Effect of Nano Hydroxyapatite in Toothpaste on Controlling Oral Microbial Viability

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Abstract. The aim of this study is to examine the effect of nano hydroxyapatite in the toothpaste and its effect on the pH and microbial activity. Nano-hydroxyapatite (nHAp) is considered one of the materials that have high biocompatibility for biomimetic material due to its chemical and morphological similarity with dental apatite. Additionally, it has been documented to possess antibacterial potentials. The present study was conducted to identify the relationship between oral microenvironment pH change and its role in the Streptococcus mutans viability, a common pathogen in the oral cavity. Change in pH is closely related to number of Streptococcus mutans as the main cariogenic organism and acid-producing bacteria. The study was carried out using 0.25%, 0.7%, and 1.5% concentration of HAp in toothpaste formulation and commercial toothpaste as control. Our studies showed that the most significant pH fall was observed in 1.5% and 0.7% nHAp. However, in 60 minutes, all nHAp groups were able to restore pH into neutral, especially in the 0.7% nHAp, which reached a pH of 7. As a comparison, the commercial toothpaste only returned to 6 within 1 hour. Experimental method of this study is In vitro oral microenvironment pH and microbiological analysis on teeth fragment. The detailed data about microstructure and antibacterial activity will be presented.

Keywords: Nano-hydroxyapatite, Streptococcus mutans, Dental, Toothpaste Formulation.

1. Introduction
Dental caries is the most common oral biofilm-associated disease caused by a complex interaction between cariogenic bacteria, hosts (saliva and teeth), and carbohydrate consumption [1]. The important factor that determines the onset of dental caries is the presence of Streptococcus mutans as the major cariogenic bacteria. S. mutans is acidogenic bacteria which has the ability to ferment carbohydrate into acid. By the time, the acid will accumulate then diffuse through the pores of enamel and dentin tubules. The acid condition could dissolve the mineral content both in dentin and enamel, thus demineralization process occur. For a prolonged time, the cavity is formed in the teeth [2]. Actually, the caries process...
consists of the dynamic continuous cycle between demineralization and remineralization. Remineralization is the natural repair process where calcium and phosphate ions deposited back into enamel or dentin. Remineralization can occur in nearly neutral conditions (> 5.5), while demineralization happens in an acid environment (< 5.5). However, in caries cases, demineralization is dominated by the remineralization process, thus leading to tooth decay [1-3].

Rajesh (2015) in his study had described the critical condition for demineralization of teeth is in acidic pH (< 7), in which a solution is saturated with a particular mineral [4]. Naturally, saliva plays a major role in recovering pH from the acid condition into neutral. Saliva also serves as a calcium and phosphate ions to repair ion loss in the enamel. Moreover, the ability of saliva is dependent on the quantity of secretion, systemic disease, and saliva flow rates [5]. Therefore, to help saliva roles in enhancing the remineralization process, fluoride is often used. However, it is important to consider that inappropriate fluoride application could cause enamel fluorosis.

Nano-hydroxyapatite (nHAp) is considered one of the materials that have high biocompatibility for bone tissue engineering and dental implant. Due to its chemical and morphological similarity with dental apatite, nHAp is widely used as biomimetic material to repair enamel or dentin defects. Hydroxyapatite could induce enamel remineralization through formation of a new layer of HAp, thus promote calcium and phosphate deposition [6]. Recently, some denitrifies has contain nHAp in their formulation, like Sangi Co. (Japan). However, to our knowledge, there is no information about hydroxyapatite effect in helping salivary pH recovery to support enamel remineralization process. The aim of this study is to examine the effect of nano hydroxyapatite (nHAp) in the toothpaste by concentration of nHAp 0.25, 0.70, and 1.5 % to the salivary pH and microbial activity.

2. Experimental method

2.1. Sample preparation
Bovine molar teeth were purchased from the slaughterhouse. The teeth were cleaned from soft tissue and debris, then the crown portion was sectioned to separate from the root, using handpiece and diamond disc. After that, the crown portion was cut into 4 × 4 mm pieces with ± 1.5 mm thickness. Teeth fragment were covered with acid-resistant nail varnish, except for enamel surface, then resulting 16 mm² open areas. Each enamel surface of the specimen was polished using sandpapers to remove the disturbance area. Then, teeth fragment was stored in 40°C prior to use. The fragment teeth were treated by the toothpaste that contains nHAp of 0.25, 0.7 and 1.5%. The samples were named 0.25nHAp, 0.7nHAp and 1.5nHAp, respectively. For comparison purpose, the fragment teeth were also treated by a commercial toothpaste (CT), distilled water (K-) and ampicillin (K+).

2.2. In vitro oral microenvironment pH and microbiological analysis
Teeth fragment was put on 96-well plate, then coated with artificial saliva for 90 minutes at 37°C. After removing unbound saliva, 200 μL of Streptococcus mutans ATCC 25175 (2 × 10⁷ CFU/mL) in Brain Heart Infusion (BHI, Oxoid Limited, Hampshire, UK) broth supplemented with 1% sucrose (Himedia Laboratories, Mumbai, India) was added. Biofilms were allowed to growth for 24 hours at 37°C, anaerobically (10% CO₂, 80% N₂, 10% H₂). After 24 hours of incubation, bacteria suspension was discharged, then the biofilm was gently washed using Phosphate Buffer Saline (PBS). The dental plaque formed in the teeth was treated with 200 μL of slurry toothpaste, correspond with the groups. Then, treated-dental plaque was incubated for 1 minutes at rotary shaker (85 rpm). Subsequently, teeth fragment was moved into 1.5 mL microtube filled with BHI broth + 1% sucrose. pH measurement was done using pH strip test, every 10 minutes in 1 hour. Furthermore, the total of Streptococcus mutans bacteria in dental plaque also counts using total plate count method at the end of pH measurement.
2.3. Remineralization capability
Fragment teeth were immersed in 20 mL demineralizing solution (2 mmol/L Ca\(^{2+}\), 2 mmol/L PO\(_4^{3-}\), 0.075 mol/L acetate) for 4 days at 37° C, to produce artificial carious lesions. After 4 days, remineralization procedure using artificial saliva and toothpaste sample was done for 14 days. A three minutes treatment with toothpaste was given twice daily (08.00 AM and 15.00 PM) during remineralizing step. Furthermore, fragment teeth were cleaned using toothbrush under distilled water, then stored again in artificial saliva at 37° C. Artificial saliva was changed every 48 hours. Finally, on the last day, fragment teeth were washed with deionized water, dried in room temperature, then observed the enamel surface under Scanning Electron Microscope (FEI Quanta 650) with an Oxford INCA/ENERGY-350 microanalysis system. Furthermore, quantification of hole area in the enamel surface was done using image processing with software ImageJ.

3. Results and discussion
Figure 1 shows the pH of dental plaque pH for 60 minutes. All treatment groups were experienced a pH decrease after 20 minutes. Significant pH fall was observed in toothpaste with the addition of 0.25, 0.7, and 1.5 nHAp. However, in 60 minutes, all nHAp samples were able to return the pH into neutral condition, showing the pH of 7. In particular of 0.7nHAp, the teeth that treated with this condition able to restore the pH to 6.7 in 50 minutes, which is the highest pH among all samples. On the other hand, CT samples only returned the pH to 6 within 1 hour, which is lower compared to nHAp samples. Indeed, the K- samples has a trend that continues to decline until reach pH 5 after 1 hour, while K+ tends to be stable in the range of 7 to 6.5. Carbohydrate fermentation by oral bacteria will result an organic acid as the final product via the glycolytic pathway [7-9]. Acidic conditions in tooth enamel can cause demineralization, because hydroxyapatite as the main component of enamel has a high solubility in acid solutions. Therefore, maintaining oral microenvironment pH in the neutral condition is an important method to prevent enamel demineralization [10,11]. According to the results are shown in Figure 1, nHAp is capable to to restore oral microenvironment pH back to neutral in order to promote enamel remineralization.
Table 1. Relationship between pH value and number of Streptococcus mutans after 60 minutes.

|            | 0.25nHAp | 0.7nHAp | 1.5nHAp | CT | K+ | K- |
|------------|----------|---------|---------|----|----|----|
| pH value   | 7        | 7       | 7       | 6  | 6.5| 5  |
| Number of  | 2.46×10^6| 6.67×10^4 | 5.56×10^5 | 6.67×10^4 | 1.26×10^7 | 3.50×10^8 |
| bacteria (CFU/mL) |          |         |         |    |    |    |

Figure 2. SEM images of the enamel surface after 14 days treatment (a) 0.25nHAp, (b) 0.7nHAp, (c) 1.5nHAp, (d) CT and (e) K-. 
Change in pH is closely related to number of Streptococcus mutans as the main cariogenic organism and acid-producing bacteria. Table 1 shows the number of Streptococcus mutans and pH after 60 minutes. It should be noted that the amount of Streptococcus mutans in the nHAp samples was lower compared to K+ and K- samples. Although the final pH of nHAp samples after 60 minutes was similar, the total Streptococcus mutans is difference. As shown in Table 1, the total Streptococcus mutans for 0.25nHAp, 0.7nHAp and 1.5nHAp was $2.46 \times 10^6$, $6.67 \times 10^4$ and $5.56 \times 10^5$ CFU/mL, respectively. This difference is more likely corresponding to pH after 50 minutes, as shown in Figure 1. 0.7nHAp has the lowest Streptococcus mutans since its has highest pH after 50 minutes, while 0.25nHAp showed the lowest pH among the nHAp samples, therefore, the amount of Streptococcus mutans is the highest compared to other nHAp samples. The total number of Streptococcus mutans in the 0.7nHAp is equal with the CT samples, despite the fact that they have difference pH number. In contrast, K+ and K- samples revealed the highest amount of bacteria Streptococcus mutans, even though in the K+ treated by ampicillin.

The observed pH dynamics graph consisted of several stages, as shown in Figure 1. The first stage, which is in the 0 to 10 minutes, is the stage of adaptation or resting phase of Streptococcus mutans, so that there is a slightly change in pH. Furthermore, in the 10 - 40 minutes, Streptococcus mutans is in the active stage that metabolizes sucrose, which was indicated by the pH begins to fall. In the K+ samples, there was a decrease in pH, however, the decrease of pH is lower compared to the toothpaste with nHAp samples. The third stage is the recovery stage where the pH starts return to normal, which is observed in 40 - 60 minutes, especially in the nHAp toothpaste group. This result is in agreement with the other reports [12].

Generally, the recovery stage is covered by the role of saliva as buffer capacity [5]. However, in this research, the buffer capacity is performed by bacterial medium and nHAp, which bound in the enamel surface. Under acidic conditions (pH ≤ 5.5) hydroxyapatite begins to dissolutions and release its ions, following the Equation (1) [13-15]:

$$\text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 \rightarrow 10\text{Ca}^{2+} + 6\text{PO}_4^{3-} + 2\text{OH}^-$$  \hspace{1cm} (1)

Calcium ions reacts with water molecules to form Ca(OH)$_2$ which increases the pH. Moreover, phosphate ions also can help a pH buffer [8, 16]. The buffer capacity is not observed in K- and K+. In
the K- and K+, the pH was observed decreasing gradually within 1 hour. This is because the acid production and growth of remaining bacteria in K+ and K-, which was shown in Table 1.

Figure 2 shows the SEM images of the enamel surface after 14 days treatment using nHAp, CT and K-. Indeed, the enamel surface of K- samples (Figure 2 (e)) showed rough surface, which might be from the demineralization since the pH of this samples is very low and had the large number of Streptococcus mutans. Surprisingly, the CT samples (Figure 2 (d) showed almost no difference compared to K- samples (Figure 2 (e)) in term of porosity and roughness. It seems that CT samples could not inhibit enamel demineralization activity after 14 days, showing very rough surface of enamel, as shown in Figure 2 (d). In fact, the pH and the number of bacteria of CT samples is comparable with nHAp samples after 60 minutes, however, for long treatment time, CT samples could not maintain the optimum condition for remineralization. In contrast, the number of porosities of the demineralized area decreased along with the increasing of nHAp content (Figure 2 (a) – (c)), showing a better appearance compared to K- and CT samples after 14 days treatment.

Anti-bacterial activity of nHAP is supported by the ability to recovery salivary pH to prevent the growth of S. mutans and inhibit adhesion of bacteria on the tooth surface [17-20]. Hydroxyapatite in nanosize is also considered as a remineralizing agent due to its morphology and chemical features more similar to biological apatite [21,22]. The application of nHAP as toothpaste may produce the outermost layer that serves as a barrier and protect the enamel against acid. nHAp will penetrate through enamel lesion and serve as a template to attract Ca2+ and PO43- from surrounding environment. As the nHAp concentration increased, the rate and amount of Ca2+ and PO43- deposition will increase [23,24]. This can be observed in 1.5nHAp, where the surface had minimal pores area, as shown in Figure 2 (c). Quantitatively, the holes area in 1.5nHAp and 0.7nHAp is not significantly different (Figure 3).

4. Conclusion
The effect of nHAp in the toothpaste on the pH and microbial activity was successfully investigated in this study. The results showed that the addition of nHAp in the toothpaste improve the ability to recover the pH in the first of 60 minutes. The pH of all nHAp samples after 60 minutes were 7, while the CT samples only 6. Moreover, the pH is correlated with the number of Streptococcus mutans bacteria, which showed the number of the bacteria is lower in the teeth that treated by nHAp compared to K+ and K-samples. The number of bacteria in the 0.25 nHAp, 0.7 nHAp and 1.5 nHAp was 2.46×10⁶, 6.67×10⁴ and 5.56×10⁵ CFU/mL, respectively. Moreover, the treatment of the enamel surface for 14 days revealed that the nHAp prevent the damage of the enamel compared to the CT or K- samples. The number of porosities in the nHAp samples lower compared to samples without the addition of nHAp, indicating the effectiveness of nHAp to enhance the remineralization of the teeth by maintain the pH and the number of bacteria.

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