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Research Report

The potency of Immunoglobulin Y anti *Porphyromonas gingivalis* to inhibit the adherence ability of *Porphyromonas gingivalis* on enterocytes

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ABSTRACT

Background: *Pophyromonas gingivalis (P. gingivalis) bacteria are the main type of bacterium that cause chronic periodontitis. Immunoglobulin Y (IgY) is a type of immunoglobulin found in poultry, such as chickens and birds. IgY can be used as an alternative method of preventing the accumulation of plaque that causes chronic periodontitis.* ***Purpose:*** *To determine the ability of IgY anti P. gingivalis to inhibit adherence of P. gingivalis.* ***Methods:*** *The samples were divided into eight groups, each group containing 10 ml of IgY anti P. gingivalis and 50 ml of enterocyte cells. The control group contained 50 ml of IgY anti P. gingivalis, and 50 ml of enterocyte cells. Serial dilution was carried out to the first seven groups, with the first group containing 90 ml phosphate-buffered saline (PBS) and 10 ml IgY anti P. gingivalis, and the second to seventh groups containing 50 ml PBS before adding 50 ml of enterocyte cells and 50 ml of bacterial suspension per group. The number of bacteria was calculated as an adherence index value using a light microscope.* ***Results:*** *This study shows that IgY anti P. gingivalis significantly reduces the adherence index value of P. gingivalis.* ***Conclusion:*** *IgY anti P. gingivalis has potency to inhibit the adherence of P. gingivalis.*

Keywords: *adherence; egg yolk; IgY; Pophyromonas gingivalis*

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INTRODUCTION

Periodontal disease or periodontitis is a bacterial infectious disease characterised by continuous inflammation, connective tissue damage, and alveolar bone destruction.[1] Severe periodontitis can lead to tooth loss. This disease can be found in approximately 5–20% of adults worldwide. Periodontal disease is divided into three types: aggressive periodontitis; chronic periodontitis; and periodontitis caused by systemic disease manifestation.[2] The bacterium that causes chronic periodontitis is *Pophyromonas gingivalis* (*P.gingivalis*).[3] This bacterium is a rod-shaped anaerobic Gram-negative bacterium. *P. gingivalis* bacteria inhabit the subgingival area. Some virulence factors of *P. gingivalis* bacteria include adhesives, capsules, lipopolysaccharides (LPS), proteases, and outer membrane proteins. Capsules can reduce the phagocytic activity for invasion; LPSs, protease enzymes and membrane proteins can aid bacterial aggregation on the cell surface and fimbriae.[4]

Pulling activity between the surface of the bacteria and the surface of the host cell is called bacterial adherence activity. There are three stages of bacterial adherence to the surface, namely transport, initial adherence (usually called bioattachment) and colonisation.[5] Antibiotics can be used to kill bacteria, but antibiotics also have a negative effect, including the resistance or increased ability of bacteria to stay alive in the presence of antibiotics.[6]

Immunoglobulin Y (IgY) technology is an innovative technology that involves the non-invasive production of polyclonal antibodies from egg yolk, and due to its non-invasive nature, IgY technology has provided new opportunities in both therapeutic and prophylactic applications in human and veterinary medicine.[7] IgY in the form of polyclonal antibodies is used as passive

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immunisation, and these antibodies derive from egg yolk, colostrum, or concentrated cow's milk.[8] IgY has the same biological role as IgG in mammals, as a major type of immunoglobulin that provides defence against infectious agents.[9] IgY can be used to prevent periodontitis by inhibiting bacterial adherence to the cell surface, inhibiting enzyme activity, and neutralising the toxins produced by the periontopathogen.[10] The aim of this research was to determine the ability of anti IgY of *P. gingivalis* in egg yolk to inhibit the adherence of *P. gingivalis* bacteria.

MATERIALS AND METHODS

This study was a laboratory in vitro experimental study with a post-test-controlled group design. IgY specific *P. gingivalis* was obtained from chicken eggs that had been injected with *P. gingivalis* (ATCC 33277) by as much as 1.5 x 10^9 bacterial colony, three times a week for three weeks. In the fourth week, the egg yolks were taken. The sample used was a mixture of enterocyte cells and IgY that had been induced with *P. gingivalis* (ATCC 33277) bacteria, and the control was a mixture of enterocyte cells with *P. gingivalis* (ATCC 33277) bacteria with three times replication.

This research required the following research tools: a centrifuge, an anaerobic jar, a shaking incubator, a measuring cup, a petri dish, a light microscope with 1000x magnification, a glass slide, a Falcon tube, a microcentrifuge tube, a micropipette, a research subject, i.e. mice with body weight 135 g, specific *P. gingivalis* (ATCC 33277) IgY serum, a culture of *P. gingivalis* (ATCC 33277) bacteria, phospate buffer saline (PBS), Mueller-Hinton broth (MHB), a solution containing PBS pH 7.4 + 1 mm dithiothreitol (DTT), a solution containing PBS pH 7.3, a solution containing PBS pH 7.4 + 1.5 mg Ethylenediaminetetraactic acid (EDTA) + 0.771 mg DTT, methanol, violet crystals, safranin, lugol, and alcohol. One colony of *P. gingivalis* (ATCC 33277) that had been grown in blood agar, was put into a test tube containing brain heart infusion (BHI) media using an oese and incubated in an anaerobic atmosphere using a gas generating kit for 24 hours at 37°C. After the incubation period, an equal concentration of bacteria was processed in another test tube containing BHI media, so that it was to the same McFarland standard 0.5 (1.5 x 10^8 CFU/ml).[11]

Isolation of enterocytes was performed using the Weisler method.[12] Enterocytes were taken from the small intestine of 6–8-week-old mice weighing 135 g. The mice were humanely destroyed and then dissected to remove a part of the small intestine. The small intestine that had been taken from the body of the mice was then cut across and minced into small pieces, then washed using a solution containing PBS pH 7.4 + 1 mm DTT, to remove dirt and mucus. After the intestinal tissue was clean, it was put into a falcon tube, then as much as 20 ml of the solution containing PBS pH 7.3 was added. It was then put into a water heater at 37°C and shaken with a shaker for 30 minutes. The discarded supernatant was then replaced with as much as 30 ml of a solution containing PBS pH 7.4 + 1.5 mg EDTA + 0.771 mg DTT, and was shaken using a water bath for 30 minutes at 37°C. After being shaken, the supernatant was removed. Falcon tubes containing enterocyte cells were washed using PBS. Next, they were left until the enterocytes had all settled at the bottom of the tube. After the enterocytes had settled, the supernatant was removed, then 20 ml of PBS was added, and the tubes were inserted into the microcentrifuge tube and centrifuged at 1500 rpm for three minutes.

Separation of the *P. gingivalis* IgY concentration was carried out using the dilution series method on the microcentrifuge tubes. The concentrations made were 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and these were put into Eppendorf tubes. Each tube was added 50 ml PBS solution except in a tube with a concentration of 1/10, in other six samples PBS solution was added as much as 90 µl and 10 µl IgY *P. gingivalis*, then all of the samples homogenised using vortex. After that, 50 µl of the homogeneous solution was taken from the tube with a concentration of 1/10 using a micropipette and put into the tube with a concentration of 1/20, after which it was homogenised using a vortex. The same procedure was carried out until the concentration reached 1/640. In the tubes with a concentration of 1/640, as much as 50µl of the solution was removed.[13] For adherence to the test procedure, cultures of bacteria were centrifuged at 6000 rpm and 4°C for 15 minutes. The precipitate was suspended in PBS containing 1% of bovine serum albumin (BSA). The bacterial content used was 10^8/ ml. Then 50 µl of the enterocyte suspension was added to each concentration and shaken using a shaker in the water bath at 37°C for 30 minutes. As much as 50 µl of the mixture was then added to the bacterial suspension (10^8/ml) The mixture was incubated in the shaking incubator for 30 minutes at 37°C. It was then centrifuged at 1500 rpm and 4°C for 3 minutes, then as much as 100 µl of the liquid was disposed of.

Each precipitate was taken and smeared on a glass slide and painted with Gram stain. The glass slides were observed under a light microscope with 1000x magnification, and the number of bacteria attached to enterocytes were counted, being calculated for each observation of 100 enterocytes.[14] The Kruskal Wallis difference test was carried out in the control group and the treatment group to determine if the

Table 1. Mean and standard deviation of the power of adherence of sample groups to *P. gingivalis*

	Adherence Pg		
	N	Mean	Std. Deviation
Control	6	12.7583	6.44508
A-1/10	6	4.0733	.53638
B-1/20	6	5.3417	.85873
C-1/40	6	5.4517	1.75848
D-1/80	6	4.8500	1.77213
E-1/160	6	6.2950	1.89505
F-1/320	6	4.1700	1.58865
G-1/640	6	9.1000	4.19729

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significance value was below 0.05 ($p<0.05$), i.e. showed significant differences between groups. Then the Bonferroni test was carried out.

RESULTS

The treatment group with *P. gingivalis* (ATCC 33277) of concentration 1/640 had the largest mean of 9.1, whereas the treatment group with *P. gingivalis* (ATCC 33277) of concentration 1/10 had the smallest mean of 4.07.

The mean and standard deviation of the power of adherence in each treatment group of the *P. gingivalis* bacteria can be seen in Table 1. The research data were analysed using the Kruskall Wallis test. The results of the Kruskall Wallis test in this study were $p = 0.000$ ($p <0.05$), so it could be interpreted that there were significant differences between the data of the entire group.The Bonferroni test results, shown in Table 2, obtained a value of 0.00 between the control group and the sample groups, except in the group 1/320. This shows that there was a significant difference between the control group and the sample groups. Comparison between the sample groups produces a value of 1.000. This can be interpreted as there being no significant difference between the sample groups. Figure 1 shows the result of the experiment in adherence of bacteria onto the enterocyte cells.

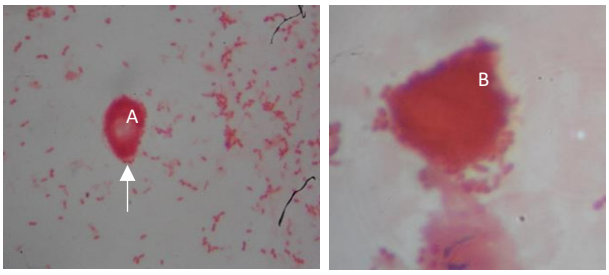

Figure 1. Bacterial adherence in the sample group to P. gingivalis (A) in enterocyte cell. (B) control group. Arrow indicates *P. gingivalis* adherence to the enterocyte cell.

DISCUSSION

IgY has several advantages compared to antibiotics, vaccines, and immunotherapy. The advantages of IgY compared to antibiotics are: 1) it is natural; 2) it is safe and is not absorbed into the body circulation (no toxic tissue residues); 3) it avoids environmental contamination with synthetic chemical drugs; 4) it does not induce specific pathogenic resistance of microorganisms since it is directed to multi epitopic antigenic targets that need multiple genes for their synthesis; 5) it is highly specific in its reactivity and controls only targeted pathogens without affecting normal bacterial flora; 6) it has a potentially broad spectrum of specificity when customised against viruses, bacteria or fungi; and 7) it does not induce adverse side effects as synthetic drugs do.[10] The advantage of IgY as passive immunotherapy is that IgY has rapid action and high specific activity; it can be given to all ages ranging from infants to adults, including babies with low birth weight (LBW), patients with immunodeficiency, and pregnant women. It is not toxic and can be stored for a long time. IgY is attractive for oral immunotherapy because some of its properties can be taken from animals without hurting them, the binding to antigens is stronger than in mammalian IgG, and it reacts more to the same antigen. IgY is also a natural ingredient that does not cause side effects when taken orally unless the recipient has an allergy to eggs.[15]

Based on the results and analysis of the data, it was found that *P. gingivalis* IgY can inhibit the adherence of *P. gingivalis* bacteria. This is likely because IgY is a polyclonal antibody that can capture various epitopes on the cell surface of the bacterium. Antibodies are host proteins found in plasma and extracellular fluids that serve as the first response and comprise one of the principal effectors of the adaptive immune system. They are produced in response to molecules and organisms, which they ultimately neutralise and/or eliminate. The ability of antibodies to bind to an antigen with a high degree of affinity and specificity has led to their ubiquitous use in a variety of scientific and medical disciplines. The formation of antibodies is not only due to the binding of the epitope with the antibodies,

Table 2. Results of Bonferroni test to *P. gingivalis*

Groups	1	2	3	4	5	6	7	8
1								
2	.000*							
3	.003*	1.000						
4	.004*	1.000	1.000					
5	.001*	1.000	1.000	1.000				
6	.017*	1.000	1.000	1.000	1.000			
7	.000*	1.000	1.000	1.000	1.000	1.000		
8	1.000	.173	1.000	1.000	.532	1.000	.200	

Note: 1) control group; 2) treatment group 1/10; 3) treatment group 1/20; 4) treatment group 1/40; 5) treatment group 1/80; 6) treatment group 1/60; 7) treatment group 1/320; 8) treatment group 1/640.
*There are significant differences between sample groups.

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but because bacteria are one type of antigen that has virulence factors that can stimulate antibody formation.[16] The main mechanism of IgY is binding to components on the surface of bacteria, such as outer membrane proteins, lipopolysaccharides,as well as colonisation tools such as vesicles and fimbriae which are virulence factors of the *P. gingivalis* bacteria.[17] The adherence value of *P. gingivalis* bacteria is significantly different between the treatment groups and the control group because of the possibility that this bacterium has expressed its virulence factor, particularly adhesion.[18]

Fragments of antigen binding (FAB) possessed by IgY anti *P. gingivalis* can bind and recognise proteins on the cell surface of the bacterium. FAB from IgY anti *P. gingivalis* can also bind to fimbriae, which are bacterial movement tools, to attach to periodontal tissue and carry out colonisation activities. As shown in this study, IgY binds to enterocytes.[19] The presence of fimbriae is a virulence factor that plays a major role in the adherence activity of the *P.gingivalis*.[20] On periodontal tissue, periodontitis carried out by imbriae from this bacterium are bound by IgY *P.gingivalis*,[21] whereas to carry out pathogenic activities, the process that must be carried on is that bacteria must be attached to the periodontal tissue and then carry out colonisation activities with similar bacteria.[21] Comparing concentration groups, based on the results of the data analysis, there were significant differences in the comparison of the control group with the concentration of the *P. gingivalis*, while in the 1/640 dilution group no significant difference could be seen, indicating IgY's ability to inhibit bacterial adherence on enterocytes at a minimum concentration. However, comparing between fellow treatment groups, there was no significant difference. This shows that, to inhibit P. gingivalis bacteria, it was sufficient to use a minimum concentration of IgY anti *P. gingivalis* in this study (1/320). The conclusion of this study is that IgY anti *P. gingivalis* has the potency to inhibit the adherence of *P. gingivalis* on enterocytes, and this potency is not dependent on the concentration.

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