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STUDY OF ADHESIN FROM *Aggregatibacter actinomycetemcomitans* LOCAL ISOLATE ON ALVEOLAR BONE DESTRUCTION IN AGGRESSIVE PERIODONTITIS DISEASE

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ABSTRACT

Adhesion is a powerful survival mechanism as well as a virulence mechanism for bacterial pathogens. Bacterial adhesin is a media for bacteria to invade the host. Bacterial adhesin,is a medium for bacteria to invade the host. Baterial adhesion, moreover, is depend on the ligand interaction as a signaling mediator that will influence the invasion process and increase pro and anti-inflammatory due tob the influence of the receptors of innate immune response. Aggregatibacter actimycetemcomitans (A.actinomycetemcomitan) have many virulence factors that may result in tissue and alveolar bone damage. One of the virulence factors is adhesin that can be isolated from the fimbriae. This research purposed to analyze the ability of adhesin protein from A.actinomycetemcomitan that cause the destruction of alveolar bone. Thus, the number of osteoblasts and osteoclasts as well as osteocalcin expression can be used as a marker of damage on the alveolar bone of Wistar rats. The research was conducted through several processes. First, the adhesin of A.actinomycetemcomitan with a molecular weight (MW) of 24 kDa is induced into Wistar rats. Next, to determine the number of osteoblasts and osteoclasts performed, hematoxylin eosin staining is conducted. Meanwhile, to determine osteocalcin expression performed, immunohistochemical techniques is used. This research shows the decreasing of the number of osteoblasts and increasing of the number of osteoclasts in the treatment groups induced by adhesin proteins, *A. actinomycetemcomitans* + adhesin protein, and A.actinomycetemcomitan compared those in the control group. It also shows the increasing of osteocalcin expressions on the alveolar bone of Wistar rats in the groups induced by adhesin proteins, *A. actinomycetemcomitans* + adhesin protein, and *A. actinomycetemcomitans* than those in the control group. It can be concluded that the adhesin protein of A. actinomycetemcomitans plays an important role in the destruction of alveolar bone through the reduction of the number of osteoblasts, the increasing of the number of osteoclasts and osteocalcin expression in aggressive periodontitis.

Keywords: *Adhesin, A. actinomycetemcomitans, osteoblast, osteoclast, osteocalcin expression*

INTRODUCTION

Aggressive periodontitis is a disease found on tissues supporting teeth, and characterized by rapid deterioration in periodontal ligament and alveolar bone. Aggressive periodontitis is usually found in young patients, who are under 30 years old. In the aggressive periodontitis, moreover, the loss of tissue attachment and the recession of gingival can occur four times (4x) faster than in chronic periodontitis (Newman et al., 2006; Velden et al., 2006). Until now, the occurence of aggressive periodontitis in young age has been a problem that cannot be explained comprehensively in dentistry.

The pathogenesis of periodontitis is actually affected by the interaction of the host and bacterial factors dominated by Aggregatibacter actinomycetemcomitans (*A. actinomycetemcomitans*). In other words, the presence of these bacteria in dental plaque can trigger the aggression of periodontal tissue destruction that may also be exacerbated by genetic and environment factors (Korman 2000). The direct contact between infectious agents and host cells is actually started with the process of adhesion (attachment). The process of adhesion is one of the virulence properties of pathogenic bacteria, which are crucial for the colonization, the invasion and the onset of infectious diseases (Doig et al., 1988). It is because *A. actinomycetemcomitans* have fimbriae which functions as the adhesion and the invasion. The fimbriae, thus, can be considered as the virulence factors in infection process in oral cavity. When periodontitis actively becomes progressive, as a result, the level of MMP-8, in gingival crevicular fluid (GCF) significantly increases, causing damage to periodontal tissues and alveolar bone. This study purposed to analyze the ability of adhesin protein from A.actinomycetemcomitan local isolate that cause the destruction of alveolar bone.

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METHODS

Culture for *A. actinomycetemcomitans*

The culture for *A. actinomycetemcomitans* should be prepared in Luria Berthani medium as much as 200 mL to be used in each group (20 mice). Thus, it must be pre-

pared at least as much as 5 ml at a density of 108 *A. actinomycetemcomitans* and given in at least 7 days.

Adhesin of *A. actinomycetemcomitans*

The adhesin of *A. actinomycetemcomitans* should be prepared with a molecular weight (MW) of 24 kDa. It means that the induction for each group is about 200 µl with 200 mg / ml protein and it is given for at least 7 days.

Inducing adhesin protein into Wistar rats

First, Wistar rats we are divided into four groups, one control group and three treatment groups. Each of the groups consisteds of ten rats. Next, the first group as a negative control was induced by 0.9% NaCl, while the second group was induced by adhesin. Meanwhile the third group was induced by adhesin and the whole cell of A.actinomycetemcomitan. The last group as the positive control was induced by the whole cell of *A. actinomycetemcomitans*. The induction of adhesin was done with 200 mL with 200 mg/ml protein at the density of 108 *A. actinomycetemcomitans*, and was given at least 7 days to acquire real aggressive periodontitis symptoms (Zhou et al., 2005). The induction was carried out in the pocket of the right first molar of the Wistar rats as explained in Dumitrescu method (Dumitrescu 2006).

Immunohistochemical technique

The use of immunohistochemical methods in examining osteocalcin expression was conducted through the following stages:

1. Making histopathology preparations based on Humason's method (1972) as quoted from Sudiana (Sudiana 2015).
2. Calculating the results of immunohistochemical staining as quoted from Soini et al., and Pizem and Cor (Soini et al.,1997 ; Pizem and Cor 2003).

RESULTS

Based on the results of calculating the number of osteoblasts on the alveolar bone, it is known that the distribution of the data is normal and homogeneous with a significance value > 0.05 (0.68). From the results of ANOVA test, it is also known that the data have p-value of 0.000 (<0.05), indicating that there is a significant difference between the number of osteoblasts in the treatment group and that in the control group. LSD test results, moreover, shows that there are significant differences between the number of osteoblasts in the control group and that in the groups induced by adhesin, adhesin + *A. actinomycetemcomitans*, and *A. actinomycetemcomitans*.

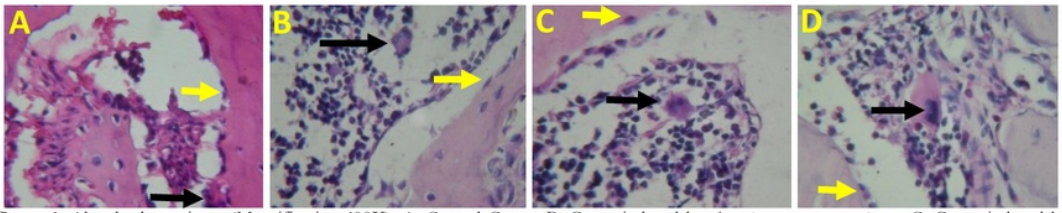

Figure 1. Alveolar bone tissue (Magnification 400X). A. Control Group; B. Group induced by *A.actinomycetemcomitans*; C. Group induced by adhesin protein; D. Group induced by A.actinomycetemcomitan + adhesin protein. Black arrow points to osteoclasts, while yellow arrow points to osteoblasts.

Table 1. Means and standard deviations of the number of osteoblasts in alveolar bone

Group	X	SD	Min	Max	Anova
Control	15[c]	2.1	11	18	F=74.716
Adhesin	4.8[a]	1.9	3	8	p=0,000
Adhesin+ *A.actinomycetemcomitans*	4.6[a]	1.6	3	8	
A.actinomycetemcomitans	6.7[b]	1.6	3	8	

Note: The superscript that is not the same indicates there is no significant difference among groups (p <0).

Table 2. Means and Standard Deviations of the Number of Osteoclasts on Alveolar Bone

Group	X	SD	Min	Max	Brown Forsythe
Control	5.7[a]	1.5	3	8	F=127.268
Adhesin	22.6[b]	2.8	18	26	p=0.000
Adhesin+ *A. actinomycetemcomitans*	24.7[b,c]	4.4	17	30	
A.actinomycetemcomitans	28.7[c]	1.8	25	31	

Note: The superscript that is not the same indicates there is no significant difference among groups (p <0.05).

Similar result is also shown among treatment group, except between that in the group induced by adhesin and that in the one induced by adhesin + A. actinomycetemcomitans. The results also show that there are significant differences between the number of osteoblasts in the control group and that in the treatment groups induced by adhesin, adhesin + *A. actinomycetemcomitans*, and A.actinomycetemcomitan as well as among the treatment groups, except between that in the group induced by adhesin and that in the one induced by adhesin + A. actinomycetemcomitans.

Furthermore, the results of calculating the number of osteoclasts on the alveolar bone show, that the distribution of the data is normal, but not homogeneous with a significance value <0.05 (0.02). Based on the results of Brown Forsy test, it is known that p-value is about 0.000 (<0.05), indicating that there are significant differences between the number of osteoclasts in the treatment groups and that in the control group. Meanwhile, the results of Games Howell test, show that there are significant differences between the number of osteoclasts in the control group and that in the treatment groups induced by adhesin, adhesin + *A. actinomycetemcomitans*, and *A. actino-*

mycetemcomitans as well as among the treatment groups, except between that in the group induced by adhesin and that in the group induced by adhesin + *A. actinomycetemcomitans*. Similar results are also between that in the group induced by adhesin + *A. actinomycetemcomitans* and that in the group induced by *A. actinomycetemcomitans*. Thus it may be concluded that there are significant differences between the number of osteoclasts in the control group and that in the group induced by adhesin, adhesin + *A. actinomycetemcomitans*, and *A. actinomycetemcomitans*, as well as among the treatment groups, except between that in the group induced by adhesin and that in the group induced by adhesin + *A. actinomycetemcomitans*, and also between that in the group induced by adhesin + *A. actinomycetemcomitans* and that in the group induced by *A. actinomycetemcomitans*. The Means and Standard Deviations of the Number of Osteoclasts on Alveolar Bone can seen in table 2 and figure 2.

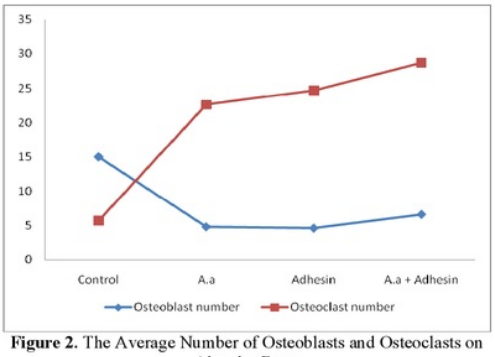

Figure 2. The Average Number of Osteoblasts and Osteoclasts on Alveolar Bone

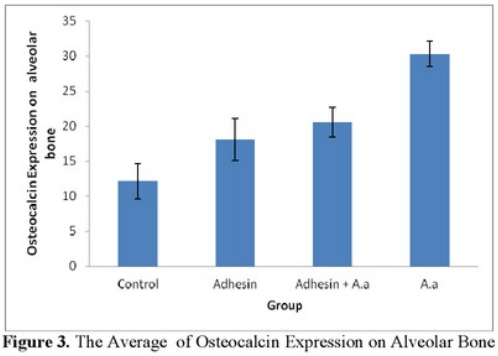

Figure 3. The Average of Osteocalcin Expression on Alveolar Bone

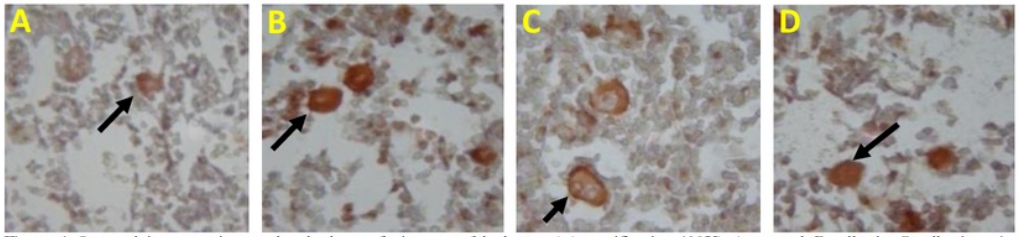

Figure 4. Osteocalcin expression on alveolar bone of wistar rats (black arrow) (magnification 400X). A. control, B. adhesin, C. adhesin + *A. actinomycetemcomitans*, D. *A.actinomycetemcomitans*

The data of positive activation of osteocalcin expressions are obtained from the observation of osteoblasts on alveolar bone by using immunohistochemical method in the control group and the treatment groups induced by adhesin, adhesin + *A. actinomycetemcomitans*, and *A. actinomycetemcomitans*. The result of the observation can be seen in Figure 3.

Osteocalcin expression can be observed through the number of osteoblasts which function as the producing cells of osteocalcin. Based on the results of Kolmogorov-Smirnov's test, it is known that the osteocalcin expressions on the alveolar bone in the rats are normally distributed ($p > 0.05$). Moreover from the results of Levene's test, it is shown that the relation among the groups has a variety of homogeneity ($p > 0.05 = 0.48$). Because the data were normally distributed and homogeneous, thus, to determine the difference of the osteocalcin expressions among the groups, ANOVA analysis is used. Based on the LSD test, the result shows that the value of p is <0.05, indicating that the expressions of osteocalcin in all four treatment groups have significant differences. The significant differences of osteocalcin expressions occur between those in the control group and those in the treatment groups induced by adhesin, adhesin + *A. actinomycetemcomitans*, and *A. actinomycetemcomitans*, as well as among those in treatment groups. However, there is no significant difference between osteocalcin expression in the group induced by adhesin and those induced by adhesin + *A. actinomycetemcomitans*. Finally, the means and standard deviations of osteocalcin expression on alveolar bone are shown in the following the figure 3.

DISCUSSION

Based on the results above, it is shown that the number of osteoblasts found on alveolar bone in the control group is significantly different from that in the treatment groups induced by adhesion, adhesin + *A. actinomycetemcomitans*, and *A. actinomycetemcomitans*. It indicates that there is no inflammation (aggressive periodontitis) in the control group. On the other hand there is inflammation in the treatment groups induced by adhesin, *A. actinomycetemcomitans* + adhesion, and *A. actinomycetemcomitans*.

Osteoblasts actually play an important role in mineralization process through the deposition of hydroxyapatite. It is because osteoblasts regulate calcium and phosphate concentrations that are useful in the formation of

hydroxyapatite. Osteoblasts also show alkaline phosphatase in high quantities in plasma membrane. This alkaline phosphatase is essential in the process of bone mineralization. Osteoblasts, moreover, also express a variety of cytokines, namely Colony Stimulating Factor 1 (CSF 1), RANKL, and Osteoprotegerin (OPG) (Manolagas 2000 ; Morawati 2009). The highest number of osteoclasts found on the alveolar bone is in the treatment group induced by *A. actinomycetemcomitans*. This condition is significantly different from the number found in the control group and in the treatment groups induced by adhesin and adhesin + *A. actinomycetemcomitans*.

That condition discussed previously indicates that the induction of *A. actinomycetemcomitans* has caused adhesion, colonization, and invasion to the host. At the time of the invasion to the host, *A. actinomycetemcomitans* secretes virulence factors, one of them is LPS. LPS is a major factor of the bacteria that has an ability to perform bone resorption by conducting osteoclasts stimulation, in which LPS activates osteoblasts to secrete factors that can attract and or activate osteoclasts. LPS, according to a research conducted by Nair et al, also inhibits the synthesis of collagen and non-collagen protein (Nair et al., 1996). In addition, LPS, according to a research conducted by Nishihara, (1994), can cause bone resorption on murine calvarial by stimulating murine macrophages. Thus, it ca ben be said that *A. actinomycetemcomitans* can cause damage to alveolar bone and create antibody responses in the three strains of mice, namely Hypertensitive Fawn Hooded (FHH), Dahln Salt Sensitive (DSS), and Brown Norway (BN) (Nishihara et al., 1994; Schreiner et al., 2006)

Basically, periodontitis occurs through four stages, namely the accumulation and the presence of bacteria in gingival sulcus (colonization), the invasion of bacteria on the epithelium and gingival tissue, the stimulation of the host response, the activation of the acquired and innate immune response (inflammation), and finally the destruction of the connecting tissue attached to tooth surface and bones causing irreversibel damage (Graves et al., 2010). The reduction of the number of osteoblasts caused by the induction of *A. actinomycetemcomitans*, *A. actinomycetemcomitans* + adhesion, and adhesin causes stimulation on RANKL. As a result, RANKL will be attached to RANK to stimulate TRAF 6, and then will activate osteoclast progenitor causing differentiation. Next, the activation of osteoclasts causes the increasing of osteoclasts. Finally, the increase of the osteoclasta. Finally, the increase of osteoclast causes alveolar bone damage.

From the research, it is known that osteocalcin expressions on alveolar bone are increasing. The research also shows that there are significant differences between osteocalcin expression found in the group induced by *A. actinomycetemcomitans* and that in the groups induced by *A. actinomycetemcomitans* + adhesin and adhesin, as well as that in the control group. It indicates that the adhesin of *A. actinomycetemcomitans* act as adhesin causing adhesion to the host. After the adhesion of the adhesin of *A. actinomycetemcomitans* to the adhesin receptor on the host, *A. actinomycetemcomitans* secrete a variety of virulence factors triggering inflammatory process and increasing osteocalcin expression on alveolar bone, which is one of indicators of bone damage.

The main function of adhesin in *A. actinomycetemcomitans*, actually, is as the adhesion factor to improve bacterial colonization, so bacteria can invade the host. In addition , it is also considered as the main component stimulating the host immune response. This is supported by the statement of Amano that serum IgG anti Flp is significantly higher in patients with periodontitis than that in healthy periodontal tissues. Flp on bacterial is identified as adhesin factor increasing the adhesion and colonization of bacteria in the host tissue. Adhesin has a primary function as an antigenic component that can evoke an immune response of the host, so it will stimulate the proinflammatory cytokines, especially TNF-α and IL-1, stimulating RANKL. RANKL itself acts as a stimulant to increase osteoclast (Amano 2010). Thus, the increasing of osteoclasts will cause the increasing of osteocalcin triggering the resorption of alveolar bone. This situation is supported by the statement of Bullon in 2007 that the increasing of osteocalcin is a marker in the absence of inhibition of bone formation. Finally, wreiter can conclude that the high level of osteocalcin in serum is associated with the rate of bone damage. It is strongly shown by a research on animals showing the role of osteocalcin in bone alveolar resorption (Bullon et al.,2007).

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