Biomimetic tooth-whitening effect of hydroxyapatite-containing mouthrinses after long-term simulated oral rinsing

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Abstract: Purpose: To investigate the tooth-whitening effects of mouthrinses containing different sizes of hydroxyapatite (HAP) particles after prolonged application time and compare them with a commercial whitening mouthrinse. **Methods:** 50 bovine incisors were stained and randomly distributed into five groups: the HAP groups with 3 μm, 200 nm and 50 nm particle size, the commercial whitening mouthrinse group and the distilled water group. The teeth underwent prolonged mouthrinse applications that were equivalent to simulated 3- and 6-month mouthrinsing. Tooth color was measured and calculated before and after mouthrinsing. The group and application time effects were analyzed with a nonparametric analysis of longitudinal data using the nparLD package in R and ANOVA-type statistic was reported. Pairwise Wilcoxon rank-sum tests with BH correction were performed to compare the tooth color changes of individual groups. The mouthrinse-treated enamel was observed by SEM. **Results:** The whitening effect of HAP mouthrinses after the prolonged application time was confirmed. The HAP mouthrinses exhibited similar whitening effects to the commercial mouthrinses. The particle size and application time could significantly affect the whitening performance of HAP mouthrinses. The 50 nm HAP group exhibited significantly higher △E values than the 3 μm group after the 6-month-equivalent application ($P= 0.024$). A longer period of application increased significantly the △E and △L values ($P< 0.05$). The HAP-treated enamel surfaces were entirely covered with HAP after the 6-month-equivalent application. (*Am J Dent* 2021;34:307-312).

Clinical significance: The HAP nanoparticles showed better tooth-whitening performance after a longer period of mouthrinsing than the microsized HAP particles. This should be taken into consideration by dental manufacturers for optimizing the particle size for their HAP-containing products. To achieve a better outcome in tooth-whitening, the patients should apply the mouthrinse regularly for an extended period of time.

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Introduction

The portrayal of perfect white smiles in the media has increased the public self-awareness of discolored teeth. The desire and demand for whiter teeth have led to a growing need for oral whitening products. In response, over-the-counter (OTC) bleaching products soon became popular when they were introduced in the market at the beginning of the 2000s.[1] Hydrogen peroxide, the active agent in the OTC bleaching products, has potential adverse effects such as post-bleaching hypersensitivity, gingival irritation, alteration in enamel micro-hardness, and even genotoxicity and carcinogenicity.[2-4] Concerning those adverse effects, the EU Council Directive 2011/84/EU stated that only the products containing up to 0.1% of hydrogen peroxide were safe and could be sold as cosmetics to consumers without the supervision of dentists on 31 October 2012. However, this concentration might be too low to have a satisfying tooth-whitening effect.[5] For safety reasons, as well as the change of regulatory framework for the OTC bleaching products, non-peroxide alternatives are in urgent need.

Hydroxyapatite (HAP) is chemically equivalent to human tooth enamel and is widely used as a biomimetic material in various areas of dentistry because of its outstanding biocompatibility, e.g., in the treatment of dentin hypersensitivity, remineralization and repair of early caries, and prevention of periodontal diseases.[6-8] In the past decade, HAP has also been proven to be a promising tooth-whitening alternative by a series of laboratory and clinical studies[9-14] as it has no irritation effect on oral mucosa and does not change the enamel micro-structure during the whitening process.

The whitening mechanism of HAP dental products is that HAP particles could adhere seamlessly to the enamel surface and form a thin white layer,[15] which could increase light reflection and thereby make the tooth appear brighter.[11,16] Considering that smaller particles could exhibit stronger attachment and bonding ability to enamel due to their higher surface charges and larger contact area,[17] it is reasonable to assume that particle size might influence the HAP tooth-whitening effect. Indeed, this was confirmed by a study[10] showing that the HAP particles with a particle size smaller than 1 μm could lead to better whitening performance than the ones that were 1- 10 μm in size. However, it was reported[9] in a different study that the particle size of HAP had no influence on the whitening ability. So far, there is no consensus on the influence of HAP particle size on its whitening effect.

The use of a tooth whitening mouthrinse usually involves a daily mouth rinsing process and a long period of application. However, previous studies focused either on different application approaches where the HAP solutions were applied to the tooth surface with a cotton pellet[9,10] or on the instant whitening effect of HAP by applying it to the enamel with the application duration ranging from 1 to 9 minutes.[11,12,18] Although these studies confirmed the feasibility of the tooth-whitening effectiveness of HAP dental products, none of them investigated the whitening effect of HAP in a condition that is comparable to the real clinical condition. Therefore, studies that take a further step towards clinical reality and investigate a clinically relevant application protocol are needed.

To bridge this gap, the current study investigated the tooth-

whitening effects of mouthrinses containing different sizes of HAP particles after prolonged application time and compared them with a commercial peroxide-free whitening mouthrinse. The prolonged mouthrinse applications were equivalent to simulated 3- and 6-month mouthrinsing. Hopefully, these findings could provide information on the whitening effect of HAP mouthrinses after an extended (3-6 months) period of daily self-application and help the dental manufacturers make a rational decision on optimizing the HAP particle size in their products. The null hypothesis states that the HAP particle size does not influence the whitening effect of HAP mouthrinses.

Material and Methods

HAP suspension preparation – Mouthrinses, containing different particle sizes of HAP were prepared. The HAP materials used in the current study were provided by the companies Fluidinova and Budenheim free of charge. The median particle sizes of the HAP materials, according to the manufacturers' descriptions, are 3 µm, 200 nm and 50 nm, respectively. Each HAP material was mixed into water[a] to form a HAP suspension with a concentration of 10 wt%. The HAP mouthrinses were freshly made before every use.

Specimen preparation - A total of 50 extracted bovine incisors were randomly assigned to five groups (n=10 for every group): 3 µm HAP group, 200 nm HAP group, 50 nm HAP group, commercial whitening mouthrinse group (Listerine Advanced White[b]) and distilled water group.

Prior to treatment, the teeth were scaled with sound-driven oscillating instruments and carefully polished to remove the unwanted connective tissue. After thorough cleaning under running tap water, the teeth were stained artificially. The staining solution was prepared by dissolving six grams of instant coffee (Nescafe Espresso[c]) in 200 ml distilled, boiling water. The coffee solution was then centrifuged at 2,000×g/minute for 10 minutes (ROTIXA/A[d]). The supernatant after centrifugation was carefully selected, in which the samples were incubated for 3 days at 37°C. This incubation time simulated 3 months of coffee consumption.[19]

After removing the samples from the staining solution, the samples were polished with a polishing paste and cleaned with ultrasound for 5 minutes followed by blotting with an absorbent paper towel. Each sample was then embedded in a 3D-printed sample holder using a self-cured polyester material (Technovit 4000[e]). The sample holder had a measuring window aligned to the middle third of the tooth's labial surface.

Mouthrinsing simulation - Samples were mounted on the inner wall of a glass beaker, which contained the mouthrinse being tested. The mouthrinse was continuously stirred at the speed of 100 rpm to simulate the oral rinsing process. The glass beaker was sealed to avoid evaporation. Continuous exposure to mouthrinse for 12 hours is equivalent to 1 year of daily application (for 1 minute twice a day).[20] Therefore, in the present study, the samples were exposed to the mouthrinse regime for 3 and 6 hours to simulate the 3- and 6-month daily uses of mouthrinses. After the mouthrinsing process, the samples were gently rinsed with distilled water.

Color measurement - Sample color was measured with a spectrophotometer (Color Eye 7000[f]). To eliminate the interference of ambient light, the color measurement was performed in darkness. Before each measurement, the spectrophotometer was calibrated with a ceramic calibration tile. The color assessment was defined by Commission Internationale de l'Eclairage (CIE) L*a*b* color system, where L* is the axis of lightness from black (0) to white (100), a* is the axis from green (−) to red (+), and b* is the axis from blue (−) to yellow (+). The color of each sample was measured before and after the simulated mouthrinsing. The average color changes (△E values) were calculated using the following equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

where △L, △a, and △b are the differences in lightness, greenness-redness, and blueness-yellowness between before and after oral rinsing. The repositioning and reproducibility of color measurement were strictly controlled with the help of a 3D-printed position locator. The enamel surface was blotted with a soft paper towel to remove the excess liquid and kept moist before the color measurement.

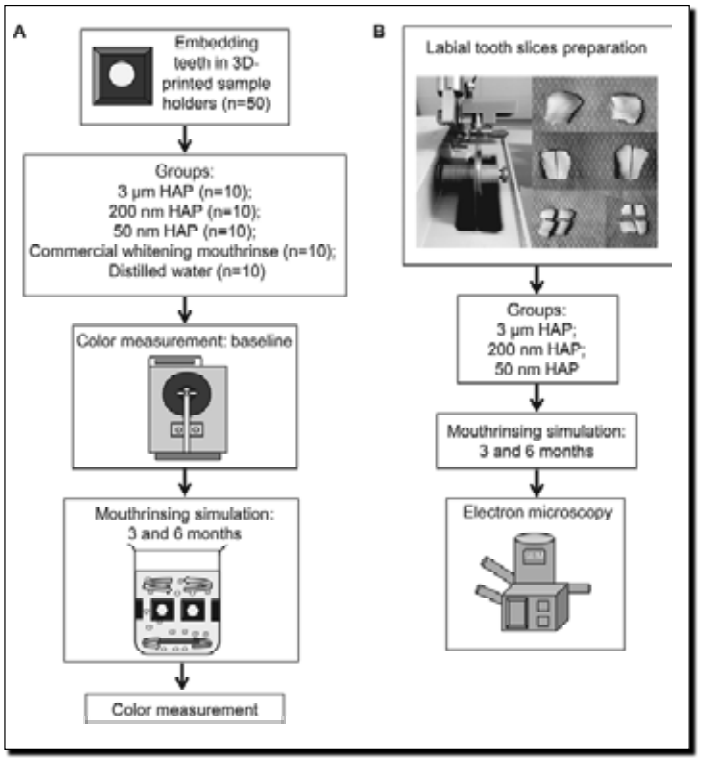

Fig. 1. The workflow of the present study. **A:** color assessment; **B:** SEM evaluation.

SEM evaluation - Bovine incisors were randomly selected, and were firstly cut along the median line into two parts. Only the labial tooth slices were chosen and randomly assigned to the three HAP groups. After the 3-month-equivalent and 6-month-equivalent mouthrinsing, the enamel surfaces were observed by field-emission scanning electron microscopy (FE-SEM, Supra 55vp[g]). After dehydration in increasing concentrations of ethanol, the specimens were sputter-coated with a 25 nm Au film using Au plasma (8*10-2 Pa, 20 mA, argon) in a vacuum evaporator (Polaron SC7620 Mini Sputter Coater[h]). Subsequently, the enamel surfaces were examined at the magnification of 1,000× and 10,000×. The workflow of the present study is presented in Fig. 1.

Statistical analysis - The data were analyzed in R. A non-parametric analysis of longitudinal data using the nparLD

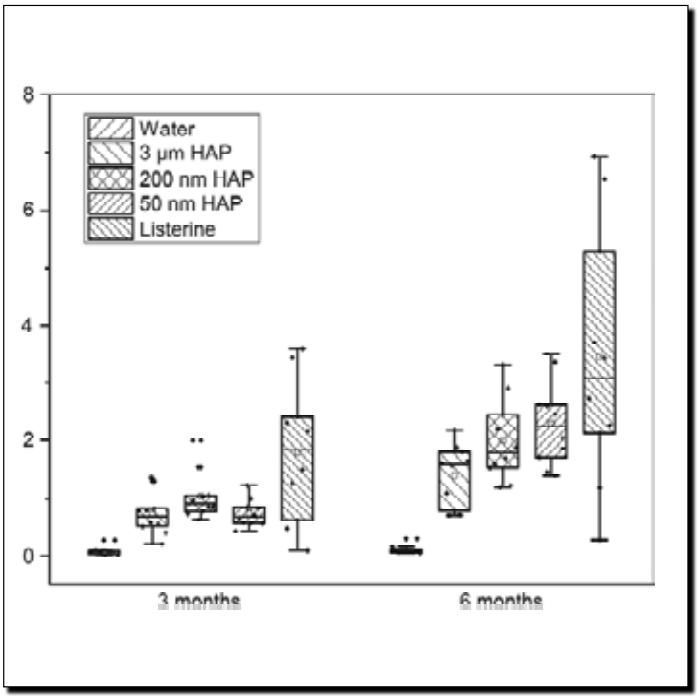

Fig. 2. The median and 95% confidence interval of △E values after 3- and 6-month-equivalent application.

package with the F1-LD-F1 design was performed to determine the group effects, the time effects and their interactions. When an interaction between the two main effects was found, a Kruskal-Wallis test was applied to confirm the group effect. Pairwise Wilcoxon rank-sum tests with BH correction were performed to compare the tooth color changes of individual groups. Paired Samples Wilcoxon tests were used to compare the color changes between the 3-month-equivalent and 6-month-equivalent applications. Statistical significance was set for all tests at $P< 0.05$.

Results

Color assessment - The ANOVA-type statistic revealed significant main effects of group and application time for the △E, △L, △a and △b values ($P< 0.00001$). Significant interactions were found between the two main effects for the △E and △L values ($P< 0.01$). The Kruskal-Wallis test confirmed the group effect on the △E and △L values ($P< 0.01$).

The △E values of each group were shown in Fig. 2. The commercial mouthrinse group exhibited the highest △E median values after the 3-month-equivalent (1.83) and the 6-month-equivalent (3.07) HAP applications. In contrast, the water group showed the lowest △E median values (0.05 and 0.08).

After the 3-month-equivalent HAP application, the △E median values of the 3 µm, 200 nm and 50 nm HAP groups were 0.67, 0.91 and 0.67, respectively. No significant differences in the △E values were found between individual HAP groups. After the 6-month-equivalent HAP application, the △E values of each HAP group increased significantly to 1.59 ($P= 0.027$), 1.79 ($P= 0.002$) and 2.25 ($P= 0.002$), respectively. In addition, the 50 nm HAP group exhibited significantly higher △E values than the 3 µm group ($P= 0.024$).

The tooth color changes of the HAP groups and commercial mouthrinse group were produced by the increased L* values and decreased a* and b* values. The median values and 95% confidence interval of the △L, △a, and △b values of each group were summarized in the Table. After the 6-month-equivalent HAP application, the △L values of HAP groups were significantly higher than those after the 3-month-equivalent application ($P< 0.05$). After the 6-month-equivalent application, the commercial mouthrinse group showed significantly higher △L values than the 3 µm HAP group ($P< 0.05$) but not the other two HAP groups.

Table. The color changes in L*, a* and b* axes (expressed as ΔL, Δa, and Δb values) at 3- and 6-month-equivalent applications. Results are shown as median value (95% confidence interval).

Groups	3 months	6 months
ΔL		
3 µm HAP	0.50 (-0.48, 0.68)a	1.07 (0.12, 1.92)b*
200 nm HAP	0.40 (-0.22, 1.21)a	1.38 (0.44, 3.27)ab*
50 nm HAP	0.34 (-0.43, 0.81)a	1.52 (-1.24, 3.43)ab*
Water	-0.01 (-0.06, 0.08)b	0.03 (-0.13, 0.08)c
Commercial mouthrinse	1.12 (-0.26, 2.49)a	2.25 (0.07, 4.36)a*
Δa		
3 µm HAP	-0.06 (-0.25, 0.38)a	-0.01 (-0.5, 0.52)ab
200 nm HAP	-0.16 (-0.31, 0.20)a	-0.45 (-0.63, -0.08)ab*
50 nm HAP	-0.07 (-0.30, 0.06)a	-0.21 (-0.65, 0.16)ab
Water	0.00 (-0.05, 0.04)a	0.01 (-0.07, 0.10)a
Commercial mouthrinse	-0.14 (-1.70, 0.06)a	-0.23 (-1.28, 0.07)b
Δb		
3 µm HAP	-0.35 (-1.18, 1.14)b	-0.86 (-1.49, 0.30)b*
200 nm HAP	-0.85 (-1.71, -0.12)c	-1.13 (-2.40, 0.31)b
50 nm HAP	-0.53 (-1.16, 0.08)c	-1.09 (-2.28, 1.12)b
Water	0.02 (-0.04, 0.27)a	0.03 (-0.07, 0.30)a
Commercial mouthrinse	-1.03 (-3.02, -0.09)c	-1.67 (-5.26, 0.33)b*

Lowercase letters indicate the differences between different groups with the same application time.
* indicates the statistical differences of the same group between the 3- and 6-month-equivalent application.
Superscripts of individual letters indicate groups that statistically differed from any other group ($P< 0.05$).
Shared superscripts suggest that groups showed no statistical differences ($P> 0.05$).
From a to c, the median value is decreased.

No significant difference in the △a values was found between the individual HAP group and commercial mouthrinse group. After the 3-month-equivalent application, the 3 µm HAP group exhibited significantly lower △b values than the other two HAP groups ($P< 0.05$).

SEM evaluation - After the 3-month-equivalent application, HAP agglomerates were identified on the enamel surfaces in all HAP groups (Figs, 3B-D, G-I). In the 3 µm group, most of the adhered HAP agglomerates were less than 1 µm (Figs. 3B and G), which were smaller than the median particle size (3 µm). The size of HAP agglomerates in the 200 nm HAP was similar to those in the 3 µm group (Figs. 3C and H). The 50 nm group exhibited a uniform size of HAP agglomerates, which connected with each other and distributed evenly on enamel (Figs. 3D and I). The enamel surfaces of the three HAP groups were not totally covered by HAP agglomerates. The enamel of the commercial mouthrinse group (Figs. 3E and J) seemed to have similar surface morphology to the untreated enamel (Figs. 3A and F).

After the 6-month-equivalent application, the enamel surfaces of all HAP groups were completely covered by thin layers of HAP (Figs. 3L-N, Q-S). Some of the HAP agglomerates of the 3 µm group grew larger and reached the

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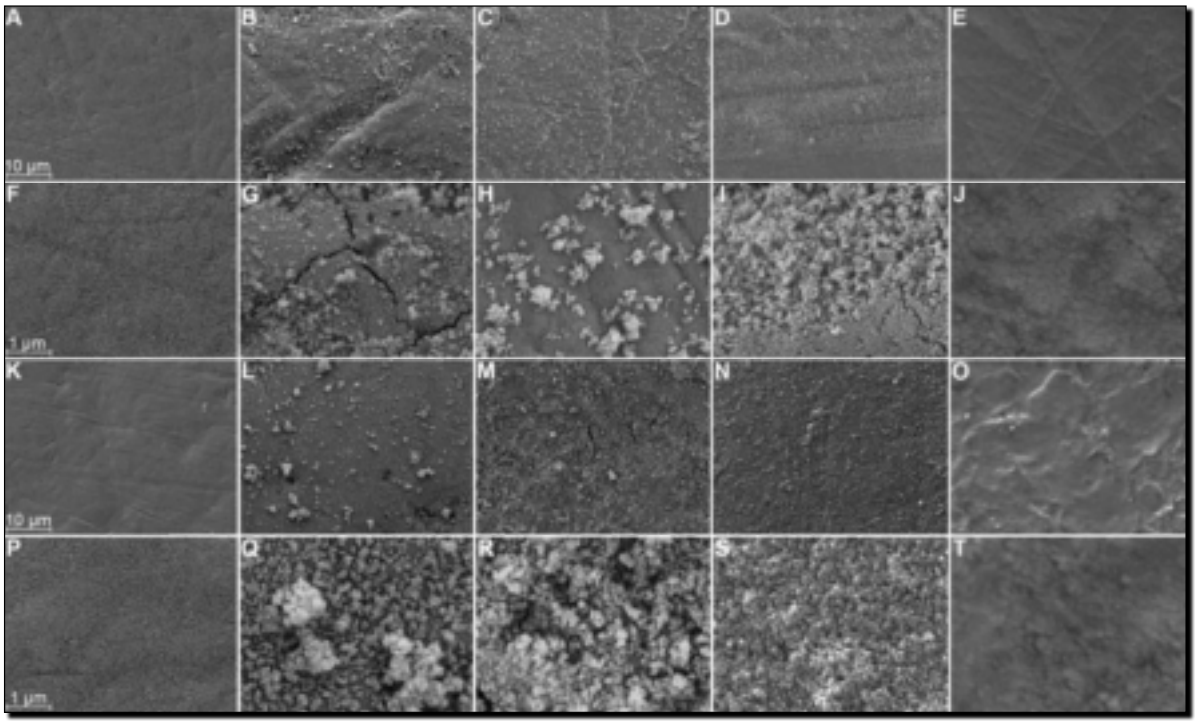

Fig. 3. The mouthrinse-treated enamel surfaces were visualized at 1,000× and 10,000× after the 3- and 6-month-equivalent applications.
A and F: untreated enamel surface at 1,000×.
K and P: untreated enamel surface at 10,000×.
B-E: the enamel of the 3 µm, 200 nm, 50 nm HAP groups and the commercial mouthrinse group at 1,000× after the 3-month-equivalent application.
G-J: the enamel of the 3 µm, 200 nm, 50 nm HAP groups and the commercial mouthrinse group at 10,000× after the 3-month-equivalent application.
L-O: the enamel of the 3 µm, 200 nm, 50 nm HAP groups and the commercial mouthrinse group at 1,000× after the 6-month-equivalent application.
Q-T: the enamel of the 3 µm, 200 nm, 50 nm HAP groups and the commercial mouthrinse group at 10,000× after the 6-month-equivalent application.

size of up to 3 µm in length (Figs. 3L and Q), which made the enamel surface seem to be rougher than the other two HAP groups. The HAP film of the 50 nm group seemed to be the least rough (Figs. 3N and S). No adhesion was observed on the enamel of the commercial mouthrinse group (Figs. 3O and T).

Discussion

HAP is a widely used biomimetic material in OTC dental products for the treatment of dental hypersensitivity and enamel demineralization. In their slogans, the "tooth-whitening effect" is often mentioned, but no studies, so far, have examined the whitening effect of HAP after prolonged application time. Here we show that the HAP mouthrinses exhibited similar tooth-whitening effects to the commercial mouthrinse throughout the observation period. We also found that the particle size and application term could significantly affect the whitening effect.

A strength of the present study is that we took a further step towards clinical reality. We applied HAP mouthrinses by the simulated mouthrinsing, which enabled us to evaluate the whitening effect of HAP mouthrinses by self-application. Second, we used pure HAP aqueous suspensions as the mouthrinses. This allowed us to investigate the whitening effect of HAP without interference from other mouthrinse additives, such as flavors, preservatives, and rheology modifiers, which may have an impact on the HAP whitening effect. Third, the vital tooth- and measurement-related interferences factors were strictly controlled. The labial middle third of the tooth was chosen for our research, which represented the best tooth color.[21] As the native tooth surface is multilayered and exhibits color transitions in all directions,[22] a reproducible positioning is crucial for accurate color measurements. We created a 3D-printed repositioning system to achieve precise repositioning. On the other hand, the study also had limitations. As with any other in vitro study, we could not reveal the influence of the complicated and non-predictable oral environment (such as variations of temperature, pH value, or nutritional habits, etc.) on the whitening effect of HAP.

The $\triangle E$ values after the 3- and 6-month-equivalent applications did not statistically differ between the commercial mouthrinse and individual HAP mouthrinse. Increased L* values and decreased a* and b* were observed in all HAP groups and the commercial mouthrinse group, indicating that both HAP and commercial mouthrinses could change the tooth color to a bright, green and blue tone. The HAP mouthrinses had lower median $\triangle E$ values but relatively narrower 95% confidence intervals than the commercial one, indicating the HAP mouthrinses had a relatively stable whitening effect. This finding may be explained by the different whitening mechanisms. According to the manufacturer, the commercial mouthrinse in the current study is non-abrasive and contains polyphosphate, which can dissolve the pigment molecules and prevent their deposition on enamel surfaces.[23,24] Therefore, the whitening performance might vary from tooth to tooth as each tooth has a different amount of pigment. Instead of dissolving pigments, the whitening effect of HAP products is caused by the adhered HAP particles, which could attach firmly to enamel

surfaces and change the light propagation, making the tooth appear whiter.[10,25]

Understanding the relationship between HAP particle size and adhesion behavior is crucial for optimizing the tooth-whitening performance of HAP whitening products. Fabritius-Vilpoux et al[26] found that the attachment of HAP to enamel surfaces relied largely on the ratio between the adhesive forces and the mass of HAP particles and that large particles tended to fall off when the sample was being washed. This finding was confirmed in the current study. After the 3-month-equivalent application, the sizes of the adhered HAP agglomerates in the 3 μm group (Figs. 3B and G) were smaller than 1 μm which suggested that smaller particles attached more strongly to the tooth surfaces compared with larger ones. The sizes of the HAP agglomerates in the 200 nm group (Figs. 3C and G) were similar to that in the 3 μm group after the 3-month-equivalent application, which might be the reason for the non-significance in the △E values. After the 6-month-equivalent application, the agglomerates in the 3 μm group grew larger (Figs. 3L and Q) and made the HAP-treated enamel surface rougher than the 50 nm group (Figs. 3N and S). In response, significantly higher △E values were found in the 50 nm HAP group compared with the 3 μm one. We assumed that the difference in the roughness of the adhered HAP layers could affect the light propagation on or through the tooth tissue and thereby change the tooth color appearance. However, no significant difference in the △E values was found between the 200 nm and 50 nm HAP groups. This result is consistent with a previous study, in which the researchers reported that the HAP particles with the length of 60-100 nm and 100-200 nm had similar whitening abilities.[27]

In the present study, prolonged application times led to increased △E and △L values of all HAP groups. The researchers of the previous studies, which focused on the HAP instant whitening effect, believed that once the enamel surface was covered with the maximal adhering HAP load, reapplication would not result in any further color changes.[10,12] They reported the maximal HAP adherence could be achieved within three cycles of application. In their studies, the HAP materials were mechanically rubbed on the teeth with the application term ranging from 30 seconds to 3 minutes. In contrast to these studies, we did not observe the maximal HAP adhering load. This suggested a different adhesion behavior for HAP when it was applied in a hydrodynamic environment induced by the simulated oral rinsing.

After the 6-month-equivalent HAP application, the △E values of the 3 μm, 200 nm and 50 nm HAP groups, compared with the values from the 3-month-equivalent application, increased significantly to 1.59, 1.79 and 2.25, respectively. Given that the △E values of higher than 1.7 could be detected by observers,[28] the tooth color changes in the 200 nm and 50 nm group after the 6-month-equivalent application were considered visually perceptible. Comparing to the tooth color changes caused by prolonged HAP applications, the previous 9-minute short-term approaches showed similar △E values, ranging from 0.91 to 2.20.9.[10] It is worth mentioning that in these studies the teeth were air-dried before the color measurement. Considering that tooth dehydration could lead to a decrease in enamel translucency and an enhancement in luminosity and therefore result in a false whiter appearance of a tooth,[29] the tooth color changes in these previous in vitro studies might be overestimated. In our study, we kept the teeth moist before the color measurement and conducted the measurement as quickly as possible to avoid the undesirable influence of dehydration[30]

The increase in the L* values has been proven to be due to the enhanced light reflection at the HAP-adhered tooth surface.[11,31] The SEM images showed that the enamel surfaces of all HAP groups were entirely covered by adhered HAP layers after the 6-month-equivalent mouthrinsing, whereas the enamel surfaces were not completely covered after the 3-month-equivalent HAP application. We assumed that more light was reflected from the 6-month-equivalent HAP-treated enamel surface and therefore made the tooth appear brighter.

The adhered HAP layers observed in the present study might protect the tooth surface from acidic attacks as the layers would interact initially with the acid.[10] From a chemical point of view, the adhered HAP layers would be dissolved in the acidic environment, resulting in the reduction of the whitening effect. However, researchers observed the HAP particles could adhere to enamel even in an acidic solution,[15] which suggested that the acidic condition might not affect the whitening ability of HAP materials. Studies, which are designed to evaluate the HAP whitening effect with complicated pH value changes in the oral environment, are needed in the future. To further augment the whitening effect of HAP mouthrinse, non-oxidizing, blue-toned agents (such as blue covarine) could be combined, which might provide teeth a yellow to blue hue color shift.[32]

In summary, this study confirmed for the first time the tooth-whitening effect of prolonged application of HAP mouthrinses. After the 3- and 6-month-equivalent mouthrinsing, the HAP mouthrinses exhibited similar tooth-whitening effects to the commercial whitening mouthrinse. It was also noticed that the tooth-whitening performance of HAP was dependent on the particle size and application time. After the 6-month-equivalent application, the 50 nm HAP mouthrinse showed significantly higher color changes than the 3 μm one. This finding should be taken into consideration by dental manufacturers for their HAP containing dental products. To achieve a better outcome in tooth-whitening, the patients should apply the mouthrinse regularly for a longer period of time.

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