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The potency of Immunoglobulin Y anti Porphyromonas gingivalis to inhibit the adherence ability of Porphyromonas gingivalis on enterocyte ABSTRACT Background: Pophyromonas gingivalis (P. gingivalis) bacteria are the main bacterium that cause chronic periodontitis. The Immunoglobulin Y (IgY) is a type of immunoglobulin found in poultry, such as: chicken and birds. IgY can be used as an alternative prevention of plaque accumulation which can cause chronic periodontitis. Purpose: To determine the ability of IgY anti P. gingivalis in to inhibit the adherence of the P. gingivalis. Methods: The samples were divided into 8 groups, each group containing 10 ml of IgY anti P. gingivalis and 50 enterocyte cells. The control group contained 50 ml of IgY anti P. gingivalis, and 50 ml of enterocyte cells. Serial dilution was performed to first group until seventh group with the first group containing 90 ml PBS and 10 ml IgY anti P. gingivalis, 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 bacterium number was calculated using a light microscope as an adherence index value. Results: This study shows that IgY anti P. gingivalis significantly reduce the adherence index value of P. gingivalis. Conclusion: IgY anti P. gingivalis has a potency to inhibit adherence of P. gingivalis. Keywords: adherence; egg yolk; IgY; Pophyromonas gingivalis INTRODUCTION Periodontal disease or periodontitis is a bacterial infectious disease characterized by continuous inflammation, connective tissue damage, and alveolar bone destruction. 1 Severe periodontitis is characterized by tooth loss. This disease can be found in around 5-20% of adults in the world. Periodontal disease is divided into 3 types, aggressive periodontitis, chronic periodontitis, and periodontitis caused 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. The habitat of the P. gingivalis bacteria is in subgingiva. Some virulence factors possessed by P. gingivalis bacteria include adhesives, capsules, lipopolysaccharide (LPS), proteases, and outer membrane proteins. Capsules can reduce phagocytic activity for invasion, LPS, protease enzyme, membrane protein can help bacterial aggregation on the cell surface, and fimbriae.4 Pulling activity between the surface of 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 colonization.5 Antibiotics can be used to kill bacteria, but antibiotics also has a negative effect, including the occurrence of resistance or increased ability of bacteria to stay alive in the presence of antibiotics.6 The 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 opened new avenues in both therapeutic and prophylactic applications in human and veterinary medicine. 7 IgY in the form of polyclonal antibodies, used as passive immunization and these antibodies come from egg yolk, colostrum, or concentrated cow's milk.8 IgY has the same biological role as IgG in mammals, namely as major immunoglobulin which provides defense from infectious agents.9 IgY can be used to prevent periodontitis by inhibiting bacterial adherence to the cell surface, inhibiting enzyme activity, and neutralize toxins produced by the periontopathogen.10 The aims of this research was to determine the ability of anti IgY of P. gingivalis in egg yolk to inhibit the adherence of the P. gingivalis bacteria. MATERIALS AND METHODS This study was a laboratory invitro 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) as much as 1.5x109 bacterial colony for 3 times per week until 3 weeks. In the fourth week egg yolk were taken. The sample used was a mixture of enterocyte and IgY that had been induced with P. gingivalis (ATCC 33277) bacteria, and a mixture of enterocyte cells with P. gingivalis (ATCC 33277) bacteria as a control study with 3 times replication. This research required research tools such as centrifuged, anaerobic jar, shaking incubator, measuring cup, petri dish, light microscope with 1000x magnification, glass slide, Falcon tube, microcentrifus tube, and micropipette, and research subject, i.e mice with body weight 135 g, specific P. gingivalis (ATCC 33277) IgY serum, culture of P. gingivalis (ATCC 33277) bacteria, Phospate Buffer Saline (PBS), Mueller-Hinton Broth (MHB), Solution containing PBS pH 7.4 + 1 mm Dithiothreitol (DTT), Solution containing PBS pH 7.3, Solution containing PBS pH 7.4 + 1. 5 mg 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 BHI media using oese and incubated in anaerobic atmosphere using gas generating kit for 24 hours at 37?C. After the incubation period, an equal concentration of bacteria was carried out in another test tube containing BHI media so that it was the same to the McFarland standard 0.5 (1.5x108 CFU/mL).11 Isolation of enterocytes using Weisler methode.12 Enterocytes were taken from the small intestine of mice 6 to 8 weeks old with mice weighing 135 g. Mice were sacrificed and then dissected to take a part of the small intestine, then the small intestine that had been taken from the body of the mice was cut across and mince into small pieces and washed using a solution containing PBS pH 7.4 + 1 mm DTT to clean from dirt and mucus. After the intestinal tissue was clean, it was put into a falcon tube, then was added, the solution containing PBS pH 7.3 as much as 20 ml, then put into a water heater at 37oC and shake with a shaker for 30 minutes. The discarded supernatant then replaced with a solution containing PBS pH 7.4 + 1.5 mg EDTA + 0.771 mg DTT as much as 30 ml, then was shaked using a waterbath for 30 minutes at 37°C. After being shaken using a shaker, the supernatant was removed. Falcon tubes containing enterocyte cells were washed using PBS. Next, leave it until the enterocytes settle all at the bottom of the tube. After the enterocytes settle, the supernatant was removed, then 20 ml of PBS was added, and inserted into the microsentrifuse tube and centrifuged at 1500 rpm for 3 minutes. Separation of P. gingivalis IgY concentration using dilution series method on microcentrifus tubes. The concentration made were 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and put into Eppendorf tube. 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 µl and 10 µl IgY P. gingivalis, then PBS and IgY P. gingivalis solution contained in the 1/10 tube homogenized using vortex. After that, on a tube with a concentration of 1/10, 50 µl 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 using vortex. The same procedure was done until to the concentration reached 1/640. In tubes with a concentration of 1/640, the solution is removed as much as 50µl.13 For the adherence test procedure, cultures of bacteria were centrifuged at 6000 rpm, at 4oC for 15 minutes. The precipitate was suspended in PBS containing 1% Bovine Serum Albumin (BSA). The bacterial content used was 108/ ml. Then 50 µl enterocyte suspension was added to each concentration and shaken using a shaker in the shaking water bath at 37oC for 30 minutes simultaneously. Then into the mixture was added as much as 50 µl bacterial suspension (108/ml) The mixture was incubated at the 'shaking incubator' for 30 minutes at 37oC simultaneously. Then centrifuged 1500 rpm, at 4oC for 3 minutes, then the liquid was disposed of as much as 100 µl. The precipitate was taken and made smeared on the glass slide and painted with Gram staining. The glass slides were observed under a light microscope with 1000x magnification, and counted the number of bacteria attached to enterocytes, calculated for each observation of 3 100 enterocytes.14 The Kruskal Wallis difference test was carried out in the control group and the treatment group was determined if the significance value was below 0.05 (p<0.05) which showed significant differences between groups and then continued with the Bonferroni test. RESULTS This research used P. gingivalis (ATCC 33277) bacteria which was inserted into microsentrifuse tube with P. gingivalis IgY as much as 50 µl and centrifuged at 1500 rpm for 3 minutes then smeared on a slide glass and manually counted using a microscope with 1000x magnification. The treatment group with P. gingivalis (ATCC 33277) in the concentration group 1/640 had the largest mean of 9.1. Whereas in the treatment group P. gingivalis (ATCC 33277) in the concentration group 1/10 had the smallest mean of 4.07. The mean and standard deviation of the power of adherence in each treatment of the P. gingivalis bacteria can be seen in Table 1. The research data were analyzed using the Kruskall Wallis test. The results of the Kruskall Wallis test in this study 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 in Table 2 obtained a value of 0.00 between the control group and the sample except in the group 1/320. This shows that there was a significant difference between the control group and the sample. On comparisons between sample groups produce a value of 1.000. This can be interpreted that there is no significant difference between sample groups. Figure 1 descript the result of experiment in adherence of bacteria onto enterocyte cells. DISCUSSION The IgY has several advantages compared to antibiotics, vaccines, and as immunotherapy. The advantage of IgY compared to antibiotics is Ig Y is 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 microorganisms resistance since it is directed to multi epitopics antigenic targets which 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 customized against viruses, bacteria or fungi, and 7) It does not induce adverse side effects unlike synthetic drugs. 10 The advantage of IgY as passive immunotherapy is that IgY has a mechanism of rapid action and high specific activity, can be given to all ages ranging from infants to adults including babies with low birth weight (LBW) or immunodeficiency patients and pregnant women, not toxic, and can be stored for a long 4 time. IgY is attractive for oral immunotherapy because some of its properties can be taken from animals without hurting animals, binding to antigens is stronger than mammalian IgG and reacting more to the same antigen, IgY is a natural ingredient that does not cause side effects when taken orally unless they have allergies against eggs.15 Based on the results and analysis of data from this study that P. gingivalis IgY can inhibit the adherence of P. gingivalis bacteria. This is likely because the IgY is a polyclonal antibodies that can capture various epitopes on the bacterium's cell surface. Antibodies are host proteins found in plasma and exracellular 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 neutralize and/or eliminate. The ability of antibodies to bind 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 antibodies, but bacteria which is one type of antigen that has virulence factors that can stimulate antibody formation.16 The main mechanism of immunoglobulin Y is binding components on the surface of bacteria such as outer membrane protein, lipopolysaccharide, colonization tools such as vesicles and fimbriae which are virulence factors of the P. gingivalis bacteria.17 The adherence value of P. gingivalis bacteria has a significant difference between the treatment groups compared to the control group because of the possibility that this bacterium has expressed its virulence factor, particularly adhesin.18 Fragments of Antigen Binding (FAB) possessed by IgY anti P. gingivalis can bind and recognize proteins on the bacterium's cell surface. Fragment of Antigen Binding from IgY anti P. gingivalis can also bind fimbriae which is a bacterial movement tool to attach to periodontal tissue and carry out colonization activities, as shown in this study, the IgY bind to enterocyte.19 Fimbriae is a virulence factor that plays a major role for adherence activity of the P.gingivalis.20 On periodontal tissue because fimbriae 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 colonization activities with similar bacteria.21 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 P. gingivalis, but in the 1/640 dilution group showed no significant difference, this indicates that IgY's able to inhibit bacterial adherence on enterocyte at a minimum concentration. Whereas for comparison between fellow treatment groups there was no significant difference. This shows that, to inhibit P. gingivalis bacteria, it is enough to use IgY 5 anti P. gingivalis with minimum concentration in this study (1/320). The conclusion of this study is that IgY anti P. gingivalis has a potency to inhibit the adherence of P. gingivalis on enterocyte and it is not dependent on the concentration. REFERENCES 1. Vargas Segura AI, Ilyina A, Segura Ceniceros EP, Silva Belmares Y, Méndez González L. Etiology and microbiology of periodontal diseases: a review. African J Microbiol Res. 2015; 9(48): 2300–6. 2. Aljehani YA. Risk factors of periodontal disease: review of the literature. Int J Dent. 2014; 2014: 1–9. 3. Ismail AD. Oral bacterial interactions in periodontal health and disease. J Dent Oral Hyg. 2014; 6(5): 51–7. 4. How KY, Song KP, Chan KG. Porphyromonas gingivalis: an overview of periodontopathic pathogen below the gum line. Front Microbiol. 2016; 7: 1–14. 5. 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Bacterial adhesion and biofilms on surfaces. Prog Nat Sci. 2008; 18(9): 1049–56. 21. Hasan A, Palmer RM. A clinical guide to periodontology: Pathology of periodontal disease. Vol. 216, British Dental Journal. Nature Publishing Group; 2014. p. 457–61. 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 A-1/10 6 4.0733 B-1/20 6 5.3417 6.44508 .53638 .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 B A Figure 1. Bacterial adherence in sample group to P. gingivalis (A) in enterocyte cell. (B) control group. Pointed with arrow is P.

gingivalis that adhere to enterocyte cell. 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. Nova Andriani Hepitaria, Indeswati Diyatri, Markus Budi Rahardjo and Rini Devijanti Ridwan Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga Surabaya – Indonesia Correspondence: Rini Devijanti Ridwan, Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjen. Prof. Dr. Moestopo 47 Surabaya 60132, Indonesia. Email: rini-d-r@fkg.unair.ac.id 1 2 6 7 8