

Increase of Enamel Hardness Score after Cow Milk Immersion of Demineralized Tooth: An *In vitro* Study

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ABSTRACT

Aim: To investigate the difference of enamel hardness score on tooth immersed in artificial saliva and cow milk.

Materials and methods: The present study was an experimental laboratory with *pre- and post-test* design. Thirty-two maxillary first premolars were collected and randomly divided into two groups, with 16 samples each immersed in artificial saliva and cow milk. Samples were immersed in an orange flavored soft drink for 5 minutes before exposing to cow milk (group 1) or artificial saliva (group 2) for 5 minutes twice a day until day 3. Enamel hardness score was measured as follows: pretreatment, post demineralization stage and posttreatment at day 1 and day 3 by Micro Vickers hardness tester. Data were analyzed using paired t-test and one way analysis of variance (ANOVA).

Results: There was a significant difference (p value = 0.000) on enamel hardness score of group 1 at day 1 (324.39 ± 20.35 VHN) and day 3 (354.80 ± 21.09 VHN) as well as group 2 at day 1 (308.06 ± 15.94 VHN) and day 3 (322.18 ± 16.94 VHN). Tooth enamel hardness on group 1 was significantly different to group 2 both at day 1 (p value = 0.018) and day3 (p value=0.000).

Conclusion: These results suggest that artificial saliva and cow milk can increase the enamel hardness score. However, the efficacy of cow milk to augment the tooth enamel hardness is much higher than that of artificial saliva.

Clinical significance: Can be used as a primary advice to the public in as precautionary measures and inhibiting tooth enamel damage from acidic drinks.

Keywords: Artificial saliva, Cow milk, Enamel hardness, Remineralization.

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INTRODUCTION

Soft drinks mainly have acidic pH lower than four that may cause demineralization on tooth enamel.¹ Demineralization is a process of enamel dissolution

when the release of hydroxyapatites occurs leading to micropores formation on enamel surface.² Enamel consists of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and fluorapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$). Hydroxyapatite has critical pH 5.5 whereas fluoride apatite falls to pH 4.5.³

Demineralization and remineralization process is associated with tooth enamel surface hardness. Hardness is defined as a resistance of solid matter to penetration.⁴ Tooth hardness is influenced by the level of phosphate and calcium of enamel, called hydroxyapatite, in which the amount of hydroxyapatite relates to the thickness of enamel.⁵

Owen et al. found that beverages with acidic pH such as sport and energy drinks, as well as orange juice initiated enamel dissolution by measuring the percentage of tooth enamel weight loss. To decrease or prevent the risk of dental erosion can be done by (a) avoiding the direct contact of acidic foods or drinks on tooth surface thus discontinuing the process of tooth demineralization; (b) applying remineralizing agents immediately after consuming foods or drinks by fluoride mouth rinse, and (c) drinking milk to accelerate remineralization process.⁶

Usually, the saliva buffering capacity neutralizes the low pH in the oral cavity when the acidic condition is present and increases saliva pH followed by remineralization. Remineralization is a process of restoring phosphate and calcium ions on tooth enamel surface when oral cavity pH returns to neutral.⁷ During remineralization, it requires minerals such as calcium, phosphate, magnesium, and fluoride to promote tooth calcification. Calcium and phosphate are commonly found in cow milk and its product (e.g., yogurt and cheese), soy milk, salmon fish and broccoli, whereas water, seafood, yogurt, and cheese are a source of fluoride.⁸

Cow milk can protect the tooth from erosion, particularly if consumed immediately after acids intake. It has been shown to enhance tooth enamel remineralization due to casein, calcium, and phosphate.^{1,9} Amoras et al. found that cow milk was able to maintain demineralization process from hydrochloric acid toward the advanced stage, which observed in an increased enamel surface hardness.⁹ Lachowski et al. investigated that cow milk could increase tooth enamel surface hardness, which decreases the potential of dental erosion. The reduction of enamel surface hardness of orange juice-immersed tooth was higher than of orange juice-immersed tooth followed by milk immersion.¹

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The aim of this study was to investigate the difference of tooth enamel hardness score after artificial saliva or cow milk immersion of demineralized tooth.

MATERIALS AND METHODS

This study was an experimental laboratory with pre- and post-test design conducted in University of Negeri Medan Mechanical Engineering laboratory and Biology laboratory. This study was approved by the Medical Research Ethics Committee, Faculty of Medicine, University of Sumatera Utara, no. 312/KOMET/FK USU/2016 on April 14, 2016.

Inclusion criteria were the extracted maxillary first premolar collected from patients age of 17–30 years for orthodontic purpose with normal size, shape, and tooth structure, and the crown must be intact. Exclusion criteria were caries tooth or erosion, attrition, and abrasion tooth, dental filling, a tooth with endodontic treatment, cracked tooth, fracture, necrosis, and whitened tooth. Samples were thirty-two maxillary first premolar extracted from patients that fulfill the inclusion and exclusion criteria.

The tooth was thoroughly cut at the cemento-enamel junction that subsequently separate the root and crown part. Palatal surface of the crown was coated with nail varnish, then was implanted in acrylic (Fig. 1). Each tooth was randomly numbered and divided into two groups. Tooth enamel hardness was measured before sample treatment.

All samples were immersed in an orange flavored soft drink for 5 minutes to demineralize teeth. Group 1 was stored in cow milk (pH 6.5) for 5 minutes twice a day at 11.00 and 17.00. Group 2 was stored in artificial saliva (pH 6.8) for 5 minutes twice a day at 11.30. Artificial saliva was produced in University of Gadjah Mada Chemical Laboratory. Later, at 17.30 samples were immersed in aquabidest and incubated at 37°C. The experiment was performed for 3 days.



Fig. 1: Tooth embedded in acrylic

Enamel hardness score of samples on pre- and post-acid immersion was assessed on day 1. Hardness score of tooth immersed in cow milk and artificial saliva on day 1 was measured on day 2, whereas tooth immersed on day 3 was measured on day 4 using Micro Vickers hardness tester with load 100 g for 15 seconds for three times (Fig. 2).

Data were analyzed using paired t-test and one way ANOVA with post hoc test.

RESULT

Table 1 shows the average of enamel hardness scores on pre- and posttreatment including orange flavored soft drink, cow milk, and saliva immersion. Enamel hardness score is decreased in all samples post acid immersion compared to pretreatment samples. However, the increased enamel hardness score is shown at day 1 and day 3 posttreatment in both groups compared with post acid treatment.

Paired t-test analysis shows that enamel hardness score in cow milk-immersed samples at day 3 is significantly higher than samples at day 1 (p value <0.05) (Table 2).

Enamel hardness score in artificial saliva treated group at day 3 is significantly increased compared to day 1 analyzed by paired t-test (p value <0.05) (Table 3).

One way ANOVA was used to determine the difference of enamel hardness score after cow milk and artificial saliva immersion in each time course with a level of significance p value = 0.000 (p value <0.05). There is a significantly different hardness score of tooth enamel soaked in cow milk and artificial saliva at each time point. Additionally, to determine which group is significantly different from the others, LSD post hoc test was used (Table 4).

DISCUSSION

Enamel hardness varies in type and location of the measured tooth. Chemical composition and enamel thickness



Fig. 2: Hardness tester

Table 1: The average of enamel hardness score on pre- and post-treatment (acid, cow milk, and saliva) at day 1 and day 3

Type of treatment	Group I (cow milk) $X \pm SD$ (VHN)	Group II (artificial saliva) $X \pm SD$ (VHN)
Pretreatment	376.23 \pm 16.94	373.22 \pm 15.08
Post demineralization	309.62 \pm 18.44	301.18 \pm 15.44
Remineralization agent treatment day 1	324.39 \pm 20.35	308.06 \pm 15.94
Remineralization agent treatment day 3	354.80 \pm 21.09	322.18 \pm 16.94

Table 2: Difference of enamel hardness score after cow milk immersion at day 1 and day 3 analyzed by paired t-test

Time of immersion	$X \pm SD$ (VHN)	ΔX (VHN)	Sig. (p value)
Day 1	324.39 \pm 20.35	30.41	0.000*
Day 3	354.80 \pm 21.09		

*level of significance, p value <0.05

Table 3: Difference of enamel hardness score after artificial saliva immersion at day 1 and day 3 analyzed by paired t-test

Time of immersion	$X \pm SD$ (VHN)	ΔX (VHN)	Sig. (p value)
Day1	308.06 \pm 15.94	14.12	0.000*
Day 3	322.18 \pm 16.94		

*level of significance, p value <0.05

Table 4: LSD test showing the enamel hardness score after cow milk and artificial saliva immersion at day 1 and day 3

Cow milk	Artificial saliva			
	Day 1		Day 3	
	ΔX (VHN)	Sig. (p value)	ΔX (VHN)	Sig. (p value)
Day 1	16.33	0.018*	2.21	0.740
Day 3	46.74	0.000*	32.62	0.000*

*level of significance, p value <0.05

may take part in each tooth. The present study was done to quantitate enamel hardness on buccal surface and the representative data were subsequently selected for each measurement. Initial hardness measurement (Table 1) shows the distinct values of standard deviation in every group. Palti et al. found the enamel hardness score in pre-treatment group ranges from 367.13 to 399.97 VHN.¹⁰ Meanwhile, the present study showed the average value ranging from 358.48 to 390.58 VHN. Palti selected premolar 4–10 years after eruption as a model sample, whereas this study used premolar from a patient in the age of 17–30 years implying that the tooth has erupted for 6–19 years.

The tooth period in the oral cavity is one of the factors affecting the enamel hardness. Enamel surface on maturation stage post-eruption eventually contact to saliva and the dynamic process between demineralization and remineralization occurs during and after tooth eruption.¹⁰

Samples were immersed in an orange flavored soft drink for 5 minutes and the hardness score was measured. The decreased enamel hardness score after acid immersion was observed, indicating that orange flavored soft drink might reduce enamel hardness. The pH value of the solution used in this study (3.6) is much lower than the critical pH of hydroxyapatite (5.5). Direct contact between hydroxyapatite and solution with low pH (<5.5) leads to enamel mineral dissolution that may influence enamel hardness.⁶

There are some factors affecting the rate of enamel dissolution, such as acidity level (pH), concentration, mineral ion composition, and saliva flow, saliva buffering capacity, diet (quantity and frequency of carbohydrate intake), and tooth structure.^{6,11}

Furthermore, samples were immersed in cow milk (group 1) and artificial saliva (group 2). Cow milk has approximately pH 6.5 and artificial saliva has pH 6.8. Enamel hardness score was increased both at day 1 and day 3, due to the presence of the remineralization process on the tooth. Remineralization is a process to restore mineral ions into hydroxyapatite structure.⁷ Adequate saliva flow rate, the presence of calcium, phosphate, as well as fluoride, and neutral pH of the oral cavity are the factors involved in remineralization.¹¹ Preserved foods and high glucose diet may predispose to inadequate remineralization. Therefore, remineralization activity needs to boost to maintain enamel strength by providing mineralization agents that release calcium and phosphate ions to enamel surface, calculus-free, resistance to lower pH (acid), stimulating remineralization in saliva and appropriate for a patient with xerostomia.¹²

Table 2 shows there is a significant difference (p value <0.05) of enamel hardness score in group 1 (cow milk) at day 1 and day 3 analyzed by paired t-test. According to these results, it indicates that cow milk could increase enamel hardness at day 1 and get much higher at day 3. Calciums and phosphors in cow milk play an important role during remineralization to increase enamel hardness. In contrast, Amoras et al. found that the enamel hardness was increased but not significant in cow milk-immersed tooth which was presoaked in HCl before milk immersion.⁹ The tooth immersion was performed for 4 times 1 minute each, which was rather shorter in time. Also, different mineral level in cow milk may affect the result.

Enamel hardness score in cow milk-immersed tooth at day 3 is higher than that at day 1 with the difference as 30.41 VHN. The longer and more frequent the enamel is exposed to calcium and phosphor containing beverages, the more minerals the enamel takes up so that may raise the hardness of enamel.

Milk is a cellular emulsion mainly containing casein that is composed of a thousand protein molecules bound to calcium and phosphate.¹³ Drinking milk immediately after tooth exposed to acid is a good alternative because of its ability to protect

the tooth from erosion. Milk acts as a remineralization agent, as well as increases mineral deposit and organic material on the enamel surface, and forms a protective film. This film is associated with casein adsorption on the surface of enamel to reduce the dissolved hydroxyapatite crystals and to prevent ions discharge.¹ High calcium beverages show the reduced erosion formation and increase remineralization. Not only to modify the potency of erosion, but the addition of calcium may also alter the basic nature of beverages such as taste and color. Therefore, drinking milk or any others with natural calcium source is preferable to consume.⁹

Carbohydrate in cow milk is composed of 80% lactose. Lactose is fermented by bacteria in the oral cavity to be lactic acid, which can lower pH into 6.0. This carbohydrate has potentially low cariogenicity in a normal condition.¹⁴ However, for particular circumstances, milk may cause caries in children if consumed using a bottle during sleeping time. The decreased saliva flow rate is associated with the bottle caries in children that reduce the saliva neutralization capacity. When the kid is falling asleep, the oral cavity is overwhelmed with milk that eventually immerses the teeth leading to prolonged fermentation time of carbohydrate. The fermented carbohydrate produces acid to initiate enamel demineralization so that caries may occur.¹⁵

Drinking milk from a bottle at night is acceptable as long as the kids must brush their teeth before sleep. Milk for daily needs may reduce pH on plaque 10 minutes after drinking (6.79) due to fermentation of lactose in the milk and yet, this pH begins to rise 20 minutes afterward as peptides and amino acids produced by the hydrolysis of casein may increase pH on plaque and prevent the tooth to demineralize.¹⁴ Fermentation of lactose in oral cavity can be dismissed by gargling with water for 5 min after drinking milk.

Paired t-test in artificial saliva groups (Table 3) shows that there is a significant difference (p value <0.05) of enamel hardness score at day 1 and day 3. Hardness score at day 3 is much higher than that at day 1 as much as 14.12 VHN.

Several mechanisms involved in the protective function of saliva against erosion that is first, the direct action of saliva against the agent of erosion by liquefying, cleansing, neutralizing, and buffering of saliva. Secondly, organic components of saliva form a thin pellicle on enamel surface that may act to defend from direct contact between acid and tooth surface, thus affecting the dissolution level of tooth hard tissue. Thirdly, the existence of calcium and phosphate ions in saliva can become natural resources to a new crystal formation.¹⁶ Those functions mentioned above indicate that saliva has

a potential ability to increase enamel hardness score as a result of remineralization.

This study shows that there is a difference of enamel hardness on tooth-immersed cow milk and artificial saliva in each time of immersion. According to one way ANOVA test, there is a difference of hardness score in each time point of all groups. LSD test (Table 4) shows the significant difference of tooth enamel hardness in cow milk-treated group compare with the artificial saliva-treated group at day 1 and in cow milk-treated group compare with an artificial saliva-treated group at day 3 (p value <0.05). The average of hardness score difference between cow milk and the artificial saliva-treated group at day 1 is 16.33 VHN, whereas at day 3 is 32.62 VHN.

According to those results, cow milk increases the enamel hardness much higher than artificial saliva does. Distinct calcium and phosphor contents in those two sources may affect remineralization so the enamel hardness score is also different in those groups. Moreover, milk, but not artificial saliva, contains casein that is rapidly adsorbed onto enamel surface to raise enamel resistance against acid.¹⁷

The limitation of the study was the results of tooth enamel hardness were different from the initial hardness before demineralization. This may be due to insufficient soaking of the samples, so it is necessary to add immersion days or by mixing cow's milk and artificial saliva to simulate the real oral conditions.

CONCLUSION

Cow milk has the better capability to raise the enamel hardness than artificial saliva does.

It can be used as a primary advice to the public in as precautionary measures and inhibiting tooth enamel damage from acidic drinks. Both cow milk and artificial saliva may enhance the enamel hardness.

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