

THE ABILITY OF IMMUNOGLOBULIN Y FROM *PORPHYROMONAS GINGIVALIS* TO PREVENT ADHESION OF *FUSOBACTERIUM NUCLEATUM* AND *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

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ABSTRACT : *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are the periodontitis bacterium. IgY is a type of immunoglobulin that found in poultry, such as: chicken and birds. IgY can be used as an alternative prevention of plaque accumulation, which can cause chronic periodontitis. IgY is attractive for oral immunotherapy due to its several properties. The aim of this study was to prove IgY in egg yolk ability that can prevent the adhesion of *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* bacteria on enterocyte cell. The sample was divided into 8 groups, each group containing 10 ml of *Porphyromonas gingivalis* IgY and 50 enterocyte cells. The control group contained 50 ml of *Porphyromonas gingivalis* IgY and 50 ml of enterocyte cells. The first group to the seventh group was performed serial dilution with the first group containing 90 ml PBS and 10 ml *Porphyromonas gingivalis* IgY, the second group to the seventh group containing 50 PBS before adding 50 ml of enterocyte cells and 50 ml of bacterial suspension per group. The inherent bacterial count was calculated using a light microscope and the adherence index value was calculated. This study shows that *Porphyromonas gingivalis* IgY can significantly reduce the adherence index value of *Aggregatibacter actinomycetemcomitans* and can reduce the adherence index value of *Fusobacterium nucleatum* but not significantly. *Porphyromonas gingivalis* IgY can inhibit *Aggregatibacter actinomycetemcomitans* adherence, but cannot inhibit *Fusobacterium nucleatum* adherence.

Key words : IgY, egg yolk, medicine, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*.

INTRODUCTION

Periodontal disease or periodontitis is a bacterial infectious disease characterized by continuous inflammation, connective tissue damage and alveolar bone destruction (Vargas *et al*, 2015). Severe periodontitis characterized by tooth loss. This incident can be found in about 5-20% of adults in the world. Periodontal disease is divided into 3 types, aggressive periodontitis, chronic periodontitis and periodontitis manifestation of systemic disease (Aljehani, 2014). The bacterium that causes chronic periodontitis is the *Fusobacterium nucleatum* (*F. nucleatum*), while the bacterium that causes aggressive periodontitis is *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) (Wilson *et al*, 2002; Raja *et al*, 2014). Both of these bacteria are a rod-shaped anaerobic gram negative bacterium. The habitat of *F. nucleatum* and *A. actinomycetemcomitans* was subgingival (Avila-campos *et al*, 2006; Gholizadeh *et al*, 2017; Popova *et al*, 2013). Virulence factors of *A. actinomycetemcomitans* bacteria were divided into three groups, virulence factors that

modulate colonization and inflammation such as fimbriae, extracellular amorphous materials such as invasion and bacteriocin. Virulence factors that induce periodontal tissue damage such as lipopolysaccharide (LPS), inhibitors of chemotactic neutrophil activity, Cytolethal Distending Toxin (CDT) and Fc binding protein. Virulence factors that inhibit periodontal tissue repair such as cytotoxins, Heat Shock Proteins (HSPs), collagenase (Malik *et al*, 2015). Virulence factors from *F. nucleatum* are adesine, outer membrane protein (OMP), lipopolysaccharide (LPS) and others (Avila-campos *et al*, 2006; Jung *et al*, 2017).

Attractive activity between the bacteria surface and the host cell surface is called bacterial adherence activity. There are three stages of bacterial surface adherence, namely transport, initial adherence (usually called bioattachment) and colonization (Sandle, 2013). To kill bacteria, we can use antibiotics, but the use of antibiotics also has a negative effect, namely the occurrence of resistance or increased bacteria ability to stay alive in the presence of antibiotics (Nami *et al*, 2015).

Egg yolk antibodies are innovative technologies that involve the formation of noninvasive polyclonal antibodies from egg yolk (Baloch *et al*, 2015). Immunoglobulin Y (IgY) in the form of polyclonal antibodies, used as passive immunization and these antibodies come from egg yolk, colostrum, or concentrated cow's milk (Bachtiar *et al*, 2016). IgY antibodies has the same biological role as IgG antibodies in mammals namely as a major immunoglobulin which provides defense from infectious agents (Munhoz *et al*, 2014). IgY has several mechanisms for prevent periodontitis by inhibiting bacterial adherence to the cell surface, suppressing viral colonization by preventing the virus spread, enzyme inhibiting activity, and neutralizing toxins (Rahman, 2013).

MATERIALS AND METHODS

This research was a laboratory experimental study *in vivo* with a post-test controlled group design. The sample used was a mixture of enterocyte and immunoglobulin Y cells that had been induced with *P. gingivalis* bacteria and a mixture of enterocyte cells with *F. nucleatum* and *A. actinomycetemcomitans* as controls research with 3 times replication.

This research requires research tools such as centrifuged, anaerobic jar, shaking incubator, measuring cup, petri dish, light microscope with 1000x magnification, glass slide, Falcon tube, microcentrifuge tube and micropipette and research materials such as mice with weight 135 g, *P. gingivalis* specific immunoglobulin Y serum, culture of *F. nucleatum* bacteria, culture of *A. actinomycetemcomitans*, Phosphate Buffer Saline (PBS), Mueller-Hinton Broth (MHB), Solution containing PBS pH 7.4 + 1 mm DTT, Solution containing PBS pH 7.3, Solutions containing PBS pH 7.4 + 1.5 mg EDTA + 0.771 mg DTT, methanol, violet crystals, safranin, lugol and alcohol.

The culture of *F. nucleatum* ATCC 25586 was inserted into a medium tube containing BHI. Incubated for 24 hours in an anaerobic atmosphere in an anaerobic jar at 37°C for 24 hours. The culture of *Fusobacterium nucleatum* ATCC 25586, which had grown on BHIB was standardized 0.5 Mc Farland (1.5×10^8 CFU/ml) (Wulandari *et al*, 2013).

Isolation of Epithelial cells

The bacterial culture of *A. actinomycetemcomitans* was obtained by taking 1 colony of *A. actinomycetemcomitans* from the stock, then incubating it on BHI (Brain Heart Infusion) media for 24 hours with a suspension containing $\pm 1.5 \times 10^8$ cells/ml (≈ 0.5 Mc Farland standard) then, from BHI media, it will be planted again in a plate containing Mueller Hinton agar

with 24-hour incubation (Sasmita *et al*, 2014).

Enterocytes isolation using the Weisler method (Nagayama *et al*, 1995). Enterocytes are taken from the small intestine of mice 6 to 8 weeks old and weighing 135 g. Mice were sacrificed and then dissected to take part of the small intestine, then the small intestine that had been taken from the body of the mice then cut across and cut into small pieces and washed from dirt and mucus using a solution containing PBS pH 7.4 + 1 mm DTT.

After the intestinal tissue clean, cut into small pieces and put into the falcon tube, then added a solution containing PBS pH 7.3 as much as 20 ml, then put into a water heater at 37°C and shake using a shaker for 30 minutes. The discarded supernatant is replaced with a solution containing PBS pH 7.4 + 1.5 mg EDTA + 0.771 mg DTT for 30 ml, then shake using a waterbath for 30 minutes at 37°C after being shaken using a shaker, the supernatant is discarded. Falcon tubes containing enterocyte cells were washed using PBS. After washing using PBS, leave it until the precipitate settles all at the bottom of the tube. After all settles at the bottom of the tube, the supernatant is removed, then PBS is added as much as 20 ml, and inserted into the microcentrifuge tube. After that centrifuged at 1500 rpm for 3 minutes.

Solution dilution

Preparation of *P. gingivalis* immunoglobulin Y concentration using the serial dilution method on microcentrifuge tubes. The total concentration made is 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640. Each tube was added 50 mL PBS solution except in a tube with a concentration of 1/10 PBS solution was added as much as 90 μ l and 10 μ g *P. gingivalis* immunoglobulin Y 10 μ l, then PBS and *P. gingivalis* immunoglobulin Y solution homogenized on a 1/10 tube using vortex. After that, on a tube with a concentration of 1/10, 50 μ l of the homogeneous solution was taken using a micropipette and put into a tube with a concentration of 1/20, after which it was homogenized again using vortex. The same is done up to the concentration of 1/640. In tubes with a concentration of 1/640, the solution is removed as much as 50 μ l (Reynolds, 2016).

Adherence test

For the adherence test procedure, cultures of bacteria were centrifuged at 6000 rpm, at 4°C for 15 minutes. The precipitate is suspended in PBS containing 1% BSA. The bacterial content used is 108 / ml. Then the enterocyte suspension was added as much as 50 μ l at each concentration and gently shake it in the shaking water bath at 37°C for 30 minutes simultaneously. Then into the bacterial suspension mixture (10⁸/ml) was added as

much as 50 μ l. The mixture was incubated at the 'shaking incubator' for 30 minutes at 37°C simultaneously. Then centrifuged 1500 rpm, at 4°C for 3 minutes, then the liquid was disposed of as much as 100 μ l, after that the precipitate was taken and made smeared on the glass slide and painted with gram staining. Preparations were observed under a light microscope with 1000x magnification and counted the number of bacteria attached to enterocytes, calculated for each observation of 100 enterocytes (Ridwan, 2012). The results of the research data were carried out by the Kruskal Wallis test, then followed by the Bonferroni test.

RESULTS

The mean and standard deviation of adherence ability for each treatment of *F. nucleatum* and *A. actinomycetemcomitans* bacteria can be seen in Tables 1 and 2. The research data were analyzed using the Kruskal Wallis test. The results of the Kruskal Wallis test on *F. nucleatum* bacteria were $P = 0.823$ ($P < 0.05$), so that it could be interpreted that there were no significant differences from all groups. The Bonferroni test results in Table 3 of *F. nucleatum* obtained a value of 1,000 among the control groups with treatment, as well as between treatment groups. This shows that there is no significant difference between the control group and the treatment and between treatment groups. While the results of the Kruskal Wallis test on *A. actinomycetemcomitans* bacteria were $p = 0,000$ ($p < 0.05$), so that it could be interpreted that there were significant differences from the data of the entire group. The Bonferroni test results of *A. actinomycetemcomitans* in Table 4 obtained a value of 0.00 between the control group and the sample except in the group 1/320. This shows that there is a significant difference between the control group and the sample. The comparison between sample groups produces a value of 1,000. This can be interpreted that there is no significant difference between

Table 1 : The mean value and standard deviation of the adherence ability of the sample group to *F. nucleatum* bacteria.

Adherence of <i>F. nucleatum</i>			
	N	Mean	Std. Deviation
Control	6	0.41	0.331
A-1/10	6	0.15	0.036
B-1/20	6	0.24	0.174
C-1/40	6	0.20	0.065
D-1/80	6	0.35	0.344
E-1/160	6	0.35	0.114
F-1/320	6	0.23	0.114
G-1/640	6	0.32	0.184

Table 2 : The mean value and standard deviation of the adherence ability of the sample group against the *A. actinomycetemcomitans* bacteria.

Adherence of <i>A. actinomycetemcomitans</i>			
	N	Mean	Std. Deviation
Control	6	7,1000	1,05010
A-1/10	6	3,1967	1,81524
B-1/20	6	3,3083	2,01111
C-1/40	6	3,5967	1,65598
D-1/80	6	,0817	,05345
E-1/160	6	3,4350	,40089
F-1/320	6	4,9983	1,75242
G-1/640	6	5,6233	1,34585

sample groups.

In Fig. 1 is a description of the adherence of bacteria attached to enterocyte cells.

DISCUSSION

Based on the results and analysis of data from this study that *Porphyromonas gingivalis* immunoglobulin Y (IgY) can reduce the adherence of *A. actinomycetemcomitans* bacteria. This is likely because *Porphyromonas gingivalis* IgY is a polyclonal antibody that can capture various epitopes that are suitable for binding to antibody receptors. The main mechanism of

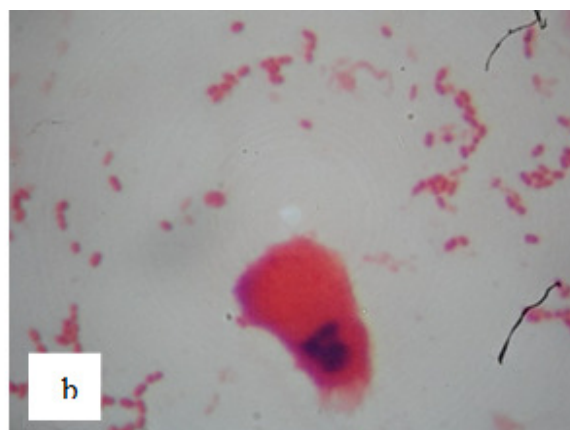
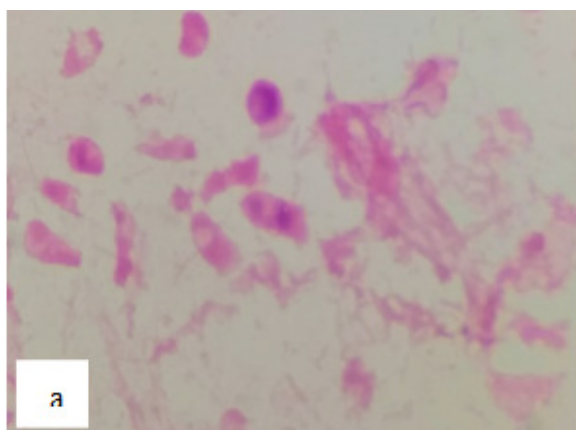


Fig. 1 : Bacterial adherence of sample groups to *F. nucleatum* (a) and *A. actinomycetemcomitans* bacteria (b).

Table 3 : Bonferroni test results on *F. nucleatum*

Group	1	2	3	4	5	6	7	8
1								
2	1,000							
3	1,000	1,000						
4	1,000	1,000	1,000					
5	1,000	1,000	1,000	1,000				
6	1,000	,409	1,000	1,000	1,000			
7	1,000	1,000	1,000	1,000	1,000	1,000		
8	1,000	1,000	1,000	1,000	1,000	1,000	1,000	

Description : 1: control group, 2: 1/10 treatment group, 3: treatment group 1/20, 4: treatment group 1/40, 5 : 1/80 treatment group, 6: treatment group 1/160, 7: treatment group 1/320, 8: treatment group 1/640, *: There are significant differences between.

Table 4 : Bonferroni test results on *A. actinomycetemcomitans* bacteria .

Group	1	2	3	4	5	6	7	8
1								
2	,001*							
3	,001*	1,000						
4	,003*	1,000	1,000					
5	,000*	,014*	,009*	,003*				
6	,002*	1,000	1,000	1,000	,006*			
7	,406	,962	1,000	1,000	,000*	1,000		
8	1,000	,148	,211	,507	,000*	,312	1,000	

Description: 1: control group; 2: 1/10 treatment group; 3: treatment group 1/20; 4: treatment group 1/40; 5: 1/80 treatment group; 6: treatment group 1/160; 7: treatment group 1/320; 8: treatment group 1/640.

immunoglobulin Y is to bind components that exist on the bacteria surface such outer membrane protein, lipopolysaccharide, colonization devices such as vesicles and fimbriae which are virulence factors of bacteria *A. actinomycetemcomitans* (Sriraman, 2014).

Adherence of the *A. actinomycetemcomitans* bacteria differed between the treatment groups compared to the probable control group because the bacteria had the same virulence factor as the antigens that stimulated the formation of antibodies such as vesicles and fimbriae. Vesicles can release proteins such as outer membrane proteins, hemagglutinin proteins, bacteriocin proteins, and adessin from bacteria (Gani *et al*, 2009). Fragments Antigen Binding (FAB) possessed by *P. gingivalis* Immunoglobulin Y can bind and recognize proteins released by vesicles found on the surface of bacteria. Fragment of Antigen Binding from *P. gingivalis* immunoglobulin Y can also bind fimbriae which is a bacterial movement tool to attach to periodontal tissue and carry out colonization activities (Garrett *et al*, 2008). Fimbriae is a virulence factor that plays a major role in the adherence activity of *A. actinomycetemcomitans*. The bacteria cannot attach to periodontal tissue because

fimbriae from these bacteria are bound by *P. gingivalis* immunoglobulin Y (Hasan and Palmer, 2014), whereas to carry out pathogenic activities, the process that must be passed is that bacteria must be attached to the periodontal tissue and then carry out colonization activities with similar bacteria (Pandit *et al*, 2015).

Unlike the *F. nucleatum* bacteria, from the analysis there was no significant difference between the control group and the treatment group. This can occur because there are differences in virulence factors which play a major role in the adherence activity between *F.nucleatum* and *P. gingivalis* bacteria. The main virulence factor for *P. gingivalis* bacteria in adherence activity is fimbriae, whereas for *F. nucleatum* bacteria is FadA. FadA is the only adhesion material identified to bind host cells and the best virulence factors identified from *F. nucleatum* bacteria. FadA is specifically produced by two species of *Fusobacterium*, namely *F. nucleatum* and *F. periodonticum* and is not present in other species in *Fusobacterium*. Because of this difference, *P. gingivalis* Immunoglobulin Y cannot inhibit the adherence of *F.nucleatum* bacteria to the *P. gingivalis* Immunoglobulin Y mechanism. Equivalentival can only bind to virulence factors similar to the virulence factors of *P. gingivalis* bacteria as antigens that stimulate the formation of *P. gingivalis* Y Immunoglobulin (Han, 2013).

For comparison between concentration groups, based on the results of data analysis there were significant differences in the comparison of the control group with the concentration of the bacteria *A. actinomycetemcomitans*, but in the 1/320 dilution group showed no significant difference, this indicates that IgY's ability to inhibit *A .actomycetemcomitans* bacterial adherence minimal at 1/160 dilution. Whereas, the comparison between fellow treatment groups there was no significant difference.

CONCLUSION

The conclusion from this study is that *Porphyromonas gingivalis* immunoglobulin Y can prevent the adherence of *A. actinomycetemcomitan* bacteria to a dilution of 1/160, but cannot prevent the adherence of *F. nucleatum* bacteria.

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