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Research Article

EFFECT OF ANCHOVY (*STOLEPHORUS SP.*) APPLICATION ON RAT ENAMEL MICROHARDNESS AND APATITE CRYSTAL SIZE: AN *IN-VIVO* STUDY

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ABSTRACT

Objectives: The study aimed to investigate the *in-vivo* effect on the enamel microhardness, apatite crystal changes, and fluorapatite formation after applying acidic anchovy solution on rat teeth.

Methods: A total of 16 Sprague-Dawley rats were divided into four groups including the untreated baseline group, demineralized distilled water (control) group, positive control (sodium fluoride) group, and anchovy treatment group. Anchovies were heated, powdered, and diluted with demineralized water to a 5% solution. The test and control solutions were applied to rat mandibular incisors twice daily for 7 days. After exposure, the teeth were subjected to microhardness testing, scanning electron microscopy, and energy dispersive X-ray spectroscopy analysis to examine enamel surface and fluoride retention, and X-ray diffraction (XRD) analysis on fluorapatite formation and changes in apatite crystal size.

Results: Anchovy treated specimens showed increase in enamel microhardness to 390±29 Vickers hardness number, decrease in apatite crystal size to 19.14±1.24 nm, higher fluoride retention on enamel (5.88±0.32%), reduction of crystal size, and increase in fluoride retention correlated with increase of enamel surface microhardness. Fluorapatite formation was demonstrated by the increased peaks at 2θ=32.67° and 33.87° in XRD analysis.

Conclusion: *In-vivo* application of anchovy solution on rat enamel surface increased enamel surface microhardness and promoted fluorapatite formation. The applied anchovy solution appears to show a clear beneficial effect as a topical fluoride agent.

Keywords: Caries, Apatite, Anchovy, Calcium fluoride, Fluorapatite.

INTRODUCTION

Dental demineralization is mainly through dissolution by acids from bacterial metabolism on carbohydrates. High incidence of dental disease caused by demineralization, such as caries, is believed to be partly due to diets rich in carbohydrates [1]. Materials containing fluorides have been widely used to reduce dental demineralization both in experimental studies and clinical applications and remain the most effective materials to protect dental enamel [1,2].

Caries prevalence in Indonesia is very high and a major challenge in dentistry [3]. There are many methods for caries prevention, but recently topical fluoride application is one method of choice [2]. A material showing good results in the topical application is calcium fluoride (CaF_2) [4]. However, CaF_2 is relatively costly, and this limits its use for caries prevention. Therefore, there is a need to obtain material that contains CaF_2 but easy to find and low in cost. One of the known natural sources to contain fluoride is anchovy (*Stolephorus* sp. of Clupeidae family) [5]. Anchovy is a slender, small ocean fish common and commercially important in Indonesia. Mineral analysis using ion selective electrode has shown anchovy to have high fluoride content (about 45 ppm), and energy dispersive X-ray spectroscopy (EDX) and X-ray diffraction (XRD) analyses have shown that it is mostly in CaF_2 form [5,6].

Enamel surface has a negative charge due to the phosphorus atom position in the apatite crystal. The negative charge will decrease fluoride reaction with the enamel since fluoride has also a negative charge. The enamel charge will invert to positive when there are enough H^+ ions surrounding the enamel surface in low pH (acidic) environment. Thus, lowering pH level will increase fluoride retention and intrusion onto enamel surface. Therefore, application of CaF_2 on enamel surface under acidic (pH 5.5) conditions is expected to provide improved fluorapatite formation [5].

Laboratory experiments using application of anchovy solution on enamel slab have shown the formation of fluorapatite and changes of crystal dimension [7]. Hydroxyapatite (HAP) of enamel is a hexagonal crystal with OH^- position in C-axis, and Ca1 takes a columnar position at the corner of the crystal. The Ca2 is found around the C-axis in double layered triangle. Substituting OH^- with fluoride as in the topical fluoride application will cause retraction of the distance between Ca1 and 2 with the C-axis (F^- position) [7]. Decreasing the interatomic distances will make the crystal more compact and decrease its solubility [8].

This study aimed to investigate the effect of acidic anchovy solution application on enamel properties and microstructure. Since rat enamel is histologically and by demineralization patterns similar to human enamel, the application was done on Sprague-Dawley rat enamel *in-vivo* [9-11]. The microhardness of enamel surface was measured after acidic (pH 5.5) anchovy solution application, and apatite crystal size changes were analyzed using bulk diffraction method [5]. The null hypotheses were that anchovy solution had no significant effect on enamel microhardness and no significant effect on the apatite crystal size.

METHODS

Animal preparation

Ethical approval for this study was provided by the Faculty of Dentistry, University of Indonesia Ethical Advisory Committee. Male 60-day-old Sprague-Dawley rats, with a mean weight of 140 grams, were bred at the Animal Laboratory of BPOM RI (Indonesian Drug and Food Agency) and used in this study. Anchovy solution was applied to two mandibular incisive teeth. After experimental application, the teeth were cut and used as analysis specimens. During the experiment, all rats remained in their individual cages and were fed with standard food pellets and demineralized distilled water [12,13]. In total, 16 rats were used in the experiment and divided into four groups of four rats each: The untreated baseline group, negative control group (treatment with demineralized distilled water), positive control group (treatment with clinical standard acidulated 2% sodium fluoride [NaF]), and anchovy treatment group. After treatment, all 32 specimens were cut and stored in individual jars and examined for microhardness, then analyzed with scanning electron microscopy (SEM) and EDX. Then, enamel specimens were taken by grinding using diamond carborundum disc (ZZLINKER Shanxi, China), and the collected enamel powder was used for XRD examination [11].

Anchovy solution

A batch of anchovy was obtained from the market and identified referring to The National Standard Index No. 01-3461/3466-1991 and 01-3471-1991. The fish were heated at 80°C for 20 minutes and powdered to 100 Mesh. About 5 g of this powder was diluted into 100 ml of demineralized distilled water to obtain anchovy solution, and adjusted to pH 5.5 using phosphoric acid. Anchovy solution was applied to lower incisive teeth of each rat of the anchovy group twice daily (9 am and 4 pm for 5 minutes), for 7 days [5,7].

Microhardness evaluation

After treatment, Vickers microhardness of surface enamel was measured from 5 indentations of each tooth sample using Buehler P-120 Hardness Tester with diamond indenter load of 50 g for 10 seconds. Special jig was made to ensure stability of the specimen during microhardness testing. The indentations of each specimen were placed sufficiently widely to avoid interference error between measurements [14,15].

SEM and EDX analyses

SEM analysis with EVO MA 10 Carl Zeiss instrument with EDX Bruker Nano X-Flash Detector 5010 to examine enamel surface and fluoride retention after anchovy application. SEM analysis on all specimens was done using back-scattering BSCD ray, at 900 mA current and 20 keV, without carbon coating. Fluoride retention examination was done using EDX to all specimens including specimens without treatment as a baseline fluoride level [5].

XRD analysis

XRD analysis of the samples was done using Philips diffractometer PW 370, Cu tube anode, λ=0.15406 nm, 40 keV and 30 mA current. The diffraction range for 2θ was 20-60° with 0.02°increments and 1.25 seconds duration per increment. Data from XRD examination were analyzed using JCPDS # 15-0876 Data, APD, and BellaV2-1 program to measure apatite crystal size [16,17].

Statistical analysis

The numeric data obtained from the study were compared using one-way analysis of variance with Bonferroni *post-hoc* analysis. Significance was assumed at $p<0.05$.

RESULTS

Microhardness evaluation

Microhardness testing results showed that both NaF (HV 394.3±24.7) and anchovy (HV 389.8±29.2) treatment groups had significantly higher microhardness compared to baseline untreated (HV 326.9±14.6) and control (HV 338.3±17.5) groups ($p\leq0.05$, Table 1).

SEM and EDX analysis

Surface analysis using SEM confirmed that rat enamel surface was similar to human enamel. Application with anchovy solution did not result in demineralization nor erosion of the enamel surfaces as the surfaces remained smooth (Fig. 1). EDX analysis showed increased fluoride retention of the enamel surface both of the NaF and anchovy group specimens compared to other groups. Surface fluoride content of the baseline (0.23±0.14) and control (0.19±0.07) groups was significantly different from both NaF (3.97±1.3) and anchovy (5.88±0.32) treated groups, respectively ($p\leq0.05$). EDX analyses were done using the normalized method (Table 1).

XRD analysis

Diffraction analysis showed the formation of fluorapatite after anchovy application (Fig. 2). There was enhanced of peak height at 2θ=±33° representing stronger crystal formation. Specimens from NaF and anchovy groups showed the formation of fluorapatite compound but not in the other groups. Diffraction analysis showed reduced apatite crystal size after application with anchovy solution (19.136±1.24 nm) compare to the baseline (24.49±2.9 nm) and control (22.85±1.46 nm) groups ($p\leq0.05$). Similarly, there was crystal size reduction of apatite after application with NaF (19.81±0.7 nm) when compared to baseline and control groups. Microhardness, SEM-EDX, and XRD analysis results are summarized in Table 1.

DISCUSSION

Enamel nanostructure composes of apatite crystals, the HAP, hexagonal crystal, which consists of calcium, phosphate, and hydroxyl ions [17]. Apatite is a hard material but unstable under acidic conditions. In oral cavity, there is a physiologic equilibrium and dynamic changes

Table 1: Vickers microhardness (VHN), fluoride retention from EDX analysis and apatite crystal size from XRD analysis for all specimen groups

Groups	n	Mean±SD		
		Microhardness VHN	Fluoride retention, % (normalized)	Crystal size nm
Baseline	8	326.9±14.6	0.23±0.14	23.49±2.90
Control	8	338.3±17.5	0.19±0.07	22.85±1.46
NaF	8	394.3±24.7	3.97±1.30	19.81±0.70
Anchovy	8	389.8±29.2	5.88±0.32	19.14±1.24

VHN: Vickers hardness number, XRD: X-ray diffraction, EDX: Energy dispersive X-ray, SD: Standard deviation, NaF: Sodium fluoride

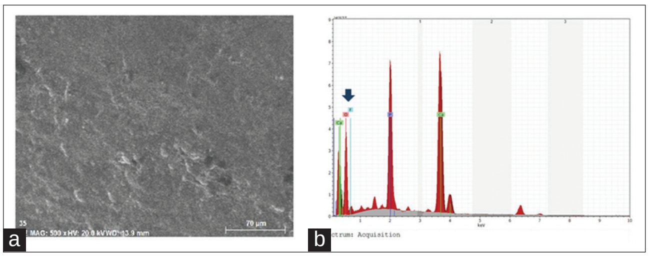

Fig. 1: (a) Enamel surface of rat incisor after application with anchovy solution, showing no demineralized or erosion effects; (b) energy dispersive X-ray spectrum from enamel treated with anchovy solution, showing the fluoride peak (arrow)

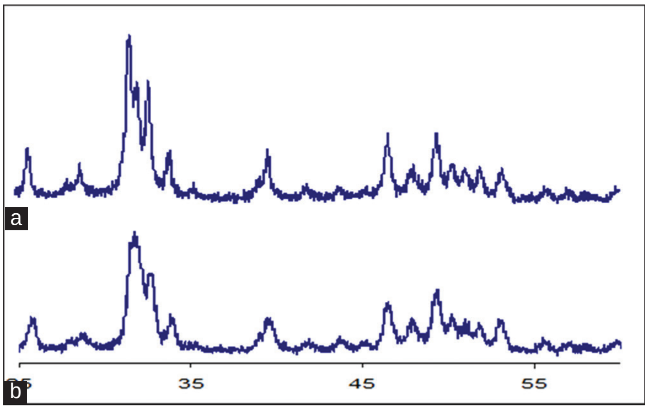

Fig. 2: Diffraction spectra, (a) Anchovy treated specimens, showing formation of fluorapatite, (b) baseline specimen

between remineralization and demineralizaton process of the enamel mineral. Saliva plays an important role to maintain this equilibrium [1]. Mineral content of saliva will precipitate on enamel surface as demineralization process is occurring. In the dental caries disease, there is a disturbance of equilibrium balance between the remineralization and demineralization processes. This imbalance will lead to tooth tissue loss, and if this process continues, dentin will be exposed. In severe condition, pulp tissue will be eventually exposed, leading to inflammation. The imbalance is caused by acid accumulation produced by the microorganism [1]. Fluoride topical application on enamel surface is used for clinical prevention of enamel demineralization [2]. Material of choice for topical application is CaF_2 that gives good results with minimal side effects, but this material is hard to produce and rather expensive [4]. In this study, fluoride containing material used as topical application material is anchovy solution. Anchovy is a common fish in Indonesia and easy to find at low cost. Fluoride application will lead to substitution of the apatite hydroxyl ion with fluoride ion, forming fluorapatite. Equation of the reaction [1] is:

$$CaF_2 + Ca_{10}(PO_4)_6(OH)_2 \rightarrow Ca_{10}(PO_4)_6F_2 + 2(OH) \quad (1)$$

The use of rat enamel in this study was based on fact that there are no histologic and crystallographic differences between human and rat enamel [10,11]. 7-day duration is based on the assumption that fluorapatite formation will occur after application [5]. Anchovy solution application to enamel surface was done according to clinical management for fluoride topical application. CaF_2 compound will break down to Ca^{++} and F^- ions, and F^- will react with hydroxyl ion of the enamel apatite to form fluorapatite. This reaction is influenced by acidity of the oral cavity. Since fluorine ion and enamel surface have negative charge, there is a need to change the enamel surface charge to positive by lowering the surface pH level. In this study, acidic anchovy solution was used to obtain positive enamel surface charge. During the study, rats were fed with carbohydrate containing standard pellets. Carbohydrate metabolism in oral cavity will decrease pH level, and on the other hand, anchovy solution also has low pH level. These factors will establish a suitable environment for fluoride precipitation and fluorapatite formation. Fluoride retention on enamel surface after anchovy application was higher than after NaF application, suggesting that CaF_2 in the anchovy solution was more effective as a fluorine releasing agent than NaF. Anchovy solution containing CaF_2 is of natural origin and gives no inflammation to rats gingiva, while in some rats gingiva showed signs of inflammation after NaF application as shown in Fig. 3. These findings imply advantages in using anchovy solution as a topical fluoride source. Microhardness of the enamel control group in this study was 326.9 Vickers hardness number on average. This is similar to that in the hardness study by Chunmuang *et al*. [14]. After anchovy application, enamel surface hardness significantly increased in comparison to the control group specimens. Fluorapatite formation appears to be reason of the observed increase in hardness of the enamel surface [17].

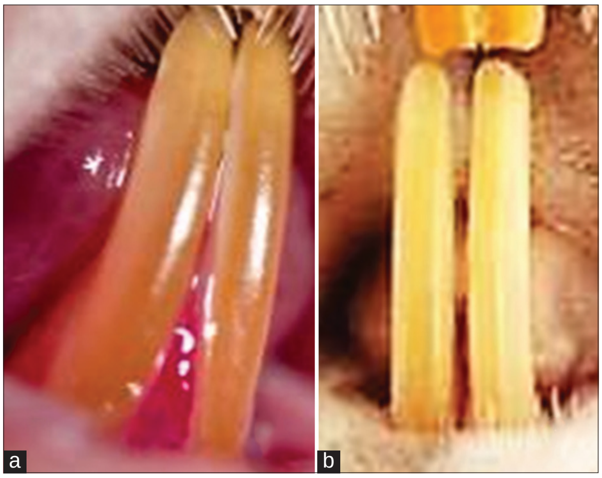

Fig. 3: Lower incisors of Sprague-Dawley rat, after application with sodium fluoride (NaF) (a) and anchovy solution, (b) Note inflammation in NaF treated gingiva

Diffraction spectra obtained from anchovy group bulk specimen analysis matched to fluorapatite diffraction data (JCDPDS 15-0876), confirming the formation of fluorapatite after anchovy treatment. Increase of the 2θ=32.67° and 33.87° peaks show fluorapatite formation enhancement. Analysis using Bella V-21 shows decrease of apatite crystal size after anchovy treatment in comparison to control or baseline groups, demonstrating that the formation of fluorapatite in anchovy specimens leads to reduced crystal size. Fluorine is the most electronegative mineral (3.98 in Pauling scale), and according to Achille *et al*., fluoride charge in fluorapatite is −0.82, compared to hydroxyl charge (−0.78) in HAP [18]. This negative charge difference makes the crystal more compact in size [19]. It also makes the crystal more difficult to break down. The reduced crystal size correlates with the increase in microhardness by anchovy treatment.

CONCLUSIONS

Application with anchovy solution on rat enamel surface was shown to significantly decrease apatite crystal size increase enamel surface microhardness and reduce enamel demineralization. Anchovy solution hence appears beneficial as a fluoride topical agent. The results suggest that topical anchovy application can provide a promising route to protect dental enamel.

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