Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion *in vitro*

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Abstract
The purpose of the research was to determine the effect of nano-hydroxyapatite concentrations
on initial enamel lesions under dynamic pH-cycling conditions. Initial enamel lesions were
prepared in bovine enamel with an acidic buffer. NaF (positive control), deionized water
(negative control) and four different concentrations of nano-hydroxyapatite (1%, 5%, 10% and
15% wt%) were selected as the treatment agents. Surface microhardness (SMH) measurements
were performed before/after demineralization and after 3, 6, 9 and 12 days of application, and
the percentage surface microhardness recovery (%SMHR) was calculated. The specimens
were then examined by a scanning electron microscope. The %SMHR in nano-hydroxyapatite
groups was significantly greater than that of negative control. When the concentration of
nano-HA was under 10%, SMH and %SMHR increased with increasing nano-hydroxyapatite
concentrations. There were no significant differences between the 10% and 15% groups at
different time periods in the pH-cycling. The SEM analysis showed that nano-hydroxyapatite
particles were regularly deposited on the cellular structure of the demineralized enamel
surface, which appeared to form new surface layers. It was concluded that
nano-hydroxyapatite had the potential to remineralize initial enamel lesions. A concentration
of 10% nano-hydroxyapatite may be optimal for remineralization of early enamel caries.

1. Introduction

Dental caries in enamel is unique amongst diseases as enamel is both acellular and avascular. Thus, in contrast to other tissues, enamel cannot heal itself by a cellular repair mechanism [1]. Nonetheless, it is now well established that the formation of incipient enamel caries is a reversible process where periods of progression alternates with periods of remineralization [2]. Given an appropriate change in conditions, remineralization may even become the predominant process, leading to apparent repair of the lesion [3].

Fluoride (F) has been a useful instrument and is one of the most effective remineralizing agents in caries prevention [4]. Over the last 25 years, the decline in dental caries

experienced in most industrialized countries can be attributed largely to the widespread use of fluoride [5]. Nevertheless, some concern has been expressed that with the wide array of both prescription and over-the-counter fluoride products now being marketed in every country, the total fluoride intake has increased to perhaps harmful levels. Chronic low-level exposure to fluoride can present problems in organ systems (gastro-intestinal, genito-urinary and respiratory) of normal individuals [6]. The prevalence of dental fluorosis, on the other hand, has increased noticeably in non-fluoridated areas and to a lesser extent in optimally fluoridated areas [6–8]. Therefore, it is still necessary to seek alternative, effective non-fluoride agents that can provide a complete cure for caries.

Hydroxyapatite (HA) is one of the most biocompatible and bioactive materials and is widely applied to coat artificial joints and tooth roots [9]. Nano-sized particles have similarity to the apatite crystal of tooth enamel in morphology, crystal

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structure and crystallinity [10]. In recent years, an increasing number of reports have shown that nano-hydroxyapatite has the potential to remineralize artificial carious lesions following addition to toothpastes, mouthwashes, etc [11–13]. Some studies have also reported that a 4% (wt%) nano-HA liquid suspension had good potential to remineralize incipient caries lesions [14, 15]. However, other studies have found no significant difference between NaF and 10% (wt%) nano-HA on the effect of remineralization of initial enamel lesions [16]. The divergence in these results is probably related to different methodologies (*in vivo* or *in vitro*, type of remineralizing agents used, time of application, etc). To date, there have been no reported studies regarding the concentrations of nano-hydroxyapatite required for protection, or which manifest the best protection under systems that reflect real-life situations. Another important factor to be considered is that when *in vitro* remineralization and demineralization cycle model systems are used to evaluate the efficacy of agents, different testing protocols may obtain distinct results. Therefore, the aim of this study was to determine the effects of nano-hydroxyapatite concentration on the initial enamel lesions over a range of time periods under dynamic pH-cycling conditions. In addition, our goal was to address the mitigating factors and thereby aid in a thorough evaluation of nano-hydroxyapatite for practical applications in the anti-caries area.

2. Methods and materials

2.1. Solution preparation

Demineralization solution: The demineralization solution used to initially form subsurface caries lesions and in the pH-cycling was a pH 4.5 acetic acid (50 mM) solution containing 2.2 mM $Ca(NO_3)_2$, 2.2 mM KH_2PO_4 and 0.1 ppm NaF.

Remineralization solution: The remineralization solution used in pH-cycling contained 20 mmol l^{-1} HEPES, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl and 1 mM NaN_3. The pH was adjusted to 7.0 with KOH; these solutions were similar to those used by Ten Cate and Duijsters [17].

Nano-hydroxyapatite power was purchased from National Incubation Base for Nano-Biomaterials Industrialization, Sichuan University, China. The nano-HA crystals were of nanometer grade and had a crystal size of 5–26.7 nm diameter by 30–84 nm in length, giving an aspect ratio of 3.1. These nano-HA crystals had also a similarity in crystallinity to apatite in bone and enamel as revealed by XRD [18–20].

Treatment solutions were 1000 ppm NaF aq. (positive control), distilled and deionized water (DDW, negative control); 1%, 5%, 10% and 15% (wt%) nano-HA suspension liquid in distilled water, pH adjusted to 7.0 using 2 M HCL.

2.2. Enamel specimen preparation

In this study, bovine incisors were used. The freshly extracted teeth were thoroughly cleaned of debris and inspected under a stereoscopical microscope for visibly observable cracks, hypoplasia or white spot lesions, and then stored in a 0.1% thymol solution until required. The crowns were separated from the roots by a diamond-coated band saw

Table 1. The pH-cycling model in the experiment.

Time	Experimental solution
8:00 a.m.–8:03 a.m.	Treatment solutions
8:03 a.m.–9:00 a.m.	Remineralization solution
9:00 a.m.–9:03 a.m.	Treatment solutions
9:03 a.m.–11:00 a.m.	Remineralization solution
11:00 a.m.–1:00 p.m.	Demineralization solution
1:00 p.m.–3:00 p.m.	Remineralization solution
3:00 p.m.–3:03 p.m.	Treatment solutions
3:03 p.m.–4:00 p.m.	Remineralization solution
4:00 p.m.–4:03 p.m.	Treatment solutions
4:03 p.m.–8:00 a.m.	Remineralization solution

The treatment solutions, remineralization solution and demineralization solution were used as described above.

under continuous water cooling (Struers Minitom; Struers, Copenhagen, Denmark). Enamel blocks (4 mm × 4 mm) were embedded in polymethyl methacrylate. The superficial enamel surface was ground flat with water-cooled carborundum discs (1200 grit; Water Proof Silicon Carbide Paper, Struers, Germany) and polished with diamond paste (15 μm Diamond Paste, Struers), thereby removing approximately 100 μm of the outermost enamel layer and yielding a flat surface.

2.3. Baseline microhardness test

Baseline surface hardness of the sound enamel after polishing was performed with a microhardness tester (Duramin-1/-2; Struers, Copenhagen, Denmark) using a Knoop indenter at 10 g load for 15 s. 126 enamel blocks with baseline surface microhardness (SMH) between 469.6 and 488.0 Knoop hardness numbers (KHN) were selected for further study.

2.4. Preparation of early artificial caries lesions

Early artificial caries lesions were produced in the enamel, basically according to Ten Cate and Duijsters [17]. Each specimen was immersed in 8 ml of demineralization solution for 72 h at 37 °C. After artificial caries preparation, the SMH of the enamel blocks was again measured (SMH 1). Indentations were spaced at 100 μm from each other and baseline measurements were made. After artificial caries preparation, 70 blocks with baseline KHN values (SMH1) between 169.6 and 189.8 were selected for pH-cycling. One half of each specimen was covered with an acid-resistant varnish to maintain the baseline lesion.

2.5. pH-cycling model

The specimens were randomly divided into six groups (10 specimens/group) according to the treatment solutions. The cycling schedule was designed to approximate the pH dynamics of the oral environment and used the regime reported by White [21]. The de- and remineralization cycles consisted of the episodes shown in table 1. Each cycle involved 2 h of demineralization in order to simulate the daily acid challenges occurring in the oral cavity.

The treatment solutions, remineralization solution and demineralization solution were used as described above.

Table 2. Surface microhardness analysis of enamel blocks at various time periods according to the treatments.

Treatments	Baseline SMH	Before pH-cycling	After pH-cycling 3 days	After pH-cycling 6 days	After pH-cycling 9 days	After pH-cycling 12 days
NaF	484.7 ± 3.3^a	187.3 ± 7.6^a	270.6 ± 8.7	334.2 ± 8.3	351.3 ± 12.1^c	362.0 ± 16.5^c
1%Nano-HA	483.7 ± 6.7^a	190.7 ± 9.9^a	218.5 ± 5.0	226.2 ± 3.0	233.5 ± 2.4	236.6 ± 4.3
5%Nano-HA	481.0 ± 3.5^a	189.6 ± 9.1^a	230.1 ± 3.0^a	252.9 ± 1.5	261.7 ± 3.4^c	263.4 ± 4.3^c
10%Nano-HA	475.7 ± 4.3^a	188.9 ± 6.3^a	$236.5 \pm 3.0^{a,b}$	$306.1 \pm 18.0^{a,c}$	$309.2 \pm 10.6^{a,c}$	$313.5 \pm 8.2^{a,c}$
15%Nano-HA	478.1 ± 6.0^a	194.3 ± 3.2^a	245.7 ± 12.3^b	$313.1 \pm 14.6^{a,c}$	$314.6 \pm 6.7^{a,c}$	$318.8 \pm 3.5^{a,c}$
DDW	483.8 ± 9.1^a	186.3 ± 7.9^a	193.02 ± 4.2	204.1 ± 2.7^c	203.3 ± 2.8^c	204.7 ± 3.2^c

[a,b] The same letter denotes values that are not significantly different within the same time period in different treatments ($p > 0.05$).
[c] Show no significant difference at different time periods for each treatment ($p > 0.05$).

The regimen was repeated for 12 days and temperature maintained at 37 °C. The de- and remineralizing solutions were freshly made every third day and the treatment solutions were made daily and used with continuous stirring throughout the experimental period. Nano-HA treatment solutions were ultrasonicated immediately after preparation, as ultrasonication is particularly effective in breaking up the aggregates and in reducing the size and polydispersity of nanoparticles [22].

2.6. Surface microhardness analysis

After 3, 6, 9 and 12 days of application, the SMH of the enamel blocks was again measured (SMHn). At each time point, five indentations were placed next to the previous measurement at 100 μm intervals, the mean values of all five measurements at different application times were then compared and the percentage SMH recovery was calculated as [after n(th) days %SMHR = 100 (SMH n–SMH 1)/(SMH–SMH 1)] [23] (n = 3/6/9/12).

2.7. SEM examination

After the SMH analysis, representative specimens from the nano-HA groups, the positive control and the negative control groups were randomly selected for SEM sample preparation. These were then examined using a scanning electron microscope (S-2460 N, Hitachi, Tokyo, Japan).

2.8. Statistical analysis

Data were computerized and analyzed using SPSS 13.0 software. SMH and percentage surface microhardness recovery (%SMHR) among treatments were analyzed by repeated measures, followed by the LSD test. The significance level was set at 0.05.

3. Results

The results of SMH analysis of enamel blocks are shown in table 2. The enamel blocks in all treatment groups had rehardened significantly after pH-cycling. Fluoride had a significantly greater effect than all the other treatments ($p < 0.05$). All treatments except the 10% nano-HA and 15% nano-HA treatments were statistically different from each
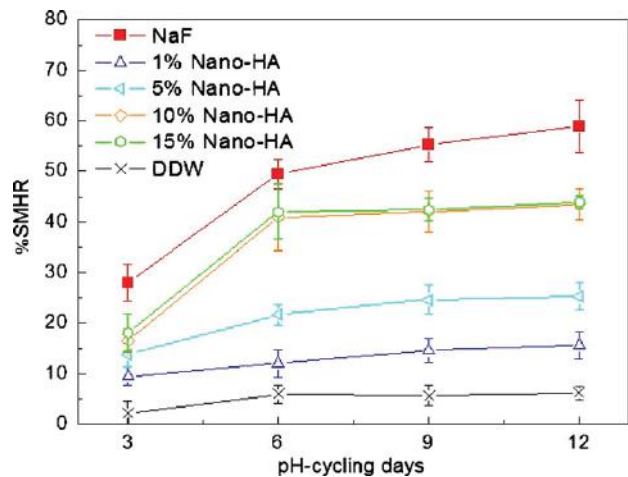

Figure 1. %SMHR of enamel blocks at various time periods according to the treatments.

(This figure is in colour only in the electronic version)

other after pH-cycling has been completed. In the nano-HA groups, %SMHR was significantly greater than that of the negative control group at each time point ($p < 0.05$). When the concentrations of nano-HA were under 10%, SMH and %SMHR increased with nano-HA concentration at each time point in the pH-cycling. The highest percentage SMHR was found for the treatment with 15% nano-HA and the lowest with 1% nano-HA. In addition, the %SMHR and time curve (figure 1) revealed that, for all treatment groups, the remineralization rate increased significantly in the first 6 days of pH-cycling. However, little further improvement happened beyond this point.

Distinct surface coatings deposited by different agents were evident by SEM on the treated anatomical enamel surfaces of the specimens under different conditions (figure 2). As shown in figure 2(*a*), a smooth and intact surface was obtained in the normal anatomical enamel surface before demineralization; however, many micropores and cellular structures appeared on the surface of the initial lesions (figure 2(*b*)). After pH-cycling, in the nano-hydroxyapatite groups, acicular crystals had sedimented on the enamel surfaces after demineralization, and the cavities and defects of the enamel surface had decreased (figure 2(*c*)). The NaF group indicated formation of different-sized globular structures in
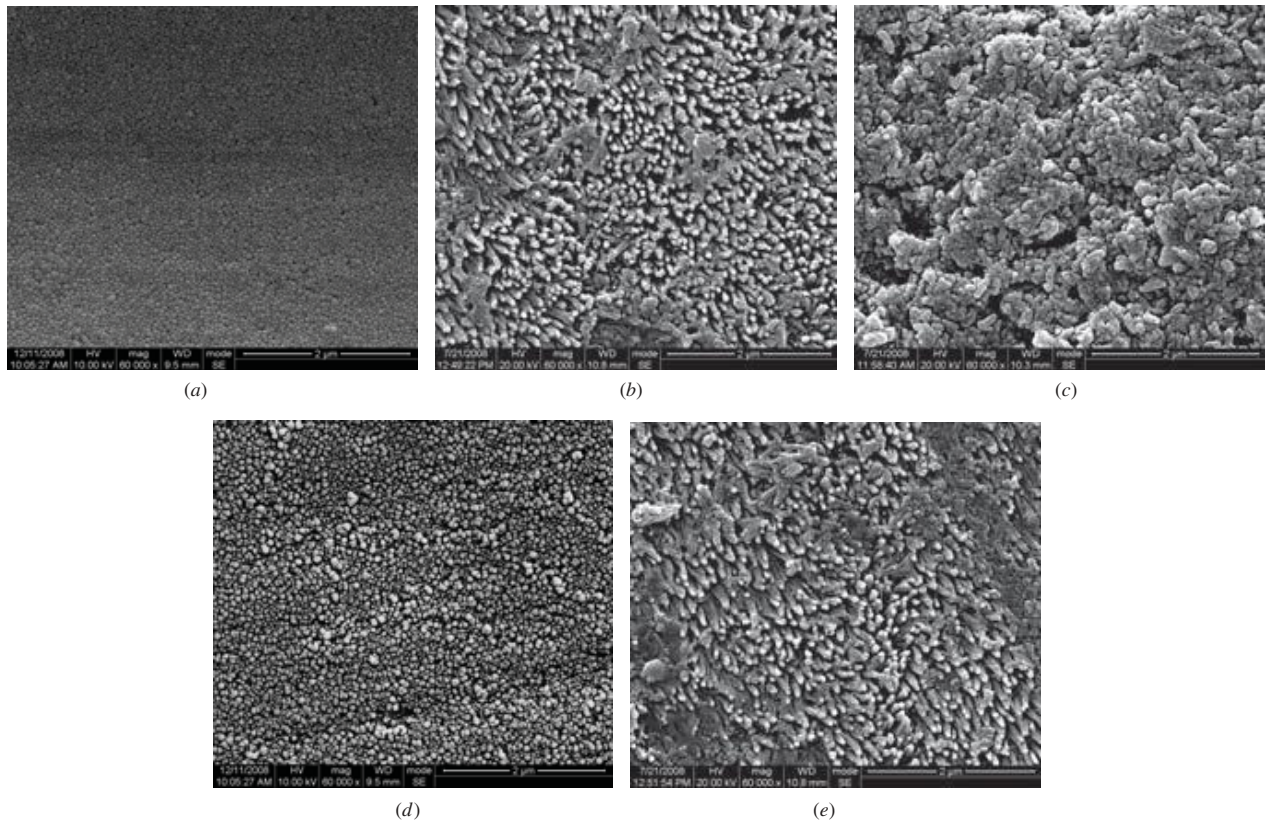
(*a*)   (*b*)   (*c*)

(*d*)   (*e*)

Figure 2. SEM images of a representative specimen in different groups before and after pH-cycling: (*a*) normal enamel before demineralization, (*b*) initial enamel lesion, (*c*) initial enamel lesion remineralized by nano-HA (10%), (*d*) initial enamel lesion remineralized by NaF, (*e*) initial enamel lesion remineralized by DDW.

the enamel surface (figure 2(*d*)); however, only a honeycomb structure was found in the DDW groups (figure 2(*e*)).

4. Discussion

In the current study, nano-hydroxyapatite was directly selected as a remineralizing agent and an *in vitro* pH-cycling model was used to evaluate the effect of four nano-HA concentrations on the initial enamel caries lesions. Although the data were obtained in a laboratory setup, the pH-cycling model provided a better simulation of the caries processes and more closely approached the oral environment, compared to separate de- and remineralization studies [24]. These results confirmed the ability of nano-hydroxyapatite to aid in remineralizing enamel; at each time point in the pH-cycling, the different concentrations were directly related to the distinct effects on remineralization. The most likely explanation of the increased remineralization effect is that it was due to the ability of nano-hydroxyapatite to promote remineralization.

In a previous study on the remineralization effect of nano-HA toothpaste on artificial caries, the solubility properties of nano-HA were found to play a significant role in remineralization when the demineralized specimens were subjected to the treatment solutions continuously for several days [11]. However, in the present study, nano-HA was applied

for only a short period during pH-cycling, and due to the low solubility of pure hydroxyapatite, not enough Ca^{2+} and PO_4^{3-} were available to increase the stability of hydroxyapatite in the enamel and to prevent dissolution of the dental enamel.

Since the surface area and proportion of atomicity increase with decreasing particle size, nano-HA has bioactive and biocompatible properties [25]. As shown in figures 2(*a*) and (*b*), incipient lesions extended into the enamel and were significantly more porous than was sound enamel, which allowed a greater penetration of solution ion constituents and a larger surface area for a subsequent reaction of enamel mineral [26]. These factors increased the potential of nano-HA to directly fill up defects and micropores on demineralized teeth. If nano-HA penetrates the enamel pores, nano-HA will act as a template in the precipitation process and will continuously attract a large amount of Ca^{2+} and PO_4^{3-} from the remineralization solution to the enamel surface to fill the vacant positions of the enamel calcium crystals. This in turn will promote crystal integrity and growth.

The surface chemical properties and morphological structure of nano-HA has been claimed to play the most important part in the remineralization of early caries lesions. As the concentration increases, the rate and amount of nano-HA precipitation would also increase, along with the deposition of extensive amounts of Ca^{2+} and PO_4^{3-}, thus

significantly promoting the remineralization effect. Kim *et al* [12] also demonstrated that surface hardness of the demineralized enamel increased with increasing nano-HA concentration when nano-HA was added to a NaF mouthwash. In the nano-HA groups, 15% nano-HA showed a good effect on remineralization; however, this concentration is a little high for practical purposes in mouthwash or toothpaste, as concentrations in this range will cause a certain level of unavoidable aggregation. However, as the data obtained for 10% nano-HA were quite similar to those for 15% nano-HA, a 10% suspension may prove to be an optimal concentration for remineralization of early enamel caries.

From the %SMHR and time curve (figure 1), it was clear that remineralization continued over an extended period of time. However, the rate of remineralization in all treatments was fastest during the first 6 days of pH-cycling and then slowed and stabilized beyond this point. A previous report [28] on mineral deposition in artificial early caries lesions also supported the data of the current study.

When the concentration of nano-HA was under 10%, the remineralization effect increased significantly along with increase in the concentration while there was a sharp change between the remineralization effect at 5% and 10%. These findings also could be due to the possible mechanisms of nano-HA in remineralization as described above. As a result of remineralization of the outer enamel region, the deposition of nano-HA on the surface layer would probably block surface pores and restrict diffusion into the lesion over the short term of remineralization. However, this deposition would eventually come to a stable level even though the concentration increased, which would result in no significant difference between the 10% and 15% nano-HA groups. Research to date has shown that lesions can be rehardened by deposition of hydroxyapatite that is initially deposited near the surface layer, but then is gradually transferred inward and finally precipitated in the dark zone in the long-term remineralization [27]. It has been well established that nano-HA has a good potential for remineralization and promotes remineralization with regular daily usage from this perspective.

In the current study, the enamel surfaces in different treatments were examined by a scanning electron microscope. The different enamel surface morphologies in the corresponding treatments may be due to different mechanisms for promoting remineralization. After the application of the higher concentrations of fluoride, calcium fluoride-like material was preferentially formed in partially demineralized human enamel [28], while it seemed to cause crystal growth of surface apatite crystals (figure 2(*d*)). Since, in the current study, we used 1000 ppm NaF as a positive control, it may be assumed that the globular structures, which sedimented in the surface layer, consisted mainly of CaF_2.

In the nano-HA groups, since the enamel surface morphologies were similar among the different concentration groups after remineralization, we selected a representative result to describe the change in the crystal morphology of specimen after application of the agent. As shown in the figure 2(*c*), the entire enamel surface was covered with finely divided particles and the products appeared to coalesce and form a surface layer microstructure. At the same time, the globules themselves appeared to be agglomerates of still smaller particles. From the aspect of a mechanism for nano-HA remineralization, acicular crystals of nano-HA sedimented onto the enamel surfaces and directly filled up defects and micropores on demineralized teeth surfaces after demineralization. This resulted in the observed decreases in cavities and defects of the enamel surface and the increased surface hardness of the enamel surface. The results of the surface microhardness analysis were supported by the observed crystal morphology.

5. Conclusions

Nano-hydroxyapatite had the potential to remineralize initial enamel caries lesions under dynamic pH-cycling conditions. A suspension of 10% nano-hydroxyapatite appeared to be the optimal concentration for remineralization of early enamel caries. Nano-hydroxyapatite of proper concentration could therefore be beneficial in promoting remineralization with regular daily usage.

Acknowledgment

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