EFFECT OF TOPICAL ANTI-*STREPTOCOCCUS MUTANS* IgY GEL ON QUANTITY OF *S. MUTANS* ON RATS' TOOTH SURFACE

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This study aims to evaluate the effect of anti-*Streptococcus mutans* IgY gel on quantity of *S. mutans* on rats' tooth surface. Sprague Dawley rats were exposed intra-orally with *S. mutans* Xc and were fed a caries-inducing diet 2000. The 24 rats were divided into four groups: group A had their teeth coated with IgY gel; group B received sterilized water as a control; group C had their teeth coated with IgY gel starting on the 29th day; and group D had their teeth coated with a gel without IgY. Plaque samples were swabbed from the anterior teeth for *S. mutans* colony quantification, and saliva was collected to measure immunoreactivity by enzyme-linked immunosorbent assay. The results indicated that the quantity of *S. mutans* in rats treated with IgY gel showed significant difference compared with the controls. After coating with IgY anti-*S. mutans* gel, the mean immunoreactivity in rat saliva was higher than that of the no treatment group. In conclusion, topical application with anti-*S. mutans* IgY gel reduced the quantity of *S. mutans* on the tooth surface.

Keywords: IgY gel, *S. mutans*, caries

Introduction

Dental caries is an infectious disease with a high estimated prevalence in Indonesia. According to Indonesian Basic Health Research conducted in 2007 by the Department of Health in Indonesia, the active caries prevalence of the

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population aged 12 years and older reached 46.50%, and 27.90% of subjects were caries free [1]. Caries involves many factors other than *Streptococcus mutans* (*S. mutans*); these include plaque accumulation, carbohydrate intake, acidic diet, and conditioning saliva [2–5]. The immune system plays a role in the prevention of cariogenic processes, along with other components that impede bacterial growth [6, 7]. Common caries prevention involves the use of fluoride and chlorhexidine. Another caries prevention method is passive immunization by inhibiting biofilm formation without eliminating the associated micro-organisms from the oral cavity [8]. This immunological approach that protects teeth from caries acts by preventing bacterial interaction with dental surfaces. Antibodies can block the interactions between *S. mutans* adhesions with receptors involved in dental surface colonization or accumulation (glucan binding) or impede the activation of the glucosyltransferase (GTF) [9]. The passive immunization method is considered to be safer and has gained more attention from experts than the active immunization method [10–12]. Various studies have shown the effectiveness of passive immunization in caries prevention both in animals and in humans. IgY antibody, in the form of monoclonal antibody, has been used in passive immunization as well, and it is obtained from egg yolk, colostrum, or cow milk concentrate [11, 13, 14]. Shimazaki et al. [10] have successfully used bovine milk containing antibody against the fusion protein antigen A of the *S. mutans* serotype c and glucan-binding (GB) domain of GTF-I (PAc-GB) as a source of passive immunization to prevent the recolonization of *S. mutans*.

The applied technology of IgY has proven to be more beneficial than conventional antibody products obtained from rabbits or other mammals. Chicken eggs produce more antibodies than do mammals. One chicken produces antibodies equal to that produced by 10–20 rabbits. Moreover, it is easier to collect eggs than to obtain blood from 10 rabbits. Each egg yolk can also be purified to produce 50–100 mg of IgY. Generally, IgY production is cheaper, and collecting eggs is non-invasive; furthermore, IgY isolation is fast and easy. Another benefit is that fewer antigens are required to titre IgY over time [15–18]. IgY as an anti-cariogenic agent that has also been examined in the form of toothpaste [19]. Research conducted by Hatta et al. [20] showed a decrease in *S. mutans* levels in saliva from a volunteer group that received mouthwash containing anti-*S. mutans* whole-cell IgY. Research conducted by Paau and Rong-Jiang [21] in 2006 showed that the IgY-anti-*S. mutans* toothpaste decreased the percentage of *S. mutans* among the total anaerobic bacteria found in both saliva and plaque samples. Similar to previous research, Otake et al. [22] used anti-*S. mutans* whole-cell IgY in the form of frozen egg yolk in the rat diet. However, the colony count of *S. mutans* was not significantly different after this intervention. This might have been caused by the shortened contact time of IgY in the mouth. IgY application in the form of gel as a protective and curative strategy against dental caries, however, has not yet been studied.

Materials and methods

Preparation of IgY gel

Anti-*S. mutans* IgY gel was produced by the Pharmacy Laboratory of the Faculty of Medicine, Universitas Indonesia. It consisted of a water-based CMC (carboxy methyl cellulose) gel that was mixed with water-soluble fraction (WSF) IgY powder. WSF powder was prepared by the Institute of Agriculture in Bogor using the egg yolks of immunized hens and was purified using the EGGSTRACT purification kit. The specificity of the IgY antibody was examined using the Ouchterlony test with rabbit antiserum against the chicken serum IgG and mouse IgG. Specific binding was only observed with the anti-chicken serum IgG. The confirmation test of the stability of gel production involved the immunoreactivity of IgY, which was checked after storage for 30 days. The enzyme-linked immunosorbent assay (ELISA) technique was used to determine the lowest concentrations of anti-*S. mutans* IgY that still showed immunoreactivity against *S. mutans* [23].

Experimental rat caries model

The experiment used a caries model in Sprague Dawley rats which were inoculated with *S. mutans* Xc (the *S. mutans* serotype c kindly provided by Yamashita, Niigata University, Japan). The coating method was conducted once per day in the labial buccal part of the rats' teeth (Figure 1). A plaque swab sample was taken from the anterior teeth for the colony forming unit (CFU) test [22]. To test the anti-cariogenic activity of IgY antibodies in vivo, the experimental rat caries model was used. Specific pathogen-free Sprague Dawley rats (aged 20 days) were treated with ampicillin and chloramphenicol for 3 days to eliminate the microbial flora. Rat chow was pulverized and mixed with 1 g of each individual antibiotic per 1 kg of diet. This treatment reduced the number of oral micro-organisms when the oral swabs were obtained from the individual rats and examined on plates containing TSB agar. *S. mutans* was not detected in the oral cavity. Twenty-four Sprague Dawley rats were selected from the initial group of rats. The rats were then randomly separated into several experimental groups, with each group containing six rats (Figure 1). All rats were fed a modified diet 2000 (M2000) consisting of 56% sucrose [24]. The rats were then infected with *S. mutans* Xc (c) strains (1 mg/ml) by pipette (50 µl of 1×1011 CFU/ml) at 24 days of age (Figure 1). Each day, two groups of rats were given coated gels containing 2% IgY anti-*S. mutans* Xc, which was started on the first day for

Group	Amount of animal	IgY antibody (gel)		Day						
		% IgY in gel	Coating commencement	0	2 4	11		14	36	78
A	6	2%	1st day							
B	6	0%	No coating							
C	6	2%	29th day							
D	6	0%	1st day							
⊢⋯⋯⊣	Administration of antibiotic									
⊢──⊣	IgY antibody coating									
•	S. mutans innoculation on rat									
○	S. mutans colony confirmation									
x	Caries sample test									

Figure 1. Summary of in vivo experiment protocol in Sprague Dawley rat

group A and on the 29th day for group C. Among the control group animals, group B received no gel coating, while group D rats were coated daily with plain gel. All rats were sacrificed at 78 days of age or on day 56 of the experimental protocol, and oral swabs were taken to confirm the colonization of the inoculum on days 11 and 34. Approval for the in vivo rat research project was obtained from the Ethics Committee of the Faculty of Dentistry, Universitas Indonesia. Twenty-four Sprague Dawley rats were then selected from the initial group of rats.

Determination of immunoreactivity of rat saliva

To determine the immunoreactivity of the non-stimulated saliva, samples were collected from each rat after daily IgY gel coating and were subsequently evaluated by ELISA. The rat saliva volume measurement was conducted while the rats were still unconscious. Saliva was obtained using pipettes with 3 suctions over 3 min. The collected material was then placed in properly labeled sterile tubes containing 50 µl of phosphate-buffered saline (PBS). The samples were stored at −20 °C before further examination and later processed at the Oral Biology Laboratory of the Faculty of Dentistry, University of Indonesia. The time course of the antibody levels in the egg yolk against *S. mutans* (c) was evaluated using the ELISA method as described by Bachtiar et al. [25]. *S. mutans* antigen was

suspended in 0.1 mol/l carbonate buffer (pH 9.6). This antigen suspension at 100 μl/well was used to coat the ELISA plate. Skim milk was used for blocking. Each well was washed three times with 200 μl of PBS containing 0.05% Tween 20 (PBS-Tween). Rat saliva was diluted 200-fold with PBS-Tween; 100 μl per well in triplicate was reacted with the antigen for 2 h at 37 °C. After each well was washed again with PBS-Tween (as mentioned above), alkaline-phosphatase-conjugated rabbit IgG anti-chicken IgG (Abcam Laboratories, Inc.), diluted 2000-fold with PBS-Tween (100 μl), was added to each well, and the plate was incubated for 2 h at 37 °C. Each well was washed with PBS-Tween, and 100 μl of the substrate solution (*p*-nitrophenyl phosphate, 1 mg/ml diethanolamine buffer, pH 9.8, Sigma, St. Louis, MO) was added. The enzyme reaction (30 min at room temperature) was stopped by adding 5 mol/l NaOH (50 μl/well), and the color that developed was read at 450 nm with a plate reader (Model 2550, Bio-Rad, Richmond, CA). As a control, saliva samples obtained from non-IgY or plain gel were used.

Results

Immunoreactivity of the IgY gel

Comparisons of immunoreactivity between anti-*S. mutans* IgY gel and pure IgY WSF powder in water were determined using ELISA. The stability of IgY gel after this study still displayed ELISA reactivity (OD 0.6) after storage for 30 days. Anti-*S. mutans* IgY gel reached the same antibody level as that of pure IgY WSF powder, even after mixing with additional gel materials (Figure 2).

The effect of the anti-*S. mutans* IgY gel on the *S. mutans* colony counts is shown in Figure 3. *S. mutans* colony counts in group A decreased after coating the rats' tooth surfaces with anti-*S. mutans* IgY gel. Similar results showing a significant difference from the control group were obtained in groups C and D, which both received gel coating. Interestingly, the most significant reduction in *S. mutans* count was observed in group A, which had the longest period of gel application. In contrast, without the coating as a control, *S. mutans* colony counts increased significantly in group B. These results showed that gel application on the tooth surfaces provided biofilm protection by reducing *S. mutans* colony formation.

The immunoreactivity of anti-*S. mutans* IgY gel in the rat saliva was evaluated using ELISA. There was no significant difference among the groups (Figure 4). These results showed that even longer gel application on the tooth surfaces resulted in an OD below 0.3, and there was no immunoreactivity in the saliva in all groups.
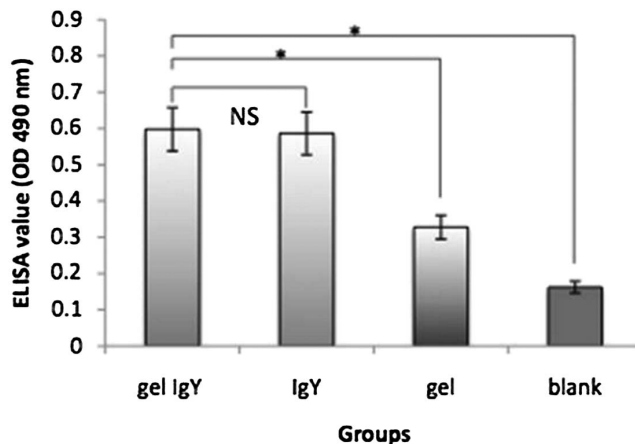
Figure 2. Immunoreactivity of IgY in gel (gel IgY), IgY in water (IgY), gel without IgY (gel), and blank control (blank) against *S. mutans* antigen. There is no significant difference in immunogenicity between IgY in the form of gel and without gel (water-soluble fraction). Gel IgY and IgY are immunoreactive against *S. mutans* antigen compared to the controls (gel and blank) ($p < 0.05$). NS: ANOVA post hoc Bonferroni was not significantly different ($p = 0.51$). *: post hoc test was significantly different ($p < 0.05$)

Oral examination of group C rats displayed the curative effect of the IgY gel. This group was coated with gel when white spots started to appear on one of the rat's teeth; on the 29th day, two of the rats were observed to have early carious lesions (data not shown).
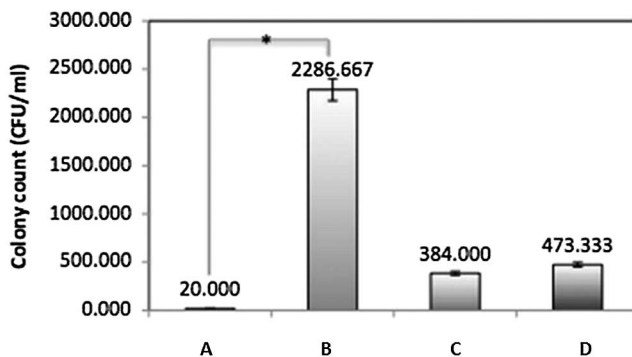

Figure 3. *S. mutans* colony counts in the tooth surface of Sprague Dawley rats. Group A: rats coated with IgY gel; group B: control group without coating; group C: rats coated with IgY gel on the 29th day; group D: rats coated with gel without IgY. *: significant difference was seen ($p < 0.05$) using ANOVA post hoc Bonferroni test
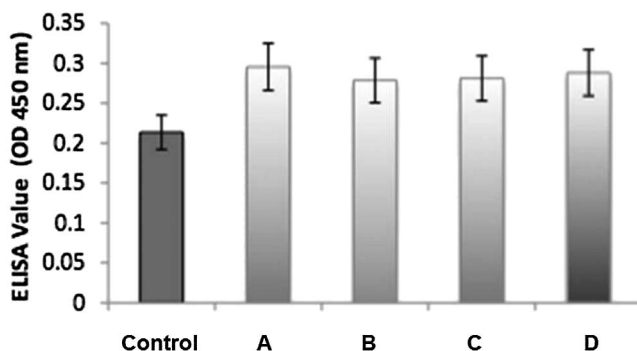
Figure 4. Immunoreactivity of IgY in saliva of the Sprague Dawley rats after 29 days. Control: medium PBS; group A: rats coated with IgY gel; group B: control animals without coating; group C: coating with IgY gel on the 29th day; group D: coating with gel without IgY. NS: ANOVA was not significantly different ($p > 0.05$)

Discussion

Our study showed that there were no differences in the antibody levels between anti-*S. mutans* IgY gel and pure IgY WSF powder in water. The mean values of *S. mutans* colony counts on the tooth surface of Sprague Dawley rats showed significant differences between the rats coated with IgY gel and the other groups, whereas no difference was observed in saliva immunoreactivity among the groups. Previous research has proved that IgY also affects bacterial colonization. The coating of gel indicated a remineralization process occurring on the teeth [26]. Wen et al. [27] reported that IgY was capable of impeding glucan synthesis by *S. mutans* and *S. sobrinus*. However, several of these studies did not use gel as a clinical application medium, instead relying on mouthwash or food and toothpaste [21, 22]. Compared with mouthwash, pure IgY is dissolved in 10 ml of water [20]. This study showed that one gargling requires 2 g or 2% of 10 ml mouthwash, whereas media in the form of gel requires only 0.2 g or 2% of 1 g of the IgY gel for a single use. As such, less IgY is required to impede the activity of *S. mutans* when gels are used.

The selection of IgY gel was also supported by in vitro research needed to evaluate the biological stability of IgY through an immunoreactivity capability test of the IgY gel. The IgY gel was stored for 30 days at room temperature [28]. Then, its immunoreactivity was compared to that of pure IgY in water using ELISA. The result of this in vitro research showed that the immunoreactivity of the IgY gel was as stable as that of the IgY without gel. Therefore, the effect of the anti-caries IgY gel was not lower than that of pure IgY. Similarly, Larsson and Sjoquist [29] studied pure IgY and confirmed that it can be stored for more than 10 years in

40 °C without any change in antibody activity. Such stability was sustained for as long as 6 months at room temperature or for 1 month at 37 °C [28]. The stability of IgY gel after this study still displayed ELISA reactivity (OD 0.6) after storage for 30 days. This was promising data, as the biological properties of IgY gel without any preservatives persisted. In addition, it was significantly different compared with the control group. The immunoreactivity properties might have been enhanced if protein preservatives had been added.

We inoculated *S. mutans* serotype c in the oral cavities of rats that were previously administered ampicillin and chloramphenicol for three days to elimi-nate microbial flora [22] and to prevent the possible impact of infections from other bacteria. In Figure 3, the group that received the IgY gel coat from the first day had lower levels of *S. mutans* bacterial colonies than did the group that received IgY starting on day 29. However, their colony counts were still lower than those of the group that received the gel without any treatment. *S. mutans* colony quantification showed the capability of the IgY gel in impeding biofilm formation at levels that were 100 times higher than those observed in samples without a gel coating.

Group D, in which plain gel was administered as a control group, showed surprising data of a significant inhibition of *S. mutans* colonization. The small number of *S. mutans* colonies in group D might be due to the role of the increased volume from rat saliva in all experimental groups which received a gel (data not shown). A thickening agent such as CMC could stimulate saliva production. The role of increased saliva production between groups A and D, which received 54 days of gel application, showed almost similar colony count ($p = 0.206$). Results of groups A and C, which received IgY gel for 54 days and 27 days, respectively, showed no significant difference ($p = 0.96$). Duration of using IgY gel did not strongly influence the inhibition effect.

We found an increase in saliva volume in all experimental groups when saliva samples were collected three times without stimulation for 3 min and immunoreactivity by ELISA was observed. As shown in Figure 4, ELISA showed that the immunoreactivity of the IgY gel in the rat's saliva in all experimental groups was not significantly different (OD 0.2–0.3). This means that IgY was not detected in the saliva after the end of the experiment. Undetected immunoreactivi-ty of the IgY gel after mixing with saliva was possibly due to enzymes in the rat's saliva that were able to destroy proteins. However, the immunoreactivity reduction of the IgY gel that was without preservatives still showed an ability to block *S. mutans*.

According to Chang's research, the addition of maltose, sucrose, glycine, and glycerol increased IgY stability [30]. On the other hand, protein destruction by trypsin, chymotrypsin, and pepsin could also be prevented by adding gum

arabic [16]. By adding various agents, the effectiveness of the IgY gel might be optimized, and further research should be conducted to enhance future IgY gel formulations.

During saliva collection, higher saliva volumes were found in the IgY gel- and plain gel-coated group. It is possible that the gel increased saliva production, as well as played a role in reducing the caries risk. To our best knowledge, other observations of rat saliva volumes have not been conducted to date [14, 22]. The increase in saliva volume could have neutralized acidic conditions, which are a cause of white spot lesions. In this study, the white spot lesions disappeared one week after application of the IgY gel.

It has been proven by numerous experts in cariology that saliva is an agent for caries prevention. According to Edgar [31], saliva contains, among other things, various immunoglobulin and non-globulin anti-bacterial agents such as amylase, lysosomes, lactoperoxidase, lactoferrin, apolactoferrin, and histatins. Saliva prevents the formation of biofilms that can cause caries. IgY gel could inhibit the growth of *S. mutans*. Our research revealed the impact of anti-*S. mutans* IgY gel and its prevention of dental caries in vitro, as well as in vivo, in Sprague Dawley rats. Anti-*S. mutans* IgY gel still displayed reactivity levels similar to that of pure IgY solution after being stored for 30 days at room temperature. The use of additional media in the form of gels would not affect the content of pure IgY nor change its biological properties. Given the various roles of IgY gel, it is expected that this research could serve as a foundation for further investigation in humans. Last, our results could be implemented in cariology experiments in the community. The safety of anti-*S. mutans* IgY gel is adequate, given its lack of side effects, and it is approved for use according to the FDA's food-grade materials' standards [32].

Conclusion

Topical application with anti-*S. mutans* IgY gel reduced the quantity of *S. mutans* on the tooth surface.

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