

Impact of Nano Hydroxyapatite, Nano Silver Fluoride and Sodium Fluoride Varnish on Primary Teeth Enamel Remineralization: An In Vitro Study

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ABSTRACT

Introduction: Dental caries is still prevailing worldwide, although different anti caries products have been introduced. Each remineralizing agent has its own shortcomings. Therefore, looking for new agents to have benefits of previous ones with lesser side effects is worthwhile.

Aim: To determine the remineralization ability of NaF varnish, nano-Hydroxyapatite Serum (n-HAP) and Nano Silver Fluoride (NSF) on enamel of primary anterior teeth.

Materials and Methods: Incipient caries were induced in primary sound anterior teeth by storing each specimen in demineralization solution for 72 hours. Then they were randomly divided into four groups of 15 samples each: (1) NaF varnish; (2) n-HAP repairing serum; (3) NSF and (4) no treatment (control). Surface Microhardness (SMH) was assessed with Vickers micro hardness tester before and after demineralization and after 10 days of pH-cycling. A total of 12 random specimens from groups 1 to 4 (3 random samples of each group) were examined

by Atomic Force Microscopy (AFM). The SMH values were analysed with one-way and repeated measures ANOVA. Level of significance was set at $p=0.05$.

Results: Post lesion SMH values significantly decreased in all groups ($p<0.001$). Post treatment SMH values significantly increased in comparison to post lesion ones ($p<0.001$) except for control group which was not different statistically ($p=0.86$). The highest SMH values were observed in NSF group (mean 222.90 ± 28.79). Statistically significant differences were shown between all groups ($p<0.05$). However, NaF varnish and n-HAP groups were not statistically different ($p=0.165$). AFM images demonstrated protective layers in all treated groups.

Conclusion: The results of this in vitro study suggest that NSF could have the greatest remineralization efficacy. NaF varnish and n-HAP serum were similar in remineralizing initial caries. Future clinical studies are recommended for selection of the most appropriate remineralizing agent in primary teeth.

Keywords: Dental caries, Silver compounds, Tooth demineralization

INTRODUCTION

Dental caries is still a major health problem despite dental care improvements [1,2]. Recent studies claim worldwide increase in both permanent and primary dental caries [3], although during the last 25 years its prevalence had declined [4]. Dental caries can be caused by continuous interchanging process of demineralization and remineralization [5]. The process can be controlled by the degree of saturation of oral fluids (saliva) and dental plaque regarding the apatite minerals [6] and in an acceptable condition, remineralization may occur [7]. Fluoride has been widely used as a remineralizing agent and based on a systematic review, 5% NaF varnish was more effective in remineralizing early enamel caries [1,8,9]. As a result to fluoride drawbacks [10,11] and also NaF varnish shortcomings [12], seeking alternatives could be valuable. Another anti caries product is hydroxyapatite, a biocompatible and bioactive material which is widely used in medicine and dentistry [6,13]. In comparison to hydroxyapatite, nano-hydroxyapatite (n-HAP) has higher solubility, surface energy and bioactivity and its structure is similar to dental apatite [14,15]. Recent reports claim that Nano-HAP can be used as a remineralizing agent [6,10,16,17].

Other alternatives for preventing dental caries are metal ions [12,18,19]. Silver has different applications in medicine and dentistry due to its anti caries, antimicrobial and anti rheumatic potentials [20,21]. A recent review reported that Silver Diamine Fluoride (SDF) can prevent and arrest caries even when applied once a year [18]. Despite its efficacy, SDF has some disadvantages such as black staining of carious tissue, slightly painful lesions which

may disappear within 48 hours and the metallic taste [18,22,23]. Therefore, Targino AG et al., has introduced nano silver fluoride. This material's safety has been approved. Also, it does not cause black staining [24]. Comparing the remineralization potential of NaF varnish with n-HAP in the form of toothpaste, have shown inconsistent results in the reviewed articles [6,25,26]. Recent studies have compared remineralization effect of fluoridated products and n-HAP on permanent enamel lesions [14,26-28] and to the best of our knowledge, none of the studies have evaluated the remineralization effect of n-HAP serum and NaF varnish on enamel of primary anterior teeth. Besides, based on our search, the remineralization potential of NSF, n-HAP in form of serum and NaF varnish has not been compared yet.

Therefore, the goal of this study was to evaluate and compare the effectiveness of three remineralizing agents: NaF varnish, n-HAP and NSF on enamel of primary anterior teeth. The null hypothesis was that there is no difference in remineralizing effect of these materials.

MATERIALS AND METHODS

Sample preparation: This experimental in vitro study was approved by Ethics Review Committee of the School of Dentistry, Shiraz University of Medical Sciences (IR.SUMS.REC.1396.595) and conducted at the School of Dentistry from March to April 2017. The study sample size was calculated according to previous experimental studies [6,26]. Primarily a total of 80 sound primary anterior teeth were collected. Primary canines were extracted due to orthodontic supervision purposes. Over retained primary incisors

were also extracted in cases of successor permanent incisor delayed eruption. The exclusion criteria were teeth with decay, crack and any enamel defect. The samples were examined using stereomicroscope (Motic K®, Wetzlar, Germany) and a number of nine samples were excluded because of these reasons. The crowns and the roots were separated from 2 mm below the Cemento-Enamel Junction (CEJ). The crowns were first washed of debris and also disinfected with 0.1% thymol. They were mounted with epoxy resin so that their buccal surfaces were exposed and to obtain a flat surface, the teeth were polished with silicon carbide paper (600-, 800-, and 2400-grit) respectively, and washed in distilled water for 20 seconds. In order to enclose the surface to an exposed area of 2 × 2 mm, two layers of nail varnish were applied in the surrounding area [29].

Baseline microhardness test and group classification: As the next step, Surface Microhardness (SMH) of the samples was tested by Vickers micro hardness tester (SCTMC®, MHV 10002, China) under a 50 gram load for 10 seconds. The surface microhardness was assessed at five random points on the enamel. The first measurement was performed at the center of the sample and the other ones were at a distance of 300 µm from the first indentation and then the mean for SMH was calculated [26,30]. SMH tests were conducted by an operator. Samples with SMH between 262.40 and 389.30 were selected and samples with SMH below that (11 samples) were excluded. A total of 60 samples were randomized into four groups of 15 samples each based on the applied remineralizing agents as the following: (1) NaF varnish (Duraphat®, Colgate, Sao Paulo); (2) n-HAP repairing serum (PrevDent International BV®, Netherlands); (3) NSF (Sigma-Aldrich®, Munich, Germany) and (4) control-no treatment.

Solution preparation: Demineralization solution: This solution was prepared with 50 mM acetic acid (pH 4.5), 2.2 mM potassium dihydrogen phosphate (KH₂PO₄), 2.2 mM calcium nitrate (Ca(NO₃)₂) and 0.1 ppm NaF.

Remineralization solution: The remineralization solution used in pH-cycling contained 20 mMol l⁻¹ HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 130 mM potassium chloride (KCl), 1.5 mM calcium chloride (CaCl₂), 0.9 mM potassium dihydrogen phosphate (KH₂PO₄) and 1 mM sodium azide (NaN₃). The pH was adjusted to 7.0 with potassium hydroxide (KOH).

Incipient caries lesion formation: Before application of the remineralizing agents, incipient caries were generated. A pH-cycling protocol was performed as said by Ten Cate et al., [31]. Each tooth sample was stored in 30 ml of demineralization solution for three days (72 hours) at 37°C. Then, the specimens were washed with distilled water for 20 seconds. SMH testing was performed again after the development of artificial caries with the same micro hardness tester machine (SCTMC®, MHV 10002, China) and the same operator, as the previous protocol [10].

Experimental groups and pH cycling model: After the second microhardness test, the samples were treated according to each groups' manufactures' instructions.

Group 1 (5% NaF Varnish): The varnish (Duraphat®, Colgate, Sao Paulo) was applied with microbrush and specimens were stored in artificial saliva for 24 hours. After that, the varnish was eliminated from the surface with a scalpel blade without scratching the enamel surface [26].

Group 2 (n-HAP Serum): The n-HAP serum (PrevDent International BV®, Netherlands) was applied with its sponge for three minutes according to manufacturer's instruction. After this, the paste was kept in contact with the enamel surface of the specimens for 20 minutes. Then the samples were washed for 20 seconds with de-ionized water.

Group 3 (NSF): The solution was prepared by dilution of NSF powder (Sigma-Aldrich®, Munich, Germany) in distilled water (Ag+

[376.5 µg/ml]). Solution was left in contact with the tooth surface for two minutes. For each specimen two drops of NSF solution were applied with a micro brush [2].

Group 4 (Control): no agent was applied.

The cariogenic challenge was accomplished with the de- and remineralizing solutions described before. Specimens were maintained in remineralizing solution for 21 hours and in the demineralizing solution for three hours.

This cycle was repeated for 10 days [32]. The re- and demineralizing solutions were changed every third day. Before each immersion in demineralization solution, the different pastes were applied according to each group. The n-HAP serum was applied once daily for 10 days while the varnish and NSF solution were applied only once to mimic their single applications in clinical settings [2,26,32].

The post treatment SMH tests were conducted by the same operator blindly and with the same conditions (load, time and the machine) applied for baseline and post lesion measurements.

The surface microhardness recovery percentage was assessed as follows: % SMHR equals 100 {(post treatment-post lesion)/(baseline-post lesion)} [17].

Atomic force microscopy: After micro hardness testing, a total of 12 random specimens of all groups (three random samples of each group) were tested using atomic force microscope (NanoWizard® II; JPK Instrument AG, Berlin, Germany). The block surface morphology was analysed in "contact mode."

Imaging was obtained by an AFM nonconductive silicon nitride cantilever (Acta-Probe, APPNano, CA) with a spring constant (13 N/m). The rate of scanning was 1 Hz and a piezoelectric scanner was used. Images of 15 × 15 µm with a resolution of 512 × 512 pixels and an operating point of 1.5 V were gathered.

STATISTICAL ANALYSIS

Data were saved on EXCEL spreadsheet and statistics were calculated using the Statistical Package for the Social Sciences (SPSS) version 17 (IBM®, Chicago, IL, USA). The Shapiro-Wilk test confirmed the data distribution normality.

The SMH values and the surface microhardness recovery percentage were compared between groups with One-way ANOVA and also the Tukey HSD Post-hoc test. Repeated measures ANOVA and the Sidak Post hoc test were used for within group comparisons. The level of significance was set at 0.05.

RESULTS

Baseline, post lesion and post treatment SMH values and also % SMHR for each group are shown in [Table/Fig-1]. At baseline, SMH values of all samples ranged from 262.40 to 389.30 (mean 330.90±25.06) and no statistically significant differences were seen between groups (p=0.906). Post lesion SMH values ranging from 100.90 to 163.70 (mean 122.88±15.41), were significantly lower than baseline SMH values (p<0.001). However, these values

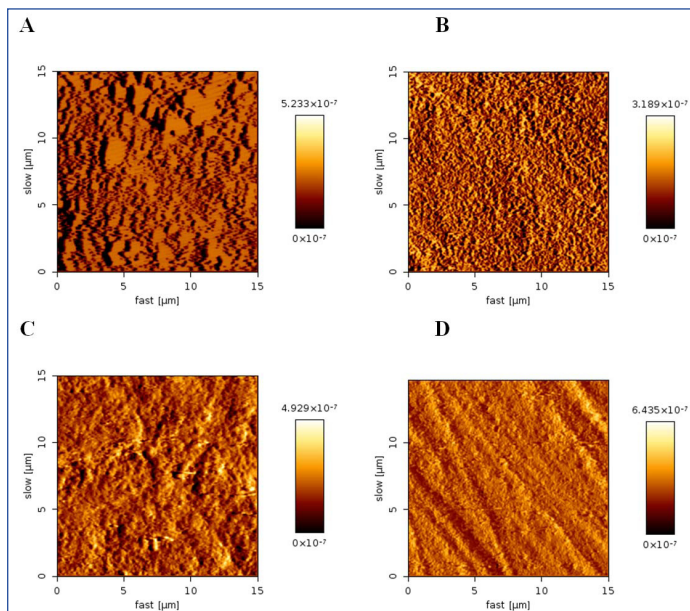
Groups	Baseline	Post lesion	Post treatment	% SMHR
NaF varnish	327.50±29.34 a, A*	120.81±18.10 a, C†	192.48±30.96 b, B	+34.94 ^b
n-HAP serum	333.41±18.29 a, A	126.03±12.71 a, C	172.65±21.68 b, B	+22.17 ^b
Nano silver fluoride	329.58±27.36 a, A	116.98±11.74 a, C	222.90±28.79 a, B	+50.70 ^a
Control	333.09±26.03 a, A	127.69±17.21 a, B	114.11±20.30 c, B	-7.21 ^c

[Table/Fig-1]: Surface microhardness values (mean±SD) and percentage of surface hardness recovery (%SMHR) of groups at baseline, post lesion and post treatment. Different lowercase letters in each column presents statistically significant differences (One-way ANOVA and Tukey post-hoc test p<0.05).

* Different uppercase letters in each row demonstrates statistically significant differences (one sample repeated measure ANOVA and Sidak post-hoc test p<0.05).

Groups	NaF varnish	n-HAP serum	Nano Silver Fluoride (NSF)	Control
NaF varnish	-	NS	p<0.05	p<0.001
n-HAP serum	NS	-	p<0.001	p<0.001
Nano Silver Fluoride (NSF)	p<0.05	p<0.001	-	p<0.001
Control	p<0.001	p<0.001	p<0.001	-

[Table/Fig-2]: Comparison between group of post-treatment surface microhardness values and %SMHR (p<0.05). NS presents non-significant difference between groups. (Post-Hoc Tukey test p<0.05).



[Table/Fig-3]: AFM images of enamel surfaces after each groups' remineralization: (A) initial enamel remineralized by NaF varnish, (B) initial enamel remineralized by n-HAP group, (C) initial enamel remineralized by NSF group, (D) No treatment (control group).

were not significantly different between groups ($p=0.209$). Post treatment SMH values were significantly different from post-lesion ones ($p<0.001$) except for control group which was not different statistically ($p=0.86$). The highest SMH values were observed in NSF group (mean 222.90 ± 28.79) followed by NaF Varnish group (mean 192.48 ± 30.96), n-HAP group (172.65 ± 21.68) and control group (mean 114.11 ± 20.30). Between groups analysis showed statistically significant differences between all groups which is demonstrated in [Table/Fig-2] ($p<0.05$). However, NaF varnish and n-HAP groups were not different statistically ($p=0.165$). Recovery of surface microhardness was observed in all interventional groups but % SMHR was negative in control group. Similarly to post treatment SMH values, pairwise comparison of % SMHR revealed significant differences statistically between groups except for NaF varnish and n-HAP groups ($p=0.74$). The result for % SMHR pairwise comparison is illustrated in [Table/Fig-2].

[Table/Fig-3] illustrates diverse AFM images obtained from each group's enamel surface after remineralization. The NaF varnish group showed development of globular arrangements which were not precisely uniform [Table/Fig-3a]. As it is shown in [Table/Fig-3b], in n-HAP group homogenous acicular crystals were formed. In NSF group, the protective layer consisted of different sized and multi shaped crystal formation [Table/Fig-3c]. No protective layer was observed in the control group [Table/Fig-3d].

DISCUSSION

In this in vitro study, different remineralizing agents were compared to evaluate their effect on demineralized enamel. The null hypothesis which was mentioned earlier was not supported. The results demonstrated that NSF had the highest SMH values. NaF varnish and n-HAP serum also increased the enamel hardness but they were not significantly different.

In the current study, like several previous studies, NaF varnish and n-HAP were effective for enamel remineralization but their comparative efficacy was controversial in reports [6,25,26]. De Carvalho FG et al., compared fluoride varnish and n-HAP paste and similar to our results, both could protect against enamel demineralization. However, in their study n-HAP was more effective than fluoride varnish [26]. This dissimilarity in their findings could be explained by the difference in the n-HAP pastes used in each study. The n-HAP paste applied in the de Carvalho FG et al., study, contained 9000 ppm fluoride while the n-HAP serum in our study did not have any fluoride. This might be the reason to the increased remineralization capability of n-HAP in de Carvalho study.

We applied the n-HAP serum once daily for 10 days. Similar to our results, another study recommended 10% n-HAP to be used daily since its efficacy was similar to 1000 ppm fluoride [27].

In another study conducted by Comar LP et al., fluoride paste (0.2% NaF) was reported to be a better agent in decreasing enamel demineralization in comparison to n-HAP pastes and n-HAP plus fluoride. This result might be due to the different study design. They focused on comparing the prevention of demineralization of the experimental pastes, not the remineralization of the pre formed enamel caries lesion [25].

Tschoppe P et al., reported that n-HAP in the form of toothpaste was more effective than amine fluoride ones in remineralizing enamel and dentin [6]. We used n-HAP (PrevDent International BV, Netherlands) in the form of serum which contained aqua, xylitol, nano-hydroxyapatite, potassium chloride, menthapiperita oil, linalool, and limonene. However, the n-HAP pastes used in Tschoppe P et al., study were in form of toothpaste with different formulations containing n-HAP with and without zinc carbonate. This difference in formulation might describe the different results. Considering fluorosis, the most important fluoride disadvantage in children, n-HAP could be recommended as an alternative. Also, Tschoppe P et al., recommended n-HAP as a substitute to fluoride which is consistent with our results [6].

In the current study, we evaluated the remineralization effectiveness of NSF as a solution. The results showed the highest SMH values and %SMHR. Zhi QH et al., demonstrated that both silver and fluoride ions were responsible for enamel remineralization. As a matter of fact silver ions could infiltrate into carious lesions and precipitate there. This could result in enamel hardening [33].

In the present study, the enamel surfaces were evaluated by AFM and diverse images were obtained as a result of different remineralization mechanisms. All treatment groups formed protective layers which are in agreement with our SMH values results. The granular structure of NaF varnish group was because of CaF_2 formation as another research had described [10]. The n-HAP group showed uniform acicular n-HAP crystals which covered the enamel pores. Its organized microstructure seemed like a woven unit protecting the enamel from demineralization attack. The protective layer caused by NSF was heterogeneous and different to the former groups. The diversity in crystals' sizes and shapes could be explained by their different nano ionic origins. No definite protective layer was observed in the control group except for minor crystals caused by the remineralization solution. The polishing procedures could be the cause to scrape like lines.

Based on the reviewed literatures, this is the first time to evaluate remineralization potential of NaF varnish, n-HAP serum and NSF on enamel carious lesions in primary anterior teeth. As mentioned in dos Santos study NSF is cost-benefit, easy to apply and it could be applied once a year [2]. Therefore, it has the potential to replace NaF varnish as an in-office fluoride therapy substance.

LIMITATION

In order to better mimic to clinical conditions, we used pH-cycling model. However, in vitro study might not reproduce the results of

an in-vivo one. Besides, the effect of oral factors such as, saliva and dental plaque on the enamel remineralization could not be considered. The antimicrobial capacity of silver products was well known but was not the aim of this study. It is also valuable to evaluate the safety of NSF before its clinical application. Therefore, further studies are recommended to evaluate the oral factors, the anti microbial potential and safety in clinical settings.

CONCLUSION

Based on the results of this in vitro study, NaF varnish and n-HAP serum were similar in remineralizing initial caries. NSF could have the greatest remineralization capability in comparison to other materials evaluated. Therefore, n-HAP serum and NSF can be introduced as substitutes for NaF varnish.

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