusobacterium nucleatum*, grade of bacterial turbidity, periapicalresorption, osteoblast, osteoclast.

INTRODUCTION

Caries is an access of microorganisms into the pulp chambers[1]. Impaired teeth with pulpal involvement may caused by physical, chemical, and bacterial agents. Bacterial agents are the main cause of pulp injury. This pulp injurycan be reversible or irreversible depends on the capability of pulp to recover itself[2]. Pulp infection could lead to inflammatory reaction followed by necrotic pulp tissue. Then, chronic infection and the spread of inflammation into the apical portion of teeth stimulate secondary immunologic response that can create periapical lesions along with periapical bone resorption.

In many cases, inflammation and bone resorption at the apical portion of teeth derived from interaction between bacterial infection and host response. The important role of bacterial in pulp disease pathogenesis has been known. One characteristic of apical periodontitis is periapical bone resorption that related to immune response of bacterial infection. In 2004, Nair defined apical periodontitis as a periradicular tissue inflammation caused by persistent bacterial infection in root canal systems[3]. Necrotized root canal system is a favorable environment for anaerobic bacteria particularly Gram-negative colonies. The lack of host defence mechanisms exhibits lost of functional circulation in the root canal systems. Moreover, dentinal tubules of root canal walls provide adequate pathway of bacterial colonization in dental pulp. One of the most prevalent anaerobic Gram-negative colonies in the root canal systems is *Fusobacterium. Fusobacterium nucleatum* (*Fn*) is occasionally associated with primary endodontic cases including endodontic abscesses. In the symptomatic cases, *Fn* mostly can be found in pre- (57.1%) and post-operative (66.6%) cases.

Bone resorption is a dynamic process in the periapical tissue. It then followed by specific cell activation that has a capability to resorp bone, called osteoclast. Osteoclast is a bone cell which has influence in the degenerative process. Along with osteoblast, it regulates dynamic balance of bone remodeling system. Imbalance boneremodeling results from more numbers of osteoclast cells than osteoblast cells. It therefore has

strong effect on bone resoption process. Bone resorption influences by osteoclast activating factor including prostaglandin, endotoxin bacterial, as well as inflammatory mediator release from human host, such as IL-1β, IL-1α, TNF-α, TNF-β, IL-6 dan IL-11. These mediators are macrophage products that mostly exist during inflammatory reaction.

It has been known that inflammation in the periapical tissue would cause bone resorption. However, the number of osteoclast and osteoblast in periapical bone resorption that induced by bacteria with different grades of turbidities is still unclear. Therefore, this study is aimed to explore the comparison between numbers of osteoclast and osteoblast on periapical bone resorption that results from *Fn* bacteria induction with different grades of turbidities.

MATERIALS AND METHOD

The study designused is an experimental laboratory. Study samples were laboratory Wistar rats from Veterinary Trial Unit Faculty of Medicine, University of Airlangga Surabaya. The samples were physically healthy mature rats with ages between 18-20 week old, body weight ± 450 grams and they have fully erupted lower incisive. Samples for treated groups were 7 rats while control group without bacterial induction consisted of 2 rats. Therefore, number of samples were16 rats. As an early preparation, rats were fixed on jaw retraction boards. Obturation of lower incisive pulpal roof using round burNo.#1/4 was conducted after the jaw fixation. Rats that have fulfilled the inclusion criteria were anesthetized with combination of 80 mg/kg Ketalar and Diazepam. Each of them received 0.2 cc/100 g body weight via intraperitoneal. If the body weight approximately 400 g, 0.8 cc of anesthetics would be given. After *Fn* induction, the cavity closure using GIC light cured was done. There were three groups of study: Group A, B and C. For Group A, tooth preparation was applied until an opening of the pulp chamber achieved. Then 10µL *Fn* was injected in sterile PBS by using micropipettes for 21 days. Its grade of turbidity was $10x10^8$.Meanwhile, there was no bacterial induction in Group B with $10x10^{10}$grade of turbidity and Group C as a control group. The following step was euthanasia procedure. Rats were placed in airproof containers filled with *ether solution* for several seconds until they were dead. The next procedure was separating rat's mandible from its cranium. To decalcify the mandible, *ethyl diaminetrichlor acetic acid* (EDTA) was applied for 7-10 days. Mandible transversal section was administered on the interdental portion of lower incisivus approximately close to anterior periapical bone. Its purpose was to obtain an optimal preparat section. After the making of HPA, numbers of osteoclast and osteoblast cells on periapical portionswere counted using a light microscope under 400x magnification. *Wilcoxon Signed Rank* and *Kruskal Wallis tests* with *Post Hoc test* were used in this study. The level of significance was settled at 0.05.

RESULTS

The study observed 7 samples of each treated group and 2 samples of control group.A distribution table and diagram below show this study results.

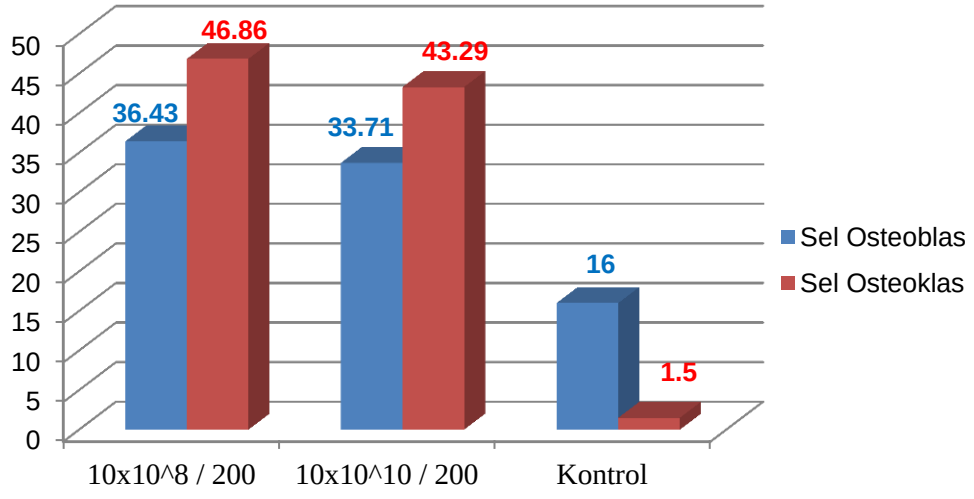

Fig-1: Distribution of osteoblast and osteoclast cells based on grades of turbidity to each group.

The study results demonstrates a higher number of osteoblast cells compare than osteoclast cells in control group without bacterial induction, while the treated group shows an increase number of osteoblast and osteoclast cells. It can be seen in $10x10^8$ turbidity group as well as in $10x10^{10}$ turbidity group. (table 1)

Table-1: Difference number of osteoblast and osteoclast cells based on grades of turbidity to each group

Grades of turbidity *CFU*	n (%)	Number of cell		Deviationosteoblastandosteoclast	*p-value*
		Osteoblast	Osteoclast		
		Mean ± SD	*Mean ± SD*	*Mean ± SD*	
$10x10^8$	7 (43.8%)	36.43 ± 4.11	46.86 ± 1.95	10.42 ± 4.27	0.018*
$10x10^{10}$	7 (43.8%)	33.71 ± 3.14	43.29 ± 2.21	9.57 ± 3.59	0.018*
control	2 (12.5%)	16.00 ± 1.41	1.50 ± 0.70	14.5 ± 2.12	
Total	16 (100%)	32.69 ± 7.41	39.63 ± 15.09	6.93 ± 9.11	

***Wilcoxon Signed Ranks test; p<0.05: significant*

In group with $10x110^8$ turbidity, mean number of osteoblast was observed 36.43. It was lower than mean number of osteoclast (46.86). Moreover, this number also higher than in group with $10x10^{10}$ turbidity, that were 33.71 for osteoblast and 43.29 for osteoclast. There was a difference 10.42 between osteoblast and osteoclast in group with $10x10^8$ turbidity and 9.57 in $10x10^{10}$turbidity. Table 1 provides the result of statistical tests (p<0.05). Group with $10x10^8$ and $10x10^{10}$ turbidities present p : 0.018. This result shows a significant difference between numbers of ostoblast and osteoclast cells in both treated groups.

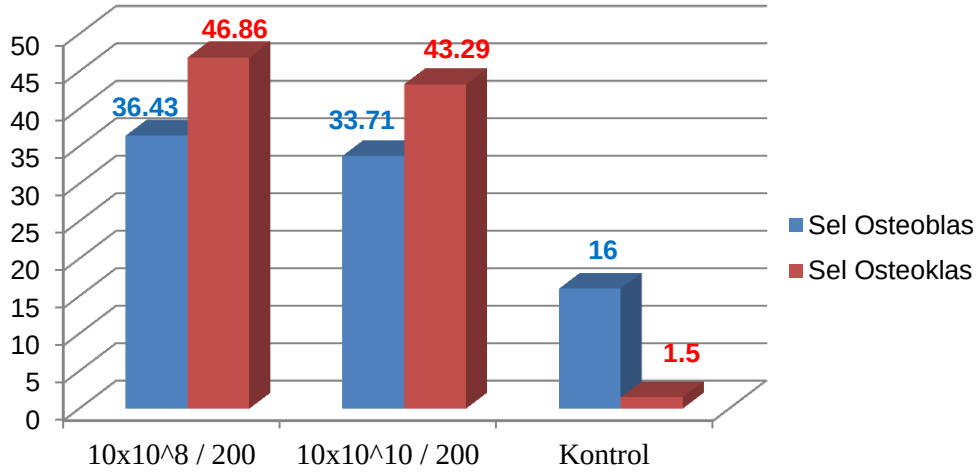

Fig-2: Comparison of osteoblast and osteoclast cells between each groups.

Comparison between numbers of osteoblast and osteoclast cells to each treated groups is shown in Picture 2. This study result also depicts that control group has the lowest number of osteoblast and osteoclast cells compare than other groups. Meanwhile, group with $10x10^8$turbidityshows the highest result. This demonstrates that osteoblast and osteoclast cells would reach the lowest number in the cases without bacterial induction. Nevertheless, the cells would increase significantly during the period of inflammation.

Table-2: Ddifferences between numbers of osteoblast and osteoclast of each group

Grades ofTurbidity *CFU*	Number of cell Osteoblast		Number of cell Osteoclast	
	Mean ± SD	*p-value*	*Mean ± SD*	*p-value*
$10x10^8$	36.43 ± 4.11	0.033*	46.86 ± 1.95	0.008*
$10x10^{10}$	33.71 ± 3.14		43.29 ± 2.21	
control	16.00 ± 1.41		1.50 ± 0.70	
Total	32.69 ± 7.41		39.63 ± 15.09	

***Kruskal Wallis test; p<0.05: significant*

Table 2 shows differences between numbers of osteoblast and osteoclast of each groups. As can be seen in Table 2, value of osteoblast and osteclast cells in $10x10^8$ turbidity group is higher than other groups, which are 36.43 and 46.86 respectively. After being tested statistically, the differences between each groups show p:0.033 (p<0.05) for osteoblast and p:0.008 (p<0.05) for osteoclast. It means that there is a significant difference between numbers of osteoblast and osteoclast cells for each treated groups.

Table-3. Ddifferential statistic test between groups with various grades of turbidity

Cell	Grades of Turbidity*CFU* (i)	Comparison(j)	*Mean Difference (i-j)*	*p-value*
Osteoblast	$10x10^8$	$10x10^{10}$	2.714	*0.175*
		Control	20.429	*0.000**
	$10x10^{10}$	Control	17.714	*0.000**
Osteoclast	$10x10^8$	$10x10^{10}$	3.571	*0.006**
		Control	45.357	*0.000**
	$10x10^{10}$	Control	41.786	*0.000**

**Pos Hoc Test: Least Significant Difference (LSD) test: p<0.05: significant*

Table 3 presents result of differential statistic test between groups with various grades of turbidity. Although 2,714 difference detected, the result could not show any significant difference between $10x10^8$ and $10x10^{10}$ turbidity groups (p:0.175; p>0.05).In comparison with control group, either $10x10^8$ or $10x10^{10}$ groups provide p:0.000 (p<0.05).This means that there is a significant difference between numbers of osteoblast in groups with $10x10^8$ and $10x10^{10}$ grade of turbidity than control group.

Numbers of osteoclast are significantly different between $10x10^8$ and $10x10^{10}$ turbidities (p:0.006; p<0.05). Besides that, Table 3 also demonstrates difference between those groups and control group (p:0.000; p<0.05).

DISCUSSION

Apical periodontitis is an inflammatory condition and periradicular tissue destruction. This condition arises as a result of many disturbances such as infection, physical trauma and iatrogenic, endodontic procedure, as well as root canal filling materials. Bacteria plays an important role in developing pulp and periapical diseases including bone resorption. Chronic inflammatory reaction stimulated by bacteria and its products around apical portion of teeth may cause bone resorption. Periapical lesions typically demonstrate the increase number of inflammatory cells. Along with other connective tissue cells, they release particular mediators to reduce the spread of infection. Pathological features after the mediators released are bone resorption around apical portion of teeth. Bone resorption is mediated by cytokines that comes from inflammatory and non-inflammatory host cells. Several cytokines are identified as bone resorption activators such as IL-1α, IL-1β, TNF-α, dan IL-6.

Development of periapical lesions depends on proinflammatory cytokines and immunomodulator that could release during the period of infection and inflammatory inhibition during chronic phase of lesions. It has been proposed that proinflammatory cytokines, such as IFN-ɣ, TNF-α and RANKL have important roles on periapical bone destruction. This bone destruction depends on RANKL production in which activate osteoclast differentiation and natural changing receptor, called osteoprotegerin (OPG).Synergistic effect of RANKL and proinflammatory cytokine occupy in the periapikal portion in order to counter bacterial stimuli. In turn, this will lead to advancement of periapical lesions. Remodeling process is a bone physiological factor that apparently balance the composition of osteoblast and osteoclast cells. Bone resorption starts happening if numbers of osteoclast higher than osteoblast. Uncontrolled bone resorption would imbalance bone remodeling process.

In this study, periapical tissue destruction of Wistar rats due to *Fn* induction appeared after three weeks. Immune-host interaction on periapical tissue started at week-1 (*acute inflammatory state*), week-2 (*severe inflammatory state*) and week-3 (*chronic inflammatory state*). These periods almost similar with periods require for human to emerge periapical lesions. Fukada S.Y et al [4] had studied periapical lesion inducted on rat teeth by using 4 types of bacteria: *Porphyromonas gingivalis, Prevotellani grescens, Actinomyces viscosus* and *Fusobacterium nucleatum*. Their result shows the formation of periapical lesion after being inducted for 21 days.

The study result shows that treated groups with $10x10^8$ and $10x10^{10}$ turbidities have average increase numbers of osteoclast and osteoblast cells compare than control group. It is mostly caused by *Fn* induction that initiate resorption process through monocyte and macrophage stimulation. The stimulation would release proinflammatory cytokine such as IL-1α, IL-1β, or TNF-α. However, the increase numbers of osteoclast is still significantly higher than osteoblast in both group $10x10^8$ and $10x10^{10}$ turbidities.

This high number of osteoclast results from toxic effect of bacteria, which is stimulated LPS by host immune response. LPS is a potent stimulator of macrophage. It can provide cell induction to produce bone resorption mediators, including IL-1 and TNF. Early inflammatory response begins when *Fn* releases endotoxin, such as LPS, that has binding effect on LBP. The binding of these components would create complex molecule known as CD-14 on targeted cells. Macrophage then could recognize it through TLR-4 receptor. The receptor finally would activate

macrophage as an adaptive immune response by releasing proinflammatory cytokines.

Tukey HSD statistic test also shows no significant difference of the increase number of osteoclast in negative control group because bacterial induction was not applied. As a result, there were no immunogens involved in periapikal bone resorption process.

CONCLUSION

In the treated group with $10x10^8$ and $10x10^{10}$ grade of turbidities, mean numbers of osteoblast and osteoclast increase significantly compare than control group (4.7 : 4.3 : 0.15). Total number of osteoclast is significantly higher in $10x10^8$ and $10x10^{10}$ turbidity groups. In conclusion, this study demonstrates the increase number of osteoclast, that is 31, when periapical bone resorption takes place as a result of bacterial induction with $10x10^8$ of turbidity.

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