The effects of an anchovy (stolephorus insularis) substrate application on the level of fluor intrusion on Sprague Dawley rat teeth (in vivo)

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Abstract. Fluoride intrusion is one of the efficacy parameters of fluoridation. Anchovy (Stolephorus insularis), which contains a high fluoride concentration in the CaF$_2$ compound, can be used as a fluoridative agent which is affordable and easily obtained. The aim of this study is to prove the effectiveness of the application of an anchovy substrate (Stolephorus insularis), either by a feeding method or a topical method, for tooth fluoridation based on the depth of fluoride intrusion on the enamel. An in vivo experimental laboratory method was used. The subjects were 14 Sprague Dawley rats divided into five groups. The groups included a baseline control, a feeding negative control, a topical negative control, an anchovy feeding method, and a topical solution anchovy method. After 15 days of treatment, the teeth were cut transversely with a 0.5 mm thickness then processed to test for fluoride intrusion using fluorescence microscopy. There was increased fluor intrusion on the enamel of the experimental groups compared to the negative control groups (p<0.05). Fluoride intrusion using the topical fluoride method is higher than with the feeding method (p <0.05). Thus, the application of an anchovy substrate, either by chewing or smearing, increases fluoride intrusion on the enamel.

1. Introduction
Dental caries cause the loss of mineral elements from tooth tissue [1]. The prevalence of dental caries has reached 90.05% in Indonesia [2]. The high rate of dental caries in Indonesia necessitates preventive actions such as fluoridation [3,4]. Fluoridation can change a hydroxyapatite compound from the enamel to a fluorapatite or hydroxyl fluorapatite that has resistance to the lower pH of the oral cavity (pH<4.5) [5]. Fluoridation can be provided systemically and topically [6]. Systemic fluoridation is only effective on pre-erupted teeth. However, topical fluoridation is proven to have an anti-caries effect if it is applied to permanent teeth since it inhibits demineralization and quickens remineralization [7,8]. Topical fluoridation also occurs in mastication, where there is contact between fluoride and the enamel surface of the tooth. In topical fluoridation, the fluoride concentration can be controlled and applied regularly. The regular and continuous application of a low fluoride concentration has an anti-caries effect because it allows more intrusion than the application of a single dose of a high fluoride concentration [5].

The success of fluoridation can be tested by examining the depth of fluoride intrusion on the enamel surface of the tooth. The depth of fluoride intrusion could indicate the quantity of fluorapatite or hydroxyfluorapatite compound formation. Furthermore, there is a relation between the fluoride
The intrusion depth and the level of enamel solubility, which is, the deeper the intrusion of fluoride, the lower the enamel solubility [9]. Successful fluoridation is shown to be affected by the form of the fluoride compound itself. The valuable compound is in the form of calcium fluoride (CaF$_2$) because this compound releases fluoride gradually and can function as a fluoride reservoir [5]. Another study also stated that a fluoride compound in the form of CaF$_2$ has better retention and intrusion on the enamel surface compared to other forms of compounds [5].

Today fluoride comes in several forms including mouthwashes, toothpastes, gels, and varnishes that contain a fluoride compound such as NaF or SnF$_2$ [8]. Fluoride in the form of CaF$_2$ is not widely used because it is usually only available as a liquid and can dissolve quickly in a low pH. It can also be quite expensive if made synthetically [5].

Based on this, the need is evident for an affordable and widely available alternative substance that contains CaF$_2$. One such natural material that has high fluoride content in the form of CaF$_2$ is anchovy. Anchovy (Stolephorus insularis) is a readily available and affordable food source in Indonesia, since Indonesia is a maritime country. Anchovy (Stolephorus insularis) has a high CaF$_2$ content, most of which can be found in its bones. Indonesian society is familiar with the anchovy as one of its daily food sources, and previous in vitro studies have proved that using an anchovy solution for topical fluoridation can increase enamel hardness and resistance to acid [5]. Therefore, this study has been done to prove the effectiveness of topical fluoridation using an anchovy substrate through feeding compared to smearing a topical solution of the same concentration. This study was performed in vivo on Sprague Dawley rats because they have a similar enamel structure to humans. Moreover, an in vivo study has the advantage of creating a comparable environment to the actual oral environment.

2. Materials and Methods
This study used an in vivo experimental laboratory method with 14 Sprague Dawley (SD) rats as subjects. The number of subjects was determined using Federer’s formula. Researchers examined five areas on every tooth from four samples of the treatment group and two samples from the baseline group. In total there were 90 areas of measurement from 18 tooth samples. The rats used were Sprague Dawley rats that passed the inclusion requirements, which were: 2 months of age, in good general health, male, no teeth anomalies, and weighed in the range of 120-140 grams. The independent variable in this study was the method of anchovy administration (smearing and feeding methods) and the intrusion of fluoride was the dependent variable. The study was conducted as follow:

The subjects were prepared with an initial examination. The rats were weighed, their teeth were examined, their gender was confirmed, and their meal times were measured. Three types of rat food were used: a commercial rat food, a food without the anchovy mixture, and a rat food that had been mixed with 5% anchovy (Table 1). Commercial rat food compositions are determined by the factory and can be found on the market. The rat food without the anchovy mixture was made by grinding sweet corn, mixing it with flour until it formed into dough, making smaller pellets, and then drying it for a day. The rat food mixed with 5% anchovy was made by crushing the corn in a blender. The anchovies were dried for two days, put in the oven at 80°C for one hour, and crushed in a blender until a powdery consistency was obtained. The corn was then mixed with flour and anchovy powder until it formed into dough, and smaller pellets were made out of it. These pellets were then dried up in the sun for a day.

The 5% anchovy solution was made fresh every day by mixing 0.5 grams of anchovy powder with 10 mL of Aquadest in a centrifuge tube. The rats were divided into five groups and given the same treatment for 15 days. Then the mandibular incisive canals of the rats from each group were cut to measure the depth of fluoride intrusion. The subject grouping was determined based on the treatments administrated. The baseline group was fed commercial pellet food with the compositions according to the factory (their maintenance was performed similar to the method from their breeding place). The control negative fed group was fed pellets with a basic composition. The distribution of these pellets was performed once a day. The control negative smeared group was smeared with Aquadest twice a day, with a duration of 15 minutes for every smearing. The fed group was fed pellets
containing 5% anchovy. The administration of these pellets was performed once a day. The smeared
group was smeared with Aquadest solution that contained 5% anchovy twice a day, with a duration of
15 minutes for every smearing.

Table 1. The composition of the intervention materials

| Composition  | Percentage | Composition  | Percentage | Composition  | Percentage |
|--------------|------------|--------------|------------|--------------|------------|
| Water        | 13%        | Carbohydrate | 52.50%     | Sweet corn   |
| Protein      | 19-21%     | Protein      | 52.50%     | Protein      |
| Fat          | 5%         | Sugar        | Sugar      |
| Fiber        | 5%         | Mineral      | Mineral    |
| Ash          | 7%         | Carbohydrate | Carbohydrate |
| Calcium      | 0.90%      | Protein      | Protein    |
| Phosphorus   | 0.60%      | Water        | Flour      |
|              |            | Sugar        | Sugar      |
|              |            | M.E.         | 3100       |
|              |            | Kcal/ kg     | (CaF₂)     |
|              |            | 3000-        |

After 15 days of treatment, a termination was done and the mandibular incisive canals of the rats
were cut away with the mandible. These teeth were then cut transversally at the incisal 1/3 and middle
1/3 at 0.5 mm thick using a diamond disc. The pieces of lower incisive were then stored in a 75%
alcohol solution for 2 seconds to clean the samples. The teeth were then washed twice with Aquadest
before they were dried. In this study, the microscope slide was not dyed fluorescent because the
fluoride penetrated into the enamel itself was already fluorescent.

The microscope examination was done using an optical evaluation and a fluorescent microscope
with 200x lens magnification. The microscope used was a Reflected Fluorescence System fluorescent
Olympus microscope with a white excitation light, resulting in a white fluorescent emission. The
optical evaluation was performed by observing the fluorescence of bright white light on the enamel
surface. After the fluorescent area was determined, a picture of it was taken with a digital camera. The
depth of fluoride intrusion was calculated by measuring the width of the white fluorescent band using
AxioVision measurement software. The border of this white fluorescent band was determined based
on calibration in between members of the group. The result of the examination was tested using the
SPSS independent samples t-test, the One-way ANOVA, the post hoc Tukey Test, and the Mann-
Whitney test to figure out the value comparison of fluor retention depth in each group. This study has
been approved by the Ethical Committee.
Figure 1. Information of fluoride intrusion examination area. (1) Fluorescent band; (2) dentin

3. Results and Discussion

3.1 Results
In this study the fluoride intrusion depth on the enamel of the rats’ teeth was measured using the white fluorescent microscope image on the sample of enamel. The average depth value of fluoride intrusion was obtained from the measurement of five areas on each rat’s enamel. The picture below shows the width of the white fluorescent band in the measurement area on the border of the fluorescent enamel.

Figure 2. Intrusion of the fluoride on the enamel surface of the baseline group
Based on the One-way ANOVA and the Tukey Post Hoc tests, it was concluded that there was no significant difference between the baseline group (3.304 ± 0.355703) and the fed control group (3.593 ± 0.571325) with \( p > 0.05 \), while there was a significant difference between the baseline group (3.304 ± 0.355703) and the Aquadest control group (2.62305 ± 0.339721) with \( p < 0.05 \). Based on the Mann-Whitney test, there was a significant difference between the anchovy fed group (6.71475 ± 1.45833) and rat food fed group (3.59345 ± 0.57132) with \( p < 0.05 \). The depth of fluoride intrusion on the anchovy fed group was higher than the rat food fed group. Based on the t-test, there was a significant difference between the anchovy solution smeared group (8.48600 ± 0.58911) and the Aquadest smeared control group (2.62305 ± 0.33972) with \( p < 0.05 \). The fluoride intrusion depth on the anchovy fed group was higher than the rat food fed group. Based on the Mann-Whitney test, there was a significant difference between the anchovy smeared group and the anchovy fed group, where the anchovy smeared group (8.48600 ± 0.58911) had a higher depth of fluoride intrusion than the anchovy fed group (6.71475 ± 1.45833) with \( p < 0.05 \).

### 3.2 Discussion

This study was performed in vivo as a continuation from a previous study which investigated the effects of anchovy substrate administration on human enamel in vitro. The results show that the administration of topical fluoride can increase retention and intrusion of fluoride, therefore increasing the enamel’s resistance to caries [5]. This mechanism occurred as fluoride bonded with the apatite group, replacing the hydroxyl group as fluorapatite. Fluorapatite compound has an affinity to bigger apatite because it has small ions and high electronegativity, making it easier to bond with apatite crystals. Electronegativity is the ability of an atom (in this case a fluoride atom) to draw electrons onto itself [10]. The fluorapatite compound also has a stronger bond compared to the hydroxyapatite compound [5]. This study used Sprague Dawley rats because they have a similar enamel structure to humans, making them easier to treat and maintain. In addition, male rats were used to prevent the hormonal changes that usually occur with female rats, affecting their saliva. One of the goals of this study was to compare the effectiveness of anchovy substrate administration with two methods, a feeding method and a smearing method. Both methods were done in consideration of the daily anchovy consumption common in Indonesia. The previous study stated that fluoride administration would be more effective topically or by smearing [5]. In the feeding method, fluoridation also occurred topically during mastication, as there was contact between the fluoride source and the teeth [7]. However, the contact that occurred in the topical method can be controlled in terms of duration and the amount of exposure to the fluoride [5].

The commercial rat food that is available on the market could not be used for this study because it has an unchangeable composition and cannot be mixed with the anchovy. Therefore, the rat food was
self-produced from nutritious ingredients that were similar to the ingredients used in rat food available on the market [11]. The composition was determined based on the previous findings, who studied the administration of corn rat food to Sprague Dawley rats [12]. An advantage of the self-produced rat food was that ingredients that might interfere with the study could be eliminated, such as fluoride and other minerals that would disrupt the fluoridation reaction. The rat food was made of corn and flour with the compositions explained in the methods section. Corn was chosen as the basic material because it does not contain fluoride or other minerals that could affect the results of this study [11]. Corn is also an appropriate food for rats to consume. The 5% concentration of anchovy in the anchovy solution was chosen based on in vitro studies that have examined the effects of anchovy. It was also chosen based on findings that when anchovy is consumed as a side dish it comprises 5% of the total serving of one meal. The anchovy content of the fed group was also similar to the concentration of anchovy in the smeared group, which was 5% in 20 grams. The subjects in this study were divided into five groups: a baseline control, a feeding negative control, a topical negative control, an anchovy feeding method, and a topical solution anchovy method. The purpose of the baseline group was to ensure that the self-made corn rat food when compared to the commercial rat food consumed by the baseline group, did not contain fluoride or other minerals that could disrupt the fluoridation reaction. The control groups were also made for the same reason. The treated group was divided into the fed group and the smeared group to find the best method for fluoride application.

The duration of this study was 15 days, which was determined based on the previous study that performed a topical application to the teeth. The previous study stated that the administration of fluoride for 15 days has a significant effect on a tooth’s resistance to caries. The duration of each application in this study was 15 minutes. This timeframe was chosen based on observations of the amount of time a rat can consume 20 grams of anchovy. This is supported by the previous study that performed fluoride topical application to human enamel using Acidulated Phosphate Fluoride in the same duration [13]. In this study, the success of fluoridation was analyzed by observing the depth of fluoride intrusion to the Sprague Dawley rats’ enamel, which was examined using a fluorescent microscope. By using a fluorescent microscope, a fluorescent band on the surface of the enamel could be seen. The width of this white band showed the fluoride intrusion depth on the enamel. The white fluorescent band is obtained from the energy that is produced by the fluoride’s photon, which results in a white fluorescence from the effect of white light excitation. There was no significant difference in the fluoride intrusion depth between the baseline group and the fed group. Since the self-made rat food did not contain fluoride or other minerals that could disrupt the reaction between apatite crystals and fluoride, it can be assumed that the results obtained in this study were purely from the addition of fluoride in the treated group, while there was a significant difference between the baseline and the Aquadest control groups. The depth of fluoride intrusion in the Aquadest control group was lower than the baseline group, and therefore it can be concluded that the Aquadest application to the enamel surface of the Sprague Dawley rat can dissolve the natural fluoride content in it, hence the decrease of fluoride content in the group.

There was a significant difference of fluoride intrusion depth between the fed control group and the anchovy fed group, therefore it can be concluded that the addition of fluoride in the anchovy rat food can increase the fluoride intrusion on the Sprague Dawley rat’s enamel. This occurred because the decrease of pH that happened during mastication that made fluoride substitute with OH- that changes hydroxyapatite crystal to be fluorapatite crystal or hydroxyfluorapatite [3]. There was a significant difference on the depth of fluoride intrusion between the Aquadest control group and the anchovy solution smeared group, where the depth of fluoride intrusion in the anchovy smeared group was higher than the control group, and therefore it can be concluded that the topical fluoride application to the enamel surface of the Sprague Dawley rat can improve the depth of fluoride intrusion. This occurred due to a potential difference and change of polarity on the enamel surface after smearing. This potential difference was due to the kinetic energy produced from the smearing process. The charge on the enamel surface was negative because the phosphate group was present on the surface, so if energy was administrated on the surface of the enamel, the charge would change from negative to
positive, and therefore cause the potential difference between the enamel surface and the fluoride with a negative charge. This potential difference could cause the fluoride to enter the enamel prism micro tunnel, causing an interstitial reaction. Then, once the fluoride is inside, it would be kept in the enamel prism micro tunnel room since the potential difference is no longer present [5].

A comparison between the anchovy fed group and the anchovy smeared group was also performed. The results showed a significant difference in the depth of fluoride intrusions in the anchovy smeared group and the anchovy fed group, where the fluoride intrusion depth in the anchovy smeared group was higher than the anchovy fed group. Therefore it can be concluded that the smearing method of anchovy solution is better than the anchovy feeding method. This occurred because during the smearing there was direct contact between the anchovy substrate and the enamel surface. Also, the anchovies were dissolved using Aquadest, which is a liquid and may have the ability to enter the enamel prism micro tunnel more easily than the anchovy rat food, which uses solid substance as the media. The topical anchovy exposure to enamel could be controlled in concentration and duration, so this group had the highest level of fluoride intrusion depth. The anchovy smeared group also had an advantage because the dosage of fluoride could be controlled so it would not have a systemic toxic effect. In the anchovy fed group the contact only occurred in intermittent and short durations, allowing fewer fluoride ions to penetrate the rats’ enamel. Most of the fluoride contained in the rat food also entered systemically rather than coming into contact with the tooth surface. Furthermore, in the anchovy fed group, the mastication process may have scraped the fluoride off the enamel surface, so that the intrusion of fluoride was not optimal. This may have caused the intrusion of fluoride in the anchovy smeared group to be deeper than it was in the anchovy fed group.

4. Conclusion

The administration of anchovy (Stolephorus insularis) with a feeding method or a smearing method resulted in a deeper intrusion of fluoride compared to the rats’ enamel that did not receive any treatment. The smearing method gave deeper fluoride intrusion compared to the feeding method. Therefore, from in vivo testing it can be concluded that the administration of an anchovy substrate, either with a feeding method or a smearing method, can increase the intrusion of fluoride on the enamel. Further study should also be done on this topic. A clinical study on the effects of anchovy administration on human enamel should be performed. Additionally, there should be an in vivo study done on the formation of fluorapatite compound on enamel and a study on the comparison between dosage forms of topical fluorides available on the market. The result of this study could be developed into a topical fluoride application product using an anchovy substrate in the form of a gel or paste.

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