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Beneficial effects of hydroxyapatite on enamel subjected to 30% hydrogen peroxide

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ABSTRACT

Objectives: To evaluate the effect of combination of hydroxyapatite (HA) and hydrogen peroxide (HP) on color, microhardness and morphology of human tooth enamel.

Methods: Forty-eight human dental blocks were obtained from 12 pairs of premolars and were randomly divided into four groups. Group DW was treated with distilled water, group HP with 30% HP, group HA + DW with HA mixed with distilled water and group HA + HP with HA mixed with 30% HP. Baseline and final color measurements and microhardness test were carried out before and after bleaching experiments. Two specimens from each group were selected for morphological investigation after final tests.

Results: The ΔE of group HP and HA + HP were significantly higher than those of group DW ($p = 0.000$ and $p = 0.000$) and group HA + DW ($p = 0.000$ and $p = 0.000$). The percentage microhardness loss of group HA + HP was significantly lower than that of group HP ($p = 0.047$), but significantly higher than those of group DW ($p = 0.000$) and group HA + DW ($p = 0.000$). The obvious variation of morphology was only observed on enamel surfaces in group HP.

Conclusions: This study suggested that combination of HA and HP was effective in tooth whitening. HA could significantly reduce the microhardness loss of enamel caused by 30% HP and keep enamel surface morphology almost unchanged.

1. Introduction

Vital tooth bleaching is a well-accepted method of treating discolored tooth.[1] The method is based upon hydrogen peroxide (HP) as the active agent.[2,3] HP may be applied directly, or produced in a chemical reaction from carbamide peroxide (CP). It acts as a strong oxidizing agent through the formation of free radicals, reactive oxygen molecules, and HP anions.[2,3] It is suggested that these reactive molecules interact with chromophore molecules and oxidize the macromolecules and pigment stains.[2,3]

Although there is little question about their efficacy, a primary concern is that the enamel structure may be weakened by the bleaching agent. Numerous studies have evaluated the effects of peroxide-containing products on the physical and chemical properties of tooth enamel. However, the research in this area has been controversial. Some studies reported that there was no evident change in enamel microhardness and morphology after bleaching treatment.[4–7] But others have found calcium loss,[8–10] alterations of surface morphology,[11–15] changes in chemical composition,[10,15–18] decrease in hardness[10,15,19–22] and fracture resistance[23] of enamel. The diver-

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gence in these results is probably related to different methodologies (in vivo or in vitro, time of evaluation, bleaching agents used, time of application, immersion of the specimens in artificial saliva between treatments, type of storage, bleaching agent pH, usage of fluoride, etc.).[6,24,25]

The oxidative effect, pH and composition of the bleaching agents are claimed to produce possible side effects during tooth bleaching.[23] As the oxidative effect is not specific, the bleaching agents may not only oxide the chromogen of the discolored teeth but also attack the organic matter of the teeth.[15,19] The acidity of HP is probably the main cause for the demineralization effect.[25] When the pH falls below 5.2, enamel demineralization[26] and root resorption[27,28] have been reported. On the other hand, however, different concentrations of CP are also capable of causing enamel surface alterations regardless of being close to neutral pH level.[13,14,29] Urea, which is the by-product of such bleaching agents, could remove enamel proteins and related mineral elements.[14,23,30] Glycerin or carbopol, the most common carriers in the bleaching formula, may also act as a demineralizing agent.[21,22]

It should be pointed out that the remineralization and protective benefits of saliva may overcome the detrimental bleaching effects in vivo.[25] And no clinical studies or case reports in the literature have documented macroscopically or clinically visible damage due to vital bleaching.[31] However, it is still necessary to minimize the risk of even minor damage caused by the agents. To achieve this goal, some ingredients such as fluoride,[31] calcium[32] or amorphous calcium phosphate[33] are added into the bleaching gel by manufacturers.

Synthetic hydroxyapatite (HA) is an attractive biomaterial for bone and tooth implants, owing to its chemical and structural similarity with natural bone and tooth mineral.[34,35] Recent research indicated that HA paste containing concentrated H_3PO_4 and HP can repair early caries lesion.[36,37] It was shown that the hydroxyapatite nanocrystals seamlessly grew in the interface between the paste and tooth enamel despite low pH of the paste.[36,37] Meanwhile, HA is an alkaline salt[38] and it may increase the pH value of the compound when it mixed with acidic HP. The increased pH may reduce the demineralization effect of acidic HP and facilitate the bleaching procedure[2] by accelerating formation of free radicals from HP. Furthermore, addition of hydroxyapatite to toothpaste could result in a marked increase in tooth whitening.[39]

Thus, we hypothesize that combination of HA and HP may bring better whitening effect and reduce enamel demineralization caused by acidic HP alone. The purpose of the study was to evaluate the effect of combination of HA and HP on color, microhardness and morphology of human tooth enamel.

2. Materials and methods

The study protocol was reviewed and approved by the Ethics Committee of the School and Hospital of Stomatology, Wuhan University. Patients from whom teeth were being extracted were asked to read and sign a consent form prior to the extraction.

2.1. Synthesis of HA

HA was synthesized in a wet condition according to the method of Boanini et al.[41] with some modifications. Twenty milliliters of 1.08 M $Ca(NO_3)_2{\cdot}4H_2O$ solution at pH adjusted to 10 with NH_4OH. The solution was heated at 90 °C and 20 ml of 0.65 M $(NH_4)_2HPO_4$ solution, pH 10 adjusted with NH_4OH, was added drop-wise under stirring. The precipitate was maintained in contact with the reaction solution for 5 h at 90 °C under stirring, and then centrifuged at 1800 *g* for 10 min. The precipitate was repeatedly washed with distilled water and centrifuged for 6 times, and then dried at 37 °C overnight.

2.2. Characterization of the precipitate

The precipitate was characterized by the scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD). Morphological investigation was performed using SEM (Fei QUANTA-200, Eindhoven, the Netherlands). For FTIR analysis, 1 mg powder was mixed with potassium bromide powder and pressed into disks. The spectra were recorded on Thermo Nicolet 5700 spectrometer (Nicolet, Madison, Wis., USA). The crystal phase was characterized by wide angle XRD (D/MAX-RB, Rigaku, Miniflex, Japan). The scanning range was 20–70°, with a scanning speed of 4°/min. The radiation utilized was CuKα at 40 kV and 50 mA.

2.3. Tooth selection

Twelve pairs of premolars, extracted for orthodontic reasons, were selected. Buccal surfaces were devoid of stain, enamel cracks or fractures, caries or other defects. The teeth were cleaned thoroughly and stored in 0.5% thymol at 4 °C until required.

2.4. Sample preparation

Two dental blocks (2 mm × 3 mm × 4 mm) were obtained from middle 1/3 of buccal halves of each tooth by a low speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. The dental blocks were individually embedded in colorless translucent acrylic resin with enamel surface exposing for bleaching applications. The specimens were serially polished by means of 600–3000 grit SiC papers with water as a cooler to obtain flat standardized enamel surfaces. Subsequently, they were polished with diamond spray (1 μm, 0.5 μm) and polishing cloths. After then the specimens were ultrasonic cleaned for 5 min with distilled water to remove smear layer. The initial color measurement and microhardness test were performed within 24 h after preparation. The specimens were stored in Hank's balanced salts solution (HBSS)[40] during the interval of the preparation and before testing.

2.5. Bleaching procedure

All specimens were washed under running distilled water for 30 s and dried by compressed air for 3 s before treatment started. Four specimens prepared from each pair were randomly divided into four groups and treated as follow:

Group DW ($n = 12$, pH ≈6.8): As a control group. The specimens were immersed in 2 ml distilled water for 15 min.
Group HP ($n = 12$, pH ≈3.2): The specimens were immersed in a 2 ml 30% solution of HP (Sinopharm Chemcial Reagent Co. Ltd., Shanghai, China) for 15 min.
Group HA + DW ($n = 12$, pH ≈7.6): HA mixed with distilled water in a ratio of 2 g powder to 1 ml liquid. A similar quantity (about 0.1 g HA and 0.05 ml liquid) of HA paste was applied to the enamel. After then the specimens were immersed in 2 ml distilled water for 15 min.
Group HA + HP ($n = 12$, pH ≈5.4): HA mixed with HP in the same ratio for group HA + DW and a similar quantity was applied to the enamel surface. Then the specimens were immersed in 2 ml 30% solution of HP for 15 min.

During the treatment period, all the specimens were stored at 37 °C. Between treatments, the residual treatment solution and HA were removed under running distilled water with cotton wool pellets. Then the next 15 min treatment was carried out immediately. Wet cotton pellets were put on the surfaces of specimens to diminish dehydration between treatments. The treatment procedure was repeated 4 times so that the total application time was 60 min. The pH values of solutions were measured with a digital pH meter (Sartorius, Göttingen, Germany).

2.6. Color measurement

Baseline and final color measurements were carried out before and after bleaching experiments. The color distributions (L^*, a^* and b^*) of each specimen were measured with a spectrophotometer (Spectrascan PR650, Photo Research, California, USA). A circular area with 1.0 mm in diameter was measured at the middle third region of the specimen. Before each measurement session, the spectrophotometer was calibrated with a white reflectance standard according to the manufacturer's protocol. A custom sample holder was used to position the specimens to insure that every measurement was on the same area of each sample.

The L^*, a^* and b^* color space system was defined by the Commission International de l'Eclairage in 1979 and is referred to as CIELAB (International Commission on Illumination, 1978). The L^* represents the value where white is 100 and black is 0. A positive a^* value indicates the red direction, a negative a^* value the green direction, a positive b^* value the yellow direction and a negative b^* value the blue direction. The difference between L^*, a^* and b^* at baseline and final of the experiment were expressed as ΔL^*, $\Delta a^*$and Δb^*. In addition, the overall color difference ΔE of the specimens in each group was calculated by the following expression:

$$\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$$

2.7. Microhardness test

Microhardness was measured with a hardness tester (HXD-1000TMC/LCD, Taiming Inc. Shanghai, China) with a load of 200 g for 15 s. Five baseline indentations were performed on each specimen and averaged. The indentations were made in five widely separated locations. The final measurements were made near the baseline indentations (space = 100 μm) to minimize interactions between neighboring marks. The percentage microhardness loss (PML) was calculated using the following calculation: PML (%) = $(VHN_{(B)} - VHN_{(F)})/VHN_{(B)}$, where $VHN_{(B)}$ is the average of the baseline microhardness measurements, and $VHN_{(F)}$ is the average of final microhardness values.

2.8. SEM analysis

To observe the morphological alterations of enamel surface after 60-min treatment, two specimens from each group were selected for SEM analysis after final microhardness measurements. Specimens were ultrasonic cleaned for 5 min with distilled water, then desiccated and sputter coated with gold in a vacuum evaporator (JEOL JFC1600, Japan). Micrographs of central areas were taken using a field emission scanning electron microscope (JEOL JSM-6700F, Japan).

To investigate the adherence ability of HA to the enamel surface, another four specimens were prepared. Two were treated the same as those in group DW + HA, the other two as those in group HP + HA. After final treatment, the specimens were washed only with running water (without cotton wool pellets or ultrasonic). Then these specimens were prepared for SEM observation.

2.9. Statistical analysis

Statistical analyses were performed with the use of SPSS 10.0 for WINDOWS. Normality was examined by using the Kolmogorov–Smirnov test, and homogeneity of variance was assessed by using Levene's test. One-way ANOVA was used for normally distributed data. This was followed by post-hoc multiple comparisons using Tukey's honestly significant difference test for cases with equal variances and Tamhane's T2 test for cases with unequal variances. Data were reported as the mean ± S.D. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of HA

The SEM micrograph of HA is shown in Fig. 1a. The precipitates are rod-like nanocrystals. The FTIR spectrum of the powder is showed in Fig. 1b. The bands at 1092, 1032, 962, 604, 564 and 470 cm^{-1} were assigned to vibrations of PO_4^{3-}, 1458, 1421 and 875 cm^{-1} to CO_3^{2-}, 3568 and 631 cm^{-1} to OH^- and 1638 cm^{-1} to water. The X-ray diffraction pattern of the precipitate was identified as HA and the peaks were in agreement with those in the standard HA diffraction pattern (Fig. 1c).

3.2. Color measurement

The L^*, a^* and b^* and ΔE values of the four groups are shown in Table 1. There was no significant difference on L^*, a^* and b^* values among the four groups at the baseline ($p = 0.996$,

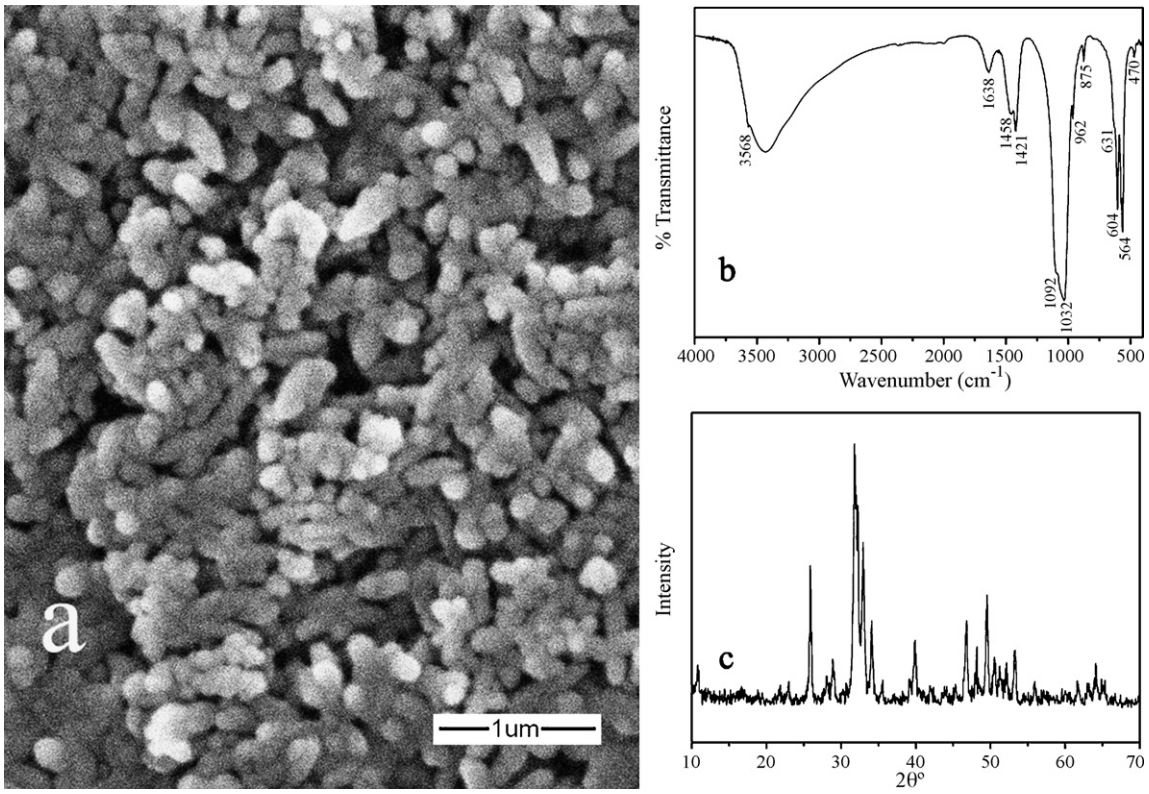

Fig. 1 – SEM micrograph (a), FTIR spectrum (b) and XRD pattern of the precipitate.

Table 1 – Mean values (standard deviations) of baseline and final L^*, a^* and b^* for each group and ΔE

Group	L^*		a^*		b^*		ΔE
	Baseline	Final	Baseline	Final	Baseline	Final	
DW	68.87 (1.71)	69.87 (1.40)	−0.61 (0.57)	−0.84 (0.55)	18.60 (2.02)	18.03 (2.28)	1.27 (0.63)[a]
HP	68.71 (2.42)	73.53 (2.73)	−0.30 (1.34)	−0.63 (1.09)	17.11 (3.14)	10.73 (2.30)	8.07 (2.32)[b]
HA + DW	68.68 (1.34)	68.95 (1.50)	−0.62 (0.42)	−0.87 (0.47)	18.29 (2.74)	19.23 (2.48)	1.70 (1.29)[a]
HA + HP	68.81 (3.08)	72.86 (2.86)	−0.23 (1.09)	−0.55 (0.93)	17.31 (3.94)	11.99 (3.84)	6.76 (3.14)[b]

Different superscripts (a and b) indicate mean values that are significantly different.

$p = 0.641$ and $p = 0.699$). The difference on ΔE value was significant among the four groups after 1 h treatment ($p = 0.000$). The ΔE of group HP and group HA + HP were significantly higher than that of group DW ($p = 0.000$ and $p = 0.000$) and group HA + DW ($p = 0.000$ and $p = 0.000$). There was no significant difference between group HP and group HA + HP ($p = 0.903$), and between group DW and group HA + DW ($p = 0.837$).

3.3. *Microhardness test*

The $VHN_{(B)}$, $VHN_{(F)}$ and PML of four groups are shown in Table 2. There was no difference on $VHN_{(B)}$ among four groups ($p = 0.905$). Significant difference on PML was found among four groups after 1 h treatment ($p = 0.000$). The PML of group HA + HP was significantly lower than that of group HP ($p = 0.047$), but significantly higher than that of group DW ($p = 0.000$) and group HA + DW ($p = 0.000$). There was no significant difference between group DW and group HA + DW ($p = 0.884$).

3.4. *SEM analysis*

Representative SEM micrographs of enamel surfaces in four groups after ultrasonic cleaned are shown in Fig. 2. The obvious variation of morphology was observed on enamel

Table 2 – Mean values (standard deviations) of baseline and final microhardness measurements for each group and the percentage of microhardness loss (%)

Group	$VHN_{(B)}$	$VHN_{(F)}$	PML (%)
DW	355.6 (15.6)	356.5 (20.1)	−0.3 (3.6)[a]
HP	357.6 (16.2)	329.3 (20.7)	7.9 (2.8)[b]
HA + DW	351.2 (31.0)	354.7 (25.5)	−1.2 (2.6)[a]
HA + HP	354.4 (19.0)	338.0 (17.5)	4.6 (3.0)[c]

$VHN_{(B)}$ is the average of the baseline microhardness measurements, $VHN_{(F)}$ the average of final microhardness values and PML percentage of microhardness loss. Different superscripts (a, b and c) indicate mean values that are significantly different.

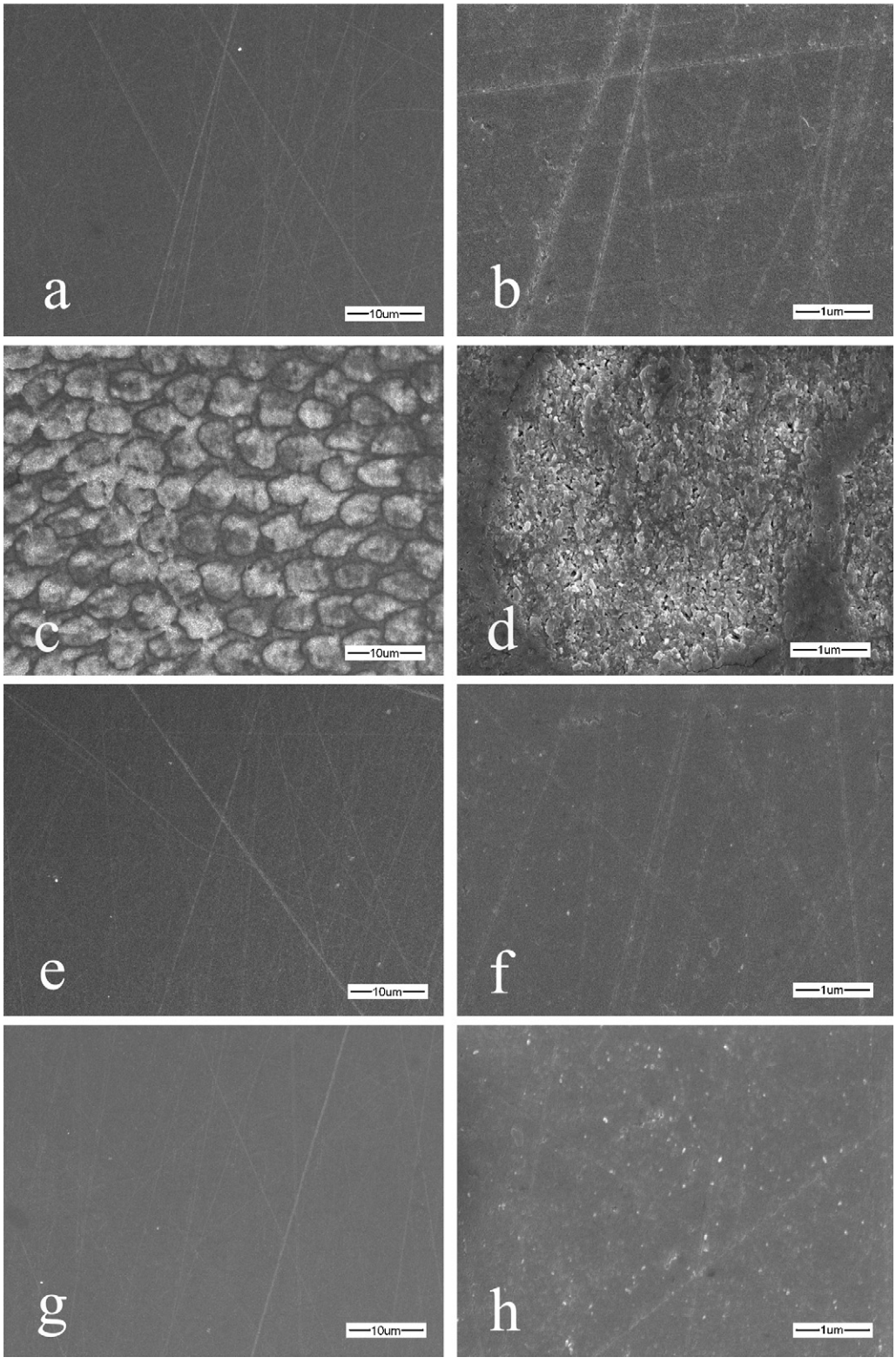

Fig. 2 – SEM micrographs of enamel after ultrasonic cleaning in group DW (a and b), group HP (c and d), group HA + DW (e and f) and group HA + HP (g and h) at 2000× (left) and 20,000× (right) magnifications.

surfaces in group HP when compared to the other three groups. It showed distinct structures of enamel, which included enamel rods and narrow interrods. Under higher magnification, the nanocrystals in rods and interrods became distinguishable from each other. No special alteration was found on the enamel surface in group HA + HP compared with those in group DW and group HA + DW under lower magnification. A smooth, flat and polished surface was

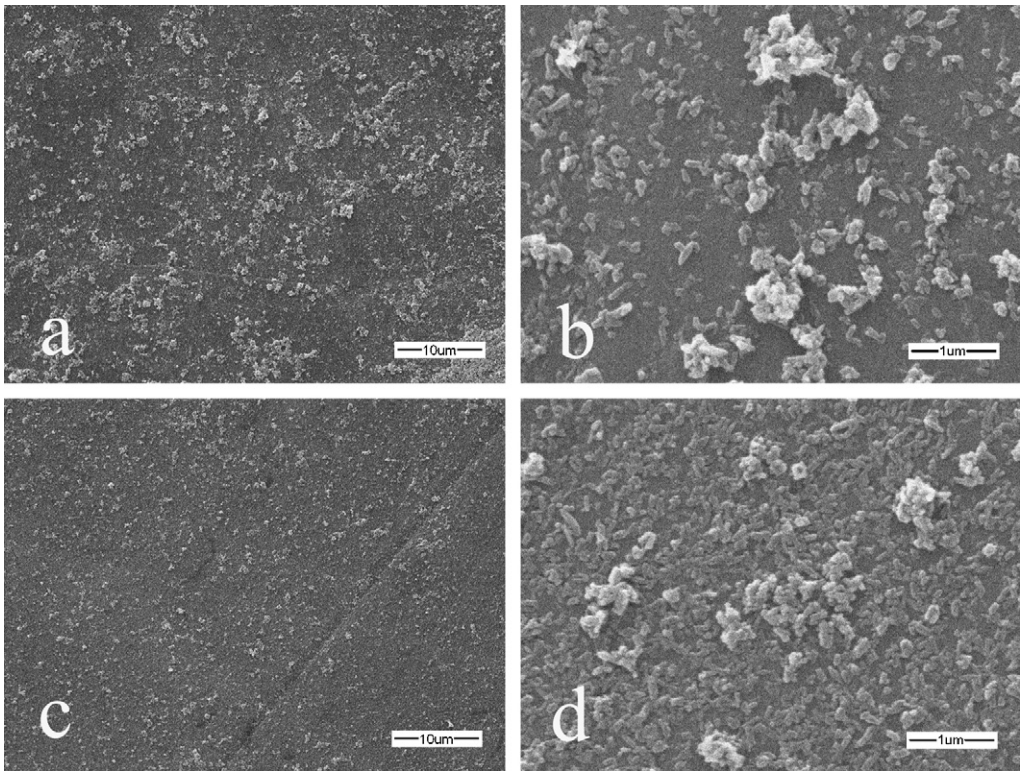

Fig. 3 – SEM micrographs of enamel after water cleaning in group HA + DW (a and b) and group HA + HP (c and d) at 2000× (left) and 20,000× (right) magnifications.

observed. Even under higher magnification, the enamel surface in group HA + HP showed little variations except some scattered nanoparticles presented.

The enamel surfaces of specimens treated with DW + HA or HP + HA and washed only with running water are shown in Fig. 3. Unlike those ultrasonic cleaned (Fig. 2e–h), the enamel surfaces were covered by a great number of HA particles. The SEM micrographs of the enamel surfaces in the two groups were similar except that the HA particles of group HP + HA spread more evenly than those of group DW + HA.

4. Discussion

Dental enamel is the most highly mineralized and hardest biological tissue. It is comprised of approximately 96% mineral, 3% water, and 1% organic matter (non-collagenous protein) by weight.[42] It is well known that mature human dental enamel crystals are carbonate-containing HA.[43] The SEM, FTIR and XRD results revealed that HA synthesized in the study was a nanosize carbonate-containing HA, which chemically and structurally resembled natural enamel.

The results of color measurement indicated that whitening effect of HA + HP was similar to that of the HP alone. We expected that HA + HP might bring better whitening effect. However, the results did not support our hypothesis. And the HA + DW did not show whitening effect either. Therefore, the whitening ability of HA + HP should be mainly attributed to the HP.

The results of microhardness test and SEM observation demonstrated that HP alone could result in significant microhardness loss and morphological change of enamel. These findings are completely consistent with our previous study[15] on the enamel exposed to 30% HP. It was suggested that morphological change of enamel is due to the demineralization caused by acidic HP, while microhardness loss to the combined effects of demineralization and destruction of organic matter by HP.[15]

The important finding in this study is that the combination of HA and HP could significantly reduce the microhardness loss of enamel and keep the enamel surface morphology almost unchanged. HA is an alkaline salt,[38] which increased the pH of HP solution (from ≈3.2 to ≈5.4) and made it less acidic. Furthermore, the HA particles adhered evenly to the enamel surface and formed a protective layer for the underlying enamel, which would lessen the direct contact of HP with enamel surface. And the solution around the enamel surface might soon become supersaturated with respect to enamel apatite.[37] All these effects of HA could lead to a great reduction in the enamel demineralization caused by HP.

It should be pointed out that there was still slight reduction in enamel microhardness in group HA + HP. Traditionally, microhardness loss means enamel demineralization has occurred.[44] However, the oxidation of enamel protein may also result in change of the mechanical properties.[15,19] Several papers have hypothesized that HP and CP cause changes to the organic component of enamel and dentin by altering of organic matrix or protein oxidation.[15,19,40,45,46] Although the

protein comprises only a minor part of enamel, it is contained in the spaces between mineral crystals, where it serves as a ''glue'' between crystallites.[47] It is reasonable to assume that the degradation of the ''glue'' will lead to the microhardness loss of enamel.

One limitation of this study was the use of the highly concentrated solution of HP. However, it had been chosen in many in vitro studies.[10,13,15,18] In present study, it was chosen instead of gel used in the clinical just because we wanted to explore the protective ability of HA under relatively rigorous condition.

Another limitation of the study is that the enamel surfaces were polished and flattened before bleaching. This procedure was performed to provide a more uniform surface to improve the precision of the indentations. However, it probably also removed the upper aprismatic surface layer from enamel, which is generally more highly mineralized than the subsurface and thus more resistant to demineralization.[48] For these reasons, we assume that the change of enamel with aprismatic surface layer would probably be less severe when treated by HP.

Some in vitro studies used artificial saliva or fluoride products between or after the treatments, for these elements are known to be an important factor to simulate clinical situations. However, the aim of this study was to investigate the protective effects of HA on the enamel surface subjected to HP. We did not employ these elements in order to prevent the influences of any other remineralization factors except HA. Nevertheless, it is necessary to involve these factors in the future studies to investigate the beneficial effects of HA under typical clinical conditions.

5. Conclusions

The 30% HP solution resulted in significant microhardness loss and morphological change of enamel. HA could significantly reduce the microhardness loss of enamel caused by 30% HP and keep the enamel surface morphology almost unchanged. However, combination of HA and HP could not bring better whitening effect than HP alone. The HA could be a potential biomaterial used for tooth bleaching.

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