

Effectiveness of anchovy substrate application on decreasing acid solubility of Sprague Dawley rats' tooth enamel (in vivo)

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Abstract. Anchovies (*Stolephorus insularis*), a natural resource of Indonesia, contain fluoride in the form of CaF_2 and can function as a fluoridation material to prevent dental caries. The aim of this study is to study the effectiveness of anchovy substrate, through food or topical application, in decreasing the acid solubility of tooth enamel. This research used 14 Sprague Dawley rats as subjects divided into the following 5 groups: baseline, experimental feeding, experimental smearing, and their negative controls. After 15 days of anchovy substrate application, lower incisors were extracted and the acid solubility of enamel was analyzed qualitatively and quantitatively using a stereo microscope and a Micro-Vickers Hardness Tester. Analysis of enamel surface destruction and enamel surface microscopic hardness shifting after a 60 sec application of H_2PO_4 (50% concentration) resulted in a decrease in acid solubility of enamel treated with anchovy substrate. This result can be seen with both the chewing and smearing method. *S. insularis* can be used as an alternative material for fluoridation.

1. Introduction

Dental caries is a disease that affects the hard tissue of teeth. It occurs due to an imbalance of tissue mass caused by loss of hydroxide ions in hydroxyapatite. This loss of hydroxide ions results from an imbalance in the process of demineralization and remineralization [1]. More than 90% of Indonesians suffer from dental caries, making a preventive program essential to maintaining good oral health [2]. One method of prevention is through fluoridation which can prevent dental caries by increasing enamel resistance to acid [1]. Resistance occurs due to a substitution reaction between hydroxide and the fluoride ions in hydroxyapatite creating fluorapatite [3]. Fluorapatite is known for its bond stability in a pH of 4.5 whereas hydroxyapatite can only survive up to a pH of 5.5 [4]. Fluoride can be given topically and systemically. Water fluoridation is the most common method of systemic fluoride [5]. However, delivering fluoride systemically is difficult to control because the volume of water needed by every person varies [6]. Nowadays, the use of systemic fluoride has decreased because of negative effects from excessive fluoride such as dental fluorosis [7].

Excessive fluoride intake can be avoided with topical fluoride [8]. Delivering fluoride topically is easier to control because the effect is localized, and continued use of topical fluoride is unnecessary. Therefore, some research recommends use of topical fluoride over systemic fluoride [9]. Topical fluoride in dentistry, through paste, gel, or mouth rinse, generally involves NaF compound [10]. Another form of fluoride is CaF_2 compound. Gunawan reports that the effect of CaF_2 compound is better than NaF compound. CaF_2 compound may release fluoride gradually and bond more easily with enamel. Although CaF_2 compound more effectively increases enamel resistance to acid, there are several problems with its use. For example, NaF compound is liquid in form and is expensive to make



synthetically. Therefore, it is necessary to develop a fluoridation method with CaF_2 compound using natural materials [4].

CaF_2 compound, as reported in Gunawan's research, can be found in *Stolephorus insularis*. *S. insularis* is an anchovy that can be readily found in the Java Sea and is sold publicly at a relatively low price. Based on Gunawan's research, a topical fluoride method using CaF_2 compound can be easily developed [4]. Earlier in vitro studies of topical solutions containing *S. insularis* have shown a reduction in acid solubility of enamel [4,11]. Practically, fluoride from *S. insularis* can be absorbed by enamel through direct contact during mastication. To date, there is no research about fluoride effectiveness from direct contact of *S. insularis* with enamel during mastication. The present study used an in vivo method of application on Sprague Dawley rats, and was aimed at reproducing oral cavity conditions that cannot be achieved by an in vitro method. Sprague Dawley rats were chosen as the experimental animal because they are relatively inexpensive, their enamel structure resembles that of humans, and they are easy to obtain and control [12].

2. Materials and Methods

This study used 14 Sprague Dawley rats. Available rats were examined based on inclusion and exclusion criteria, weight, tooth conditions, gender, and meal time. Duration of mastication was used to determine the duration of daily topical solution application. Rats need 30 minutes to chew 20 grams of pellet, so the duration of topical solution application was 15 minutes twice a day. There were 3 types of food used in this study. The first one was manufactured commercial rat food. The second one was blended sweet corn mixed with wheat flour that was shaped into small size balls and then sun-dried for a day. The third food had the same base ingredients as the second one, but 5% *S. insularis* (sun-dried for 2 days) was added before the food was baked in an oven for an hour at 80 °C. After heating, the dough was shaped into small sized balls and sun-dried for a day. The topical solution was made every day with 0.5 grams of *S. insularis* powder and 10 ml of distilled water mixed into a centrifuge tube. The number of *S. insularis* contained in the food and topical solution was determined based on the assumption that every human food portion contained 5% of *S. insularis* from total rice and side dishes.

The Sprague Dawley rats were divided into 5 groups: 1st group (baseline) was given manufactured commercial rat food; 2nd group (food control) was given corn pellet with basic composition; 3rd group (negative topical solution control) was treated with an application of distilled water; 4th group (food containing *S. insularis*) was given pellet with 5% *S. insularis*; and 5th group (topical solution containing *S. insularis*) was treated with an application of topical solution with 5% *S. insularis*. The topical solution was applied twice a day for 15 minutes while 20 grams of pellet was given once a day. This experiment took a total of 15 days. After that, the rats' lower left incisors were cut to examine the micro hardness of enamel. Samples were collected from all groups using ether. The lower jaws were separated from other body parts using a scalpel, tweezers, and scissors. The lower jaws were rinsed with 90% alcohol, soaked in 70% formalin, and dried. The jaws were then placed in a plastic pot with silica gel.

Preparation of the specimens was done with teeth planted inside acrylic. Acrylic powder and liquid were mixed until they reached the consistency of dough. The acrylic mixture was then poured into a pipe 1.5 cm in diameter as a medium for the specimens. The lower jaws were placed into a mold filled with acrylic with the tip of the teeth leaning on the mold's shoulder. Excess acrylic was disposed of, and the pipe surface was arranged so that there was no acrylic past the teeth. After the acrylic was set, the bottom part of the acrylic was flattened and smoothed using a grinding and polishing machine. Preparation of the specimens for the enamel damage test was done with 50% phosphoric acid applied using a brush to wash teeth for 60 seconds. After 60 seconds, the tooth surfaces were washed using running water and then dried using a chip blower. Surface damage was tested using a stereo microscope with 100 times magnification. Samples were then tested for hardness using a Micro-Vickers Hardness Tester. A weight of 50 grams was chosen for this Vickers test based on earlier studies. The results of the test were compared between teeth exposed to acid and teeth not exposed to

acid using One-Way ANOVA method with Post-Hoc Tukey Test and Independent Samples t-test to find the difference in the Vickers hardness value between the acid and no-acid groups.

3. Results and Discussion

3.1 Results

3.1.1 Enamel surface damage test results

There were differences in the microscopic pictures of the rats' enamel surface between groups. The extent of the destruction on the enamel surface was shown by enamel surface roughness. Enamel surface roughness was based on the number of black shadows in the pictures, with rough surfaces having more shadows because the light source was located on the side.



Figure 1. Enamel surface of 1st group (baseline) with 100 times magnification using light microscope



Figure 2. Enamel surface of 2nd group (negative food control) with 100 times magnification using light microscope



Figure 3. Enamel surface of 3rd group (negative topical solution control) with 100 times magnification using light microscope

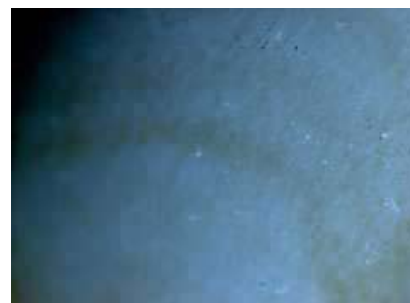


Figure 4. Enamel surface of 4th group (food containing *S. insularis*) with 100 times magnification using light microscope



Figure 5. Enamel surface of 5th group (topical Solution containing *S. insularis*) with 100 times magnification using light microscope

3.1.2 Micro hardness test results

The average results of the enamel surface micro hardness test for specimens not exposed to acid from each group are as follows: 1st group baseline (326.89 VHN), 2nd group with negative food control (315.80 VHN), 3rd group with negative topical solution control (347.28 VHN), 4th group with food containing *S. insularis* (440.30 VHN), and 5th group with topical solution containing *S. insularis* (510.31 VHN). The average results of the enamel surface micro hardness test for specimens exposed to 50% phosphoric acid from each group are as follows: 1st group baseline (193.62 VHN), 2nd group with negative food control (193.62 VHN), 3rd group with negative topical solution control (217.63 VHN), 4th group with food containing *S. insularis* (166.39 VHN), and 5th group with topical solution containing *S. insularis* (133.32 VHN). Based on the microscopic pictures, there was no qualitative difference in enamel surface roughness between the 1st group baseline (Figure 1) and the 2nd group with negative food control (Figure 2). Quantitatively there was no difference in the average result of enamel surface micro hardness between the 1st group baseline and the 2nd group with negative food control. Statistically, there was no significant difference in enamel surface micro hardness between the 1st group baseline and the 2nd group with negative food control ($p = 0.409$). Based on these analyses, food made by the researcher did not increase enamel resistance to acid.

Based on the microscopic pictures, there was no qualitative difference in enamel surface roughness between the 1st group baseline (Figure 1) and the 3rd group with negative topical solution control (Figure 3). Quantitatively, there was a difference in the average result of enamel surface micro hardness between the 1st group baseline (193.62 VHN) and the 3rd group with negative topical solution control (217.63 VHN). Statistically, there was no significant difference in enamel surface micro hardness between the 1st group baseline and the 3rd group with negative topical solution control ($p=0.953$). Based on these analyses, distilled water as a *S. insularis* powder solvent did not increase enamel resistance to acid. Statistically, there was no significant difference in the average results of enamel surface micro hardness.

Qualitatively, based on the microscopic pictures, enamel surface roughness of the 4th group with food containing *S. insularis* (Figure 4) was smoother than the 2nd group with negative food control (Figure 2). Quantitatively, the average result of enamel surface micro hardness of the 4th group with food containing *S. insularis* (166.39 VHN) was less than the 2nd group with negative food control (193.62 VHN). Statistically, there was a significant difference in enamel surface micro hardness between the two groups ($p = 0.02$). Based on these analyses, addition of *S. insularis* into food can increase enamel resistance to acid.

Qualitatively, based on microscopic pictures, enamel surface roughness of the 5th group with topical solution containing *S. insularis* (Figure 5) was smoother than the 3rd group with negative topical solution control (Figure 3). Quantitatively, the average result of enamel surface micro hardness in the 5th group with topical solution containing *S. insularis* (133.32 VHN) was smaller than the 3rd group with negative topical solution control (217.63 VHN). Statistically, there was a significant difference in enamel surface micro hardness between the two groups ($p = 0.001$). Based on these analyses, addition of *S. insularis* into a topical solution can increase enamel resistance to acid.

Qualitatively, based on microscopic pictures, enamel surface roughness of the 5th group with topical solution containing *S. insularis* (Figure 5) was smoother than the 4th group with food containing *S. insularis* (Figure 4). Quantitatively, the average result of enamel surface micro hardness in the 4th group with food containing *S. insularis* (166.39 VHN) was greater than the 5th group with topical solution containing *S. insularis* (133.32 VHN). Statistically, there was a significant difference in enamel surface micro hardness between the groups with topical solution and food containing *S. insularis* ($p = 0.025$). Based on these analyses, addition of *S. insularis* into a topical solution could increase enamel resistance to acid better than addition of *S. insularis* into food.

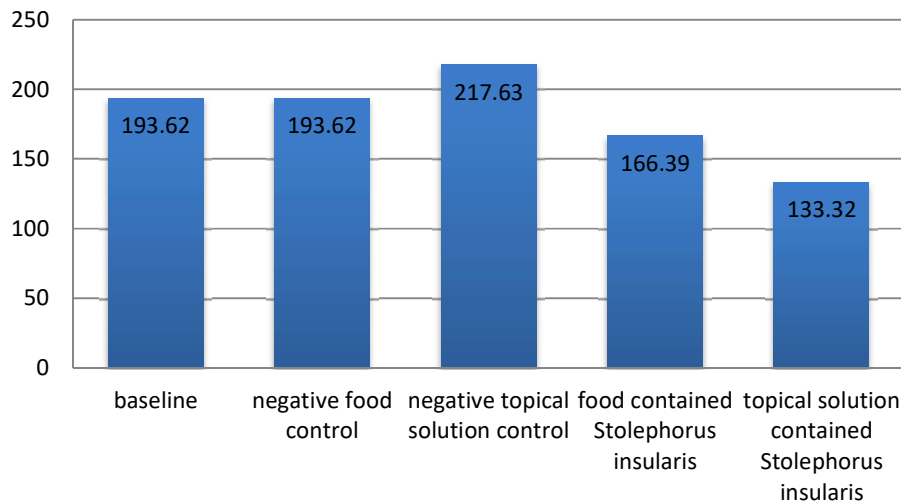


Figure 6. The average results of enamel surface micro hardness test comparison of 1st group baseline and 2nd group with negative food control. Y axis is the average results of enamel surface micro hardness test (VHN)

3.2 Discussion

The amount of food given to the specimens in this study was based on the results of a one-day observation of 9 rats. Rats weighing 120-140 grams consumed 20 grams of food a day. Duration of the topical solution application was determined by observation results. Rats need 30 minutes to finish 20 grams of food. Based on Lennon's research, application of topical fluoride for 2 weeks applied for 15 minutes can result in anti-caries [13]. Therefore, in this study, the duration of the topical solution application was 15 minutes twice a day. One-third of each incisor was tested for enamel surface damage and micro hardness. This amount was based on the rats' tooth length and rapidity of growth. A rat's lower incisor is 7 mm long and grows 2.2 mm per week. This rate of growth caused the rats to gnaw their incisors down by 2.2 mm per week. Therefore, one-third of each incisor received treatment throughout the study.

The foods used in this study were made by the researcher to avoid unwanted variables from commercial foods. Commercial foods contain calcium that might affect enamel surface hardness in such a way that it would be difficult to differentiate whether any increase in hardness was from the commercial food content or from *S. insularis*. Unlike commercial foods, the food made by the researcher did not contain calcium to eliminate this variable. In this study, enamel resistance to acid was measured using 2 parameters, enamel surface damage and micro hardness. Enamel surface damage indicates the size of the demineralization process after exposure to acid. Surface damage was tested using a stereo microscope with 100 times magnification. The demineralization process results in an increase in enamel tags which lower the enamel surface hardness value. Enamel surface micro hardness was tested 5 times using Vickers. Enamel surface roughness is directly proportional to enamel surface damage and the extent of the demineralization process [4].

Enamel resistance to acid was increased by food and a topical solution containing *S. insularis*. *S. insularis* contains CaF_2 compound which acts as topical fluoride. In CaF_2 compound hydroxyapatite is changed into fluorapatite, which is more stable than hydroxyapatite. This happens because fluor has a strong affinity for apatite. The addition of *S. insularis* into a topical solution can increase enamel resistance to acid better than the addition of *S. insularis* into food. It was assumed that the duration of fluoride exposure from *S. insularis* was different between the two methods. In the topical solution application, the rats' mouths during topical solution application, the rats' mouths remained open. This resulted in 15 minutes of contact between *S. insularis* and enamel. The duration of exposure from food

was less than the topical solution application because not all of the rats finished their food. Moreover, every time the rats drank water, the *S. insularis* would wash away. These are two reasons why the food method did not give maximum results.

4. Conclusion

Addition of *S. insularis* into foods or topical solutions increases enamel resistance to acid. Addition into a topical solution resulted in better enamel resistance to acid than addition into food. This study could be improved by using acids from oral biofilm before testing on humans. For comparison, this study could also be conducted with topical fluoride distributed in the market. In future studies, a method of scoring should be made to increase objectivity. Lastly, to obtain better pictures, a scanning electron microscope should be used.

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