The effect of nano-hydroxyapatite toothpaste on artificial enamel carious lesion progression: an in-vitro pH-cycling study

ABSTRACT

Objectives. To test whether the modified in-vitro pH-cycling model would work successfully as a means of investigating the de-/re-mineralization effects of nanoparticle hydroxyapatite toothpaste, and to compare such effects with those of sodium fluoride toothpaste. Methods. Sound extracted molars were painted, leaving a 1-mm wide window, and placed in a demineralizing solution for 96 hours to produce 100 to 120 µm deep artificial carious lesions. The teeth were sectioned longitudinally (100 to 150 µm thick) and divided into three groups (n=10 in each). Group A specimens were treated with a non-fluoride toothpaste containing 10% hydroxyapatite; group B (negative control) specimens were treated with a non-fluoride toothpaste containing 0% hydroxyapatite; while group C (positive control) specimens were treated with a fluoride (950 ppm sodium fluoride) toothpaste with 0% hydroxyapatite. The pH-cycling model was utilized for a period of 10 days. Results. For groups A and C, the mean depth (P<0.01) and the mineral content (P<0.05) of the lesions after treatment decreased significantly compared with baseline (paired t test), while the corresponding values for group B were significantly higher (P<0.001, paired t test). Furthermore, the maximum mineral content of the surface zone decreased significantly in group B but not in groups A and C. Conclusions. The modified in-vitro pH-cycling model successfully revealed progression and mineral changes in initial enamel lesions. Application of both the 10% hydroxyapatite and the 950 ppm sodium fluoride toothpastes reduced the rate of lesion progression when compared with application of toothpaste without either active ingredient.

Key words: Dental caries; Dentifrices; Tooth remineralization; Toothpaste

Introduction

Dental caries is a complex disease that affects a large proportion of the world's population, regardless of gender, age and ethnicity 1, though higher prevalence and incidence values have been reported in groups of lower socioeconomic status 2. Toothpastes have become one of the main therapeutic agents widely used for preventing dental caries. The remineralization of initial carious lesions has been well documented 3,5, and for more than 6 decades, anticaries agents, such as sodium fluoride, sodium monofluorophosphate and stannous fluoride have been added to toothpastes for caries prevention. Moreover, a high-quality meta-analysis has clearly demonstrated the efficacy of fluoride-containing...
toothpastes 4-6. It has therefore been recommended that every effort should be made to develop affordable fluoride-containing toothpastes for use in developing countries 7.

The possibility of fluorosis has always been a concern when fluoridated toothpastes are used, both in children 8,9 and adults 10,11. The main risk factors are fluoride in the drinking water, infant formula milk reconstituted with fluoridated water, fluoride supplements, and fluoridated toothpastes 12.

In recent years, alternatives to fluoride such as nano-hydroxyapatite (HA) and casein phosphopeptide (CPP) have been proposed for their anticariogenic properties. It has been shown that CPP with calcium and phosphorus, which has a high affinity for the HA of tooth enamel, can enhance tooth remineralization and is safe for clinical use 13,14.

Hydroxyapatite is the major inorganic constituent of mineralized biological tissues. It has been used in medicine as a component of artificial bone. In dentistry, it is used in artificial roots to support implants, apatite-containing cement, and as a dental alveolar bone substitute 15. Hydroxyapatite has been shown to adsorb various organic substances, such as proteins, lipids, saccharine and bacteria 16-18. It is therefore believed that these adsorptive properties can be utilized with benefit in a range of medical and dental products 19,20.

Nanoparticle HA–containing toothpastes were first introduced and tested in Japan in the 1980s (e.g. Apadent, Apagard, and others by Sangi Co., Ltd., Tokyo). Since then there have been several studies, including field trials, to test their efficacy in caries prevention 21,22, leading to their approval as antacaries agents by the Japanese Government in 1993. These studies, however, were carried out at the manufacturer’s request, and the results were published only in Japanese-language journals 21,22.

An in-vitro pH-cycling technique was introduced, over 20 year ago, to study the effect of caries-preventive regimens and treatments 23. Many researchers have utilized and modified this pH-cycling model to suit their own studies to test different caries-preventive agents 24-27. To our knowledge, however, no study has utilized the pH-cycling model to test the effect of HA-containing toothpaste.

The objectives of this study were therefore to test whether the modified in-vitro pH-cycling model, using the single-section technique, would work successfully as a means of investigating the de-/re-mineralization effects of toothpaste containing HA, and to compare its efficacy with the use of fluoride-containing toothpastes.

Methods

Preparation of demineralizing and remineralizing solutions

The buffered de-/re-mineralizing solutions were prepared using analytical grade chemicals and deionized water. The demineralizing solution contained 2.2 mM calcium chloride, 2.2 mM sodium phosphate, and 0.05 M acetic acid; the pH was adjusted with 1 M potassium hydroxide to 4.4. The remineralizing solution, which contained 1.5 mM calcium chloride, 0.9 mM sodium phosphate, and 0.15 M potassium chloride, had a pH of 7.0 23.

Preparation of artificial carious lesions

Sound extracted third molar human teeth were cleaned of soft tissue debris and inspected for cracks, hypoplasia, and white spot lesions. The teeth were coated with acid-resistant nail varnish (Revlon, New York, USA), leaving a transversely oriented ‘window’, approximately 1 mm wide, on the sound, intact surface of the buccal and lingual enamel. Each tooth was subsequently immersed in the demineralizing solution (10 mL) for 96 hours to produce lesions approximately 100 to 120 µm deep. The teeth were then sectioned longitudinally through the lesions, using a hard-tissue microtome (Leica 1600 Saw Microtome; Wetzlar, Germany) to produce specimens 100 to 150 µm thick. Each section was measured using a micrometer. Thirty sections were randomly selected and equally divided into three groups. Polarized light microscopy (PLM) [Orthoplan; Leitz, Wetzlar, Germany] and microradiography (MRG) [Softex ISR-20; JIRA, Tokyo, Japan] were utilized to record the depth and mineral content of the lesions before and after the 10 days of treatment with the selected toothpastes. Prior to the pH-cycling model, each section was carefully painted, under a stereomicroscope (Zeiss, Germany), with acid-resistant nail varnish (Revlon), leaving only the lesion surface exposed to the solutions. The sections were stored in an atmosphere of 100% humidity...
until used. This was achieved by suspending the specimens, using dental floss, in a beaker containing deionized water sealed with paraffin (Parafilm, USA).

**Toothpaste preparation**

Dentifrice supernatants were prepared by suspending 12 g of the respective dentifrice in 36-mL deionized water to create a 1:3 dilution. The suspensions were thoroughly stirred with a stirring rod and mechanically agitated for 1 minute by means of a vortex mixer (Thermolyne; Cole-Parmer, USA). They were then centrifuged at 3500 rpm (g-force=2278 g; Beckman J-25I, USA) for 20 minutes at room temperature.

**Test groups**

There were three test groups in this study, including: (1) group A: toothpaste containing 10% HA (HA particle size <100 nm; Sangi Co., Ltd., Japan); (2) group B (negative control): similar toothpaste used in group A but not containing HA or fluoride; and (3) group C (positive control): toothpaste containing 950 ppm sodium fluoride as an active ingredient.

**The pH-cycling model**

The specimens were placed in the pH-cycling system on an orbital shaker (GFL, Germany) for 10 days. Each cycle involved 3 hours of demineralization twice a day with a 2-hour immersion in a remineralizing solution approximated to human saliva in between. A 1-minute treatment with a toothpaste solution of 3:1 deionized water to toothpaste, after centrifugation (5 mL/section), was given before the first demineralizing cycle and both before and after the second demineralizing cycle, and sections were placed in a remineralizing solution overnight. All solutions (demineralizing, remineralizing, toothpaste supernatant) were freshly prepared for each cycle, and separate containers were used for each group throughout the experimental period. The pH of the demineralizing and remineralizing solutions were measured before every cycle.

**Evaluation techniques**

**Polarizing light microscopy**

Polarizing light microscopy was used to make pretreatment and post-treatment records. This was accomplished by immersing the sections in water, which normally produced a clear demarcation between the sound enamel and the initial lesion. Standardized photomicrographs were taken at a fixed magnification both before and after the experiment.

**Microradiography**

Each section was mounted and exposed to Cu (Kα) X-rays (Softex ISR-20; JIRA, Tokyo, Japan) at 12 kV and 3 mA for 1 minute. The high-resolution Kodak Electron Microscope Film 4489 (Kodak, USA) was subsequently developed using standard Kodak chemistry. All exposed films were subjected to the same developing process. After developing, each film was mounted and its image was captured using a flatbed color image scanner (Epson Perfection 3200 Photo; Epson, Japan) which enabled lesion depths, mineral changes and mineral distribution before and after treatment to be determined. The lesion depth of each section was measured in three different areas. With the aid of an image analysis system (ImageJ, USA), the depth of the lesion on each section was then calculated. The digitized images were analyzed using a specially designed computer program which yielded relative mineral densities across the lesions, based on data from sound enamel.

Two parameters that described the mineral content distribution of the lesion, namely the maximum mineral content of the surface zone ($V_{max}$) and the difference between the mineral content before and after the treatment ($\Delta Z$), were then calculated from the profiles. The mineral content profile and lesion parameters were determined for three windows on each single-section specimen. These values were averaged to give the mean $\Delta Z$ for each section. The mean $\Delta Z$ values from each section were then averaged to give the mean $\Delta Z$ for the group. The changes in $\Delta Z$ for a given window before and after treatment were calculated as follows: $\Delta Z = \Delta Z_{2} - \Delta Z_{1}$, where $\Delta Z_{2}$ and $\Delta Z_{1}$ referred to the $\Delta Z$ values of the same window area of a single-section specimen before and after the treatment, respectively. The changes in the lesion depths were similarly calculated.

**Statistical analysis**

Results for lesion depth, $V_{max}$ and $\Delta Z$ obtained from PLM and MRG, were submitted to analysis of variance (ANOVA) using
the Student-Newman-Keuls (SNK) multiple comparisons test. The differences between mean values were compared using the paired t test. A P value of less than 0.05 was considered statistically significant.

**Results**

**Observations by polarized light microscopy**

Photomicrographs of all sections before and after the treatment were taken under the PLM (Figure). Groups A and C showed decreases in lesion depth and some remineralization was evident; while group B showed an obvious increase in lesion depth.

**Observations by microradiography**

**Lesion depth**

The mean (standard deviation [SD]) pretreatment lesion depth in each group ranged from 103 (3) to 106 (2) µm (Table). Among these pretreatment lesion depths, there was no statistically significant difference (P=0.35, ANOVA).

Lesion depth measurements after the 10-day pH-cycling showed that they had decreased by 15% (106 [2] to 90 [11]) and 23% (104 [3] to 80 [9]) in groups A and C, respectively; group B lesions showed an increase of 78% (103 [3] to 183 [25]) [Table]. Using ANOVA with the SNK multiple comparisons test, the changes for groups A and C showed a statistically significant difference from that in group B (P<0.001).

**Maximum mineral content and mineral distribution**

The mean (SD) $V_{\text{max}}$ of all pretreatment sections in each group ranged from 45 (13) to 51 (11). The results after the 10-day pH-cycling showed that the $V_{\text{max}}$ increased by 19% and 28% in groups A and C, respectively, while in group B it had decreased about 48% (Table).

When the $V_{\text{max}}$ values among the treatment groups were compared, the differences between group B and groups A and C were statistically significant (P<0.05; ANOVA and SNK test). However, there was no statistically significant difference between groups A and C (P>0.05; ANOVA and SNK test) [Table].

Similar to the lesion depth results, the $\Delta Z$ values in groups A and C after treatment were significantly lower than that before treatment, while the post-treatment $\Delta Z$ values for the lesions in group B were significantly higher than that before treatment. When the $\Delta Z$ values between the three groups were compared, groups A and C were not significantly different from each other, but each had values significantly different from those in group B (P<0.001;
Effect of nano-hydroxyapatite toothpaste on enamel lesions

Even though all the specimens were sectioned from different teeth, the variations among them did not yield any major effect on the progression of demineralization. This was confirmed by the P value obtained for all the lesion depth measurements (P=0.35, ANOVA) before the in-vitro pH cycling commenced, which showed no statistically significant difference between the artificially created lesions. It was therefore reasonable to disregard such variations when analyzing the data after pH cycling.

The results of this pH-cycling study indicate that the HA toothpaste produced similar results to the positive control involving a fluoride toothpaste. Both of these toothpastes showed a similar ability to reduce the progress of demineralization, while simultaneously enhancing the remineralization of the artificial caries-like lesions. The supernatant obtained from the HA toothpaste slurry had the same pH as that of controls. It was free of fluoride and contained high calcium and phosphate ion concentrations, making the solutions highly supersaturated with respect to HA in enamel. In contrast to fluoride, nanoparticle HA is believed to affect the remineralization of tooth enamel at both the nanocrystalline and ionic levels. In our present study, supernatant of a toothpaste solution was used. If HA is hypothesized to be active in the oral cavity when in the form of a slurry of nanoparticles mixed with saliva, then

### Discussion

While the healing effects of fluoride-containing toothpastes are well established, only limited literature is available for HA-containing toothpastes. As most of the research has been carried out in Japan, the findings have only been published in Japanese-language journals. Toothpastes containing abrasive grade HA (micron-sized particles) are available in Japan and South Korea; however, investigators have only studied the abrasive or whitening characteristics of the toothpaste, rather than their remineralization efficacy.

Rather than using the traditional pH-cycling method, a modified version was utilized in our study, in an attempt to simulate the real-life situation. This included a 3-hour demineralizing cycle twice daily, with one 2-hour and one intervening overnight remineralizing cycle, respectively. And to replicate early morning, midday and before bed-time toothbrushing, toothpaste was applied thrice daily, before the first demineralization, as well as both before and after the second demineralization. The remineralizing solutions used in the study were created to replicate supersaturation by apatite minerals found in saliva and were similar to those previously utilized by ten Cate and Duijsters.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lesion depth (µm)</th>
<th>Maximum mineral content in the surface zone (% mineral)</th>
<th>ΔZ value (% mineral x µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Before</td>
<td>106 (2)</td>
<td>48 (21)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>90 (11)</td>
<td>57 (12)</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>-15% (10)</td>
<td>19% (40)</td>
</tr>
<tr>
<td>Group B</td>
<td>Before</td>
<td>103 (3)</td>
<td>51 (11)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>183 (25)</td>
<td>27 (7)</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>78% (26)</td>
<td>-48% (27)</td>
</tr>
<tr>
<td>Group C</td>
<td>Before</td>
<td>104 (3)</td>
<td>45 (13)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>80 (9)</td>
<td>57 (12)</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>-23% (8)</td>
<td>28% (28)</td>
</tr>
</tbody>
</table>

* Data are shown as mean (standard deviation). For each parameter investigated, groups identified with the same letters are not significantly different (P>0.05; analysis of variance with Student-Newman-Keuls multiple comparisons test)

† P<0.01
‡ P<0.05
§ P<0.001
the present findings may not completely reflect the full remineralizing potential of HA toothpaste. In order to confirm this hypothesis, further studies should be performed using a slurry toothpaste preparation rather than a supernatant.

In conclusion, based on the data obtained, it is shown that the modified pH cycle can be successfully applied to test the de-/re-mineralization effects of HA toothpaste. In addition, both the HA-containing and the fluoridated toothpastes demonstrated similar abilities to slow down the demineralization process and enhance the remineralization of artificial carious lesions.

References

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