The effects of chitosan mouthrinse on enamel caries *in vitro*

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Abstract

Objective: This study aimed to evaluate the effect of chitosan-containing mouthrinse on de-remineralization process by evaluating surface microhardness (SMH) measurement.

Method: 32 human premolars were cut and embedded in self-cure acrylic resin. After polishing, all samples with window4x4-mm^2 were immersed in the demineralizing solution to create artificial caries and then were randomly divided into 4 groups; deionized water as negative control, 0.5% of chitosan, 0.5% of chitosan + 0.05% NaF and 0.05% NaF mouthrinse. They were entered into a 10-day pH cycling twice daily treated with tested mouthrinse. The surface microhardness (SMH) was measured 3 times; at baseline, before and after pH cycling using the microhardness tester (FM700 Future Tech Corp. Japan). The percentages of ΔSMH were compared using One-way ANOVA and LSD test.

Results: The percentages of ΔSMH in each group were 21.85±8.3, 34.94±15.1, 54.00±16.9, and 51.45±18.1VHN respectively. All treatment groups had significantly higher rehardening effects than the control group and no difference between the chitosan added NaF mouthrinse and NaF mouthrinse.($p<0.05$)

Conclusion: Chitosan mouthrinse showed a significant better effect on rehardening of enamel caries than negative control. However, the chitosan combined with NaF mouthrinse showed no difference effect from NaF mouthrinse.

Keywords: Enamel caries, Chitosan mouthrinse, Fluoride mouthrinse, Demineralization, Remineralization

Introduction

Dental caries is known as the multi-factorial disease and has high prevalence worldwide. In 2012, the caries prevalence in Thailand of this age group in overall average is 52.3% [1].The dental decay is considered a consequence of the imbalanced de and remineralization of tooth structure. The progression of dental caries begins with the reduction of tooth surface hardness resulted from mineral loss and the formation of cavities.In many cases, an incipient lesion can be improved in the hardness aspect by several agents [2-6]. Among several chemical agents used as caries prevention, fluoride is the most effective agent that can enhance remineralization and provide high resistance to acidic solution. The recommendation for 0.05% NaF mouthrinse is 1-2 minute-rinsing once a day at least 30 minutes from tooth brushing and avoiding eating or drinking for 15 minutes [7]. The review of trials about fluoride mouthrinse stated that regular rinsing with fluoride containing mouthrinse can reduce dental caries in permanent teeth compare with non-fluoride rinsing and brushing alone [8]. Apart from fluoride, chitosan is also considered as an active ingredients in mouthrinse to prevent and inhibit caries progression.

Chitosan is a biopolymer obtained by deacetylation process of chitin. The properties of chitosan such as solubility, reactivity with other agents and thermal stability depend on its degree of deacetylation, molecular weight and N-acetyl

arrangement in the polymer chain [9]. Chitosan has been introduced in dentistry for more than 20 years in all fields of dentistry including preventive dentistry. Recently, there are several chitosan containing products that are available including chitosan toothpaste, chewing gum and chitosan mouthrinse. Chitosan mouthrinse showed the potential to be caries preventing agent because it has a great anti-microbial effect. From a randomized crossover clinical trial revealed that by rinsing 0.5% w/v of chitosan-containing mouthwash for 30 seconds twice a day after tooth brushing for 14 days can reduce plaque accumulation and *Streptococcus mutans* count in saliva [10]. Furthermore, from a recent study about toxicity of chitosan showed that chitosan mouthrinse has lower cytotoxicity than another commercial mouthrinse [11,12].

Moreover, chitosan mouthrinse also showed an interesting result on demineralization and remineralization process. Regarding a study from Arnaud TMS *et al*. on the effects of chitosan solution in term of de-remineralizing process *in vitro*, human teeth samples that were applied with chitosan solution at the concentration of 2.5 mg/mL and 5.0 mg/mL for 60 and 90 seconds had the maximum inhibition of net phosphorous loss and minimum change of surface microhardness (SMH). Additionally, chitosan at concentration of 5.0 mg/mL can penetrate through enamel surface to dento-enamel junction (DEJ) and then, acts as the physical barrier on enamel surface resulting in lower chance of acid penetration and decreased demineralization of enamel [7].

This study aimed to evaluate the effects of chitosan mouthrinse and chitosan added NaF mouthrinse on artificial caries *in vitro*. The de-remineralization were measured by the change of surface microhardness.

Materials and Methods

Tooth preparation

Human premolars that were extracted for orthodontic reason were collected and kept in normal saline solution. Sound human premolars were randomly selected from a pool of teeth collection. The teeth were cut at the buccal surface and embedded in self-cured acrylic resin in a plastic tube (10 mm-diameter, 10 mm-height). The samples were polished with abrasive papers size 600, 800 and 1000 grit until flat and smooth surface were obtained and then polished with 0.5 μm ý-Al_2O_3 paste. The samples were coated with an acid resistant nail varnish (Revlon, New York, USA), leaving a narrow window 4x4 mm^2 uncovered. The width and length of each sample were measured by a digital vernier calliper and then the active surface areas were calculated. The baseline surface microhardness was measured by using the microhardness tester (Future-Tech Corp., Tokyo, Japan) with a Vicker diamond under 100-g load for 15 sec. An indentation was made at the center of sample to check and standardize before experiment. The samples with hardness value between 240-340 Vickers hardness number (VHN) were recruited in experiment. The samples were kept in distilled water at 25ºC.

Lesion formation

The demineralizing solution 1 (D1) solutions was comprised of 0.1 M of lactic acid, 20 g of Carbapol C907 (MW = 45 kDa) and 500 mg of synthetic calcium hydroxyapatite at final pH 5. The samples were individually immersed in 3 ml. of D1 solution at 37ºC for 96 hours to produce artificial carious lesions approximately 120-200 μm in depth [13].

Chitosan containing mouthrinse preparation

Solutions containing chitosan and chitosan-NaF were prepared from 890 kDa in molecular weight of commercially available food-grade chitosan samples, Marine Bio Resources Co., LTD (Thailand) with degree of deacetylation above 90%. The chitosan powder was derived from shrimp shells with the sizes less than 150 μm. A liter of 0.5% w/v chitosan solution was prepared by dissolving 5gof chitosan powder in 200mlof 1M acetic acid and stirring with magnetic stirrer at

room temperature for 3 hours until chitosan powder was completely dissolved. Deionized water was added to the expected volume and then 1M NaOH solution was gradually added to pH 5.5. In chitosan-NaF group,0.5 g of NaF was added to the chitosan solution.

The pH cycling model

The samples were randomly divided into 4 groups (n=8). The tested solutions of each group are as follows:

Control group : Deionized water

CH-group : 0.5 % Chitosan mouthrinse

CHF- group : 0.5% Chitosan and 0.05% NaF mouthrinse

NaF-group : 0.05% NaF mouthrinse

Each sample was submerged individually for 6 hours, in 5 ml per block in demineralizing solution (DE solution) at 37ºC. They were then washed with deionized water for 10 sec and dried with paper towels. Subsequently, each samples was submerged individually for 18 hours in 5 ml per block of remineralizing solution at 37ºC. Each sample was washed with deionized water and submerged in the tested mouthrinse 2 times a day (before and after submerging in demineralization) for 1 min. This sequence was repeated for 10 consecutive days[11,12].The compositions of DE and RE solutions were listed below:

Demineralizing solution : 2.2 mM CaCl2, 2.2 mM KH2PO4, 0.05 mM acetic acid, pH 4.4

Remineralizing solution: 1.5 mM CaCl2, 0.9 mM NaH2PO4 and 0.15 M KCL, pH 7.0

Surface microhardness measurement (SMH measurement)

After lesion formation, the surface hardness was determined by using a 100-g load was used for 15 sec with a Vicker diamond as baseline, pre-treatment and post-treatment measurement. Four indentations, 1 mm spaced, from the corner were made as the pre-treatment then new four indentations were made next to the previous one as the post-test measurement [16,17]. Then, the mean values of SMH were calculated. The percentage of SMH change (%ΔSMH) of each samples was calculated from the following equation.

$$\%\Delta\mathrm{SMH} = \left(\frac{\mathrm{SMH}\ before - \mathrm{SMH}\ after}{\mathrm{SMH}\ before}\right)\ \mathrm{X}\ 100$$

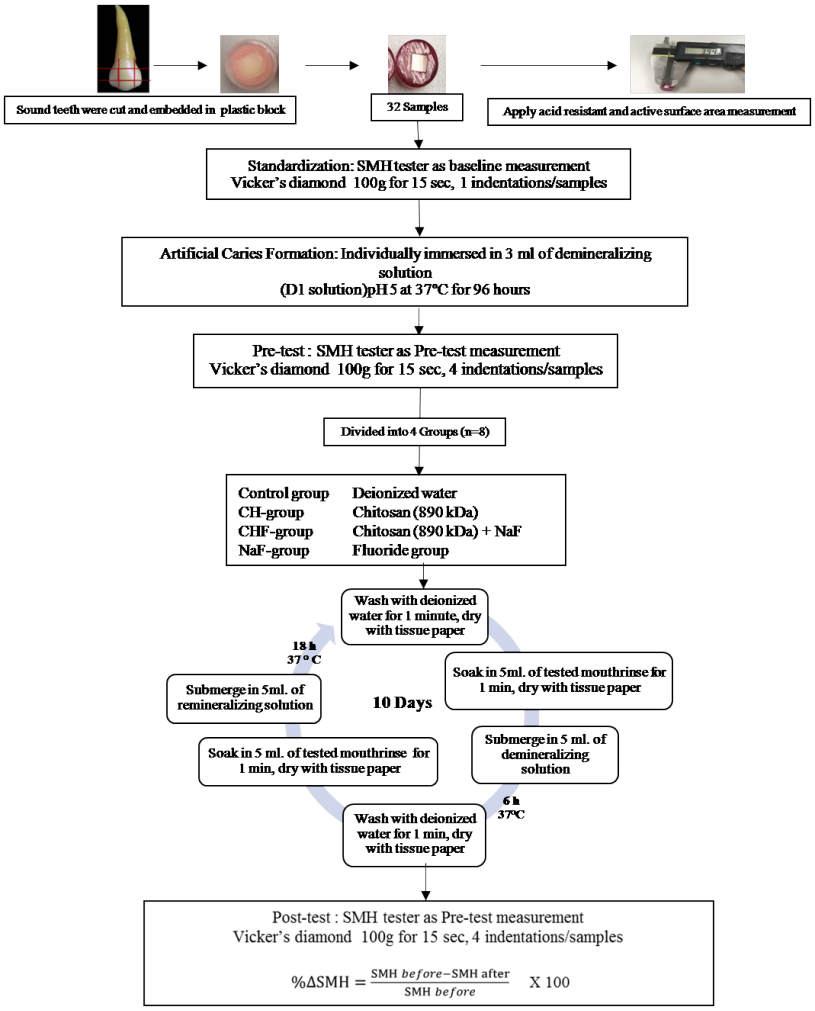

Figure 1 Diagram of the methods.

Results

Statistical Analysis

The mean values of the percentages of the surface microhardness changes (%ΔSMH), were analyzed by using Komorov-Smirnov test followed by One-way ANOVA and Least-significant different (LSD) test with significance level at 5%.

Surface Microhardness Analysis

The mean values of surface microhardness(SMH) and the percentages of relative changes were shown in table 1.The baseline measurements were made for standardization. The means SMH before experiment were between 300.02 - 315.86 VHN and there was no significant difference among groups.

The percentage of relative change of control group was the lowest and significantly different from other groups. Among groups of tested mouthrinse, the percentages of relative changes showed a statistically significant difference between the group of fluoride added mouthrinse (CHF and NaF group) and non-fluoride added mouthrinse (control and CH group) at $p<0.05$.

Table 1 Surface microhardness values and the percentage of relative changes after pH cycling (Mean ± SD).

	N	Pre-test SMH (VHN)	Post-test SMH (VHN)	Percentage of Change (%)
Control group	8	39.02 ±7.1	47.55 ±9.38	21.85[a] ±8.31
CH-group	8	39.54 ±9.25	54.43 ±14.83	34.94[b] ±15.13
CHF-group	8	41.67 ±7.97	64.23 ±13.99	54.00[c] ±16.95
NaF-group	8	38.28 ±6.99	56.01 ±12.60	51.45[c] ±18.12

Different letters indicate statistically significant difference among groups (p<0.05)

[a] indicates a significant difference when compare to CH-group, CHF-group and NaF-group

[b] indicates a significant difference when compare to Control group, CHF-group and NaF-group

[c] indicates a significant difference when compare to Control group and CH-group

Discussion

Recently, many studies have revealed the effects of chitosan mouthrinse regarding the excellent anti-microbial activity and anti-inflammation activity [9-11,18-19]. However, there were a few studies about chitosan mouthrinse on demineralization and remineralization process [7,20] and none of these studies demonstrated about the effects of chitosan-containing NaF mouthrinse.

Based on previous studies about chitosan mouthrinse, there were several concentrations of chitosan mouthrinse that have been used. The concentrations of chitosan mouthrinse were 0.4% w/v to 5%w/v regarding the purpose of the study. Arnaud TMS. *et al* [7] also reported the effect of chitosan solution on hardness of human teeth, initially sound enamel. From the *in vitro* study, 2.5% w/v and 5%w/v of chitosan revealed the minimum value of surface microhardness loss comparing with negative control. Moreover, from optical coherence tomography, the study also demonstrated that the high concentration of chitosan can penetrate through enamel surface and act as physical barrier.

In this study, we applied 0.5% w/v chitosan mouthrinse prepared from food grade of chitosan powder, with molecular weight of 890 kDa and a deacetylation degree more than 90%. Based on the percentage of surface microhardness change (%ΔSMH), the results showed that the %ΔSMH in 0.5%w/v of CH and CHF groups were greater than control. Moreover, based on the lower %ΔSMH, the results from combination of 0.5% w/v chitosan and NaF mouthrinse was as effective as in NaF group.

Apart from the benefits of de and remineralization, 0.5% w/v chitosan also has a great antimicrobial effect. Even though the mechanism of action of chitosan on antimicrobial activity is not yet clearly explained, the possible mechanism is

suggested. Based on the study of Chung et al [21], in the case of gram positive bacteria, high molecular weight of chitosan was able to form films around cell wall and impede the nutrients absorption.

According to the guideline of fluoride therapy from The American Academic of Pediatric Dentistry (AAPD), fluoride mouthrinse is recommended to use as an adjunct to fluoride toothpaste in high caries risk group. Several studies, both *in vitro* and *in vivo*, have been shown that fluoride mouthrinse, both 0.2% and 0.05% of fluoride, can promote the remineralization and treat white spot lesion [2-4,8]. In this study, we use the preparation of a 0.05% of NaF mouthrinse as the positive control and add on the combined preparations. Generally, fluoride ion is a highly reactive ion and is easily deactivated by other positive charge molecule, such as chitosan. It is important to evaluate the effect of fluoride in any products after adding any other agents. From our study, even though the concentrations of fluoride active ion in CHF mouthrinse were significantly different from NaF mouthrinse, fluoride concentration in the CHF mouthrinse was greater than 200 ppm. However, it has been shown in previous studies that even small concentration of fluoride ions was effective in promoting remineralization [22].

In our study, our products had been prepared and stored at 4°C for approximately 4 months. All of chitosan mouthrinse showed no alteration in terms of color, texture and smell. However, prior to clinical application of chitosan mouthrinse, it is essential to go through the process of product developments for example, determining the shelf life, adding some flavors to improve taste and evaluating of product's safety and stability.The shelf life testing of food or products can be evaluated in many aspects such as microbial growth, pH change, discoloration and rancidity.

Conclusions

All of the chitosan mouthrinse showed a significant higher effect on rehardening enamel caries than negative control. Moreover, the chitosan combined with NaF mouthrinse showed equal effectiveness in de-remineralization to NaF mouthrinse.

Ethic Committee

This research protocol was approved by Faculty of Dentistry / Faculty of Pharmacy, Mahidol University, Institutional Review Board. Certificate of Exemption COE. No. MU-DT/ PY-IRB 2016/025.2609.

Acknowledgements

The authors would also like to thanks to the department of Pediatric dentistry, the department of Oral Biology, Faculty of Dentistry and the department of food science and technology, Faculty of Science Mahidol University for their helpful laboratory facilities.

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