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Protective effect of phosphates and fluoride on the dissolution of hydroxyapatite and their interactions with saliva

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Short title: Salivary pellicle modulates hydroxyapatite dissolution

Key words: Fluoride, Polyphosphates, Saliva, Pellicle, Hydroxyapatite.

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Abstract

This study aimed to investigate the effect of phosphates and fluoride, alone or in combination, and the influence of salivary pellicle on hydroxyapatite (HA) dissolution. Baseline dissolution rate of HA discs was measured using a pH-stat system (0.3% citric acid, pH 3.2). In the first series of experiments, HA discs (*n*=8/group) were treated with a placebo solution (PLA, deionised water); sodium trimetaphosphate (TMP), sodium tripolyphosphate (TRI) and sodium pyrophosphate (PYRO) at 1% or 8%; 500 ppm F (500F); 1100 ppm F (1100F); 1100F/1% TMP; 1100F/8% TMP; 1100/1% TRI; 1100F/8% TRI. In the second phase, HA discs were immersed in pooled human saliva (37º C/ 2h) and treated with PLA; 1100F/1% TMP; 1100F/8% TMP; 1100F/1% TRI; and 1100F/8% TRI. After treatments, final dissolution rate were measured from three consecutive 30-min assays. Statistical analysis were performed using 2-way ANOVA followed by Fisher's test (α=0.05). The type and concentration of phosphate tested significantly influenced HA dissolution; 8% TRI showed the highest reduction (36.9%) among all treatment solutions. Fluoride alone (1100F) significantly reduced HA dissolution by 20.7%. When fluoride and phosphates were associated, 1100F/1%TMP, 1100F/8%TMP and 1100F/8% TRI showed the highest percentage reductions of dissolution (40.3 to 46.1%). Salivary pellicle led to a greater and more sustained protective effect of the treatment solutions compared to their counterparts without salivary coating. It was concluded that the association of phosphate and fluoride enhanced their protective effect against HA dissolution when compared with these compounds alone, especially in the presence of salivary pellicle.

Introduction

Dental erosion is defined as the softening of the tooth surface followed by its gradual bulk removal, caused by exposure to acids of non-bacterial origin [Lussi et al., 2011]. Surface softening and enamel loss are most often caused by the consumption of acidic soft drinks containing citric and/or phosphoric acids [Zero and Lussi, 2005].

Conventional fluoride preparations such as toothpastes or mouth rinses have a limited effect on erosion [Wiegand and Attin, 2003; Magalhães et al., 2011] and significant inhibition requires either the application in high concentrations or at high frequency [Ganss et al., 2004], or the use of preparations containing titanium or stannous ions [Ganss et al., 2010; Wiegand et al., 2010] that might be unsuitable for routine use because of their low pH and their propensity to stain the teeth. Nonetheless, promising results have been described for fluoridated products associated with sodium trimetaphosphate on erosion *in vitro* [Moretto et al., 2010; Manarelli et al., 2011; Manarelli et al., 2013] and *in situ* [Moretto *et al.*, 2013]. Similarly, sodium tripolyphosphate and sodium pyrophosphate tetrabasic have also been shown to promote significant reductions on ~~HA discs~~ the dissolution of HA discs [Barbour et al.,2005] and ~~on hydroxyapatite crystals dissolution~~ HA cyrstals [Scaramucci *et al.*, 2011; 2015] using a pH-stat approach ~~[Barbour et al., 2005; Scaramucci *et al.*, 2011; 2015]~~, despite these phosphates were not able to reduce enamel and dentine wear *in vitro* [Scaramucci *et al.*, 2011].

Saliva contains phosphates, proteins and bicarbonate buffers and is supersaturated with respect to tooth minerals, such as calcium and phosphate. It is known that proteins can protect the teeth by the formation of a salivary pellicle when teeth are exposed to saliva [Siqueira et al., 2007]. This pellicle of adsorbed salivary proteins might act as a diffusion barrier or a selective permeable membrane reducing direct contact between acids and tooth surface and thus reducing demineralization of the surface [Wetton et al., 2007; Jager et al., 2011; White et al., 2012].

Based on the above, the aim of the present study was to investigate the effect of fluoride and phosphates, alone or in combination, on the dissolution of hydroxyapatite (HA), as well as the ~~. The~~ influence of the salivary pellicle on this process ~~was also assessed~~. The study hypothesis was that HA dissolution would be significantly reduced by the presence of fluoride and phosphates, and that this effect would be influenced by the presence of salivary acquired pellicle.

Materials and Methods

Materials

Discs of compressed HA, mean diameter 12.1 mm, mean thickness 1.23 mm, were purchased from HiMed Inc., Old Bethpage, N.Y., USA. All products were obtained from Sigma-Aldrich (Poole, Dorset, UK). Solutions were prepared using deionised water.

Groups

Treatment solutions included: Placebo (PLA, deionised water); sodium trimetaphosphate at 1% and 8% (1% TMP and 8% TMP, respectively); sodium tripolyphosphate at 1% and 8% (1% TRI and 8% TRI, respectively); sodium pyrophosphate tetrabasic at 1% and 8%) (1% PYRO and 8% PYRO, respectively); fluoride (500 ppm F and 1100 ppm F, as sodium fluoride); 1100 ppm F with sodium trimetaphosphate at 1% and 8% (1100 ppm F/1% TMP and 1100 ppm F/8% TMP, respectively); 1100 ppm F with sodium tripolyphosphate at 1% and 8% (1100 ppm F/1% TRI and 1100 ppm F/8% TRI). ~~Fluoride concentrations were based on those found in conventional and low-fluoride toothpastes.~~Fluoride concentrations were chosen based on those present in conventional formulations (1100 ppm F), as well as in low-fluoride toothpastes (500 ppm F) available in some countries.

Measurement of Dissolution Rate

Dissolution in 0.3% citric acid, pH 3.2, was measured using a pH-stat model (718 Stat Titrino: Metrohm UK, Runcorn, Cheshire, UK) with a 50 ml double-walled glass reaction vessel fitted with a lid with 3 inlet ports. Water was pumped by a circulating water bath (Type GD120; Grant Instruments, Cambridge, UK) through the water jacket to maintain the reaction temperature at 37 °C. Prior to use in the pH-stat, batches of discs were conditioned to ensure consistency of response. The discs were first ultra-sonicated in deionized water in a bath with an ultrasonic power of 40 kHz for 15 min to remove loose HA particles. They were then exposed to gently stirred 0.3% citric acid, pH 3.2, for 30 min at room temperature, then washed in deionised water and finally air-dried. For use in the pH-stat, the back of the discs were coated with nail varnish to leave an area of 161.4 mm^2 available for reaction. Discs were then fixed with sticky wax to the tip of glass tubes fitted with a cone suitable for the inlet ports.

In each experiment, 30 ml of citric acid solution was introduced into the reaction vessel and the pH electrode and burette tip fitted. After the system had reached thermal equilibrium, the pH was adjusted to 3.2 by adding small quantities of concentrated KOH or HCl solution and finally adjusted using the pH-stat. The reaction was initiated by introducing the specimen on its holder and addition of titrant (50 mM HCl) was recorded for 30 min. A control measurement of dissolution rate was made after the conditioning step and the disc was removed from the holder, washed in deionised water and dried. After exposing the disc to the chosen treatment, the disc was reattached to the glass specimen holder and measurement of dissolution rate was made.

Saliva Collection

Mixed saliva was collected from a panel of 2 volunteers, who had previously registered at the saliva bank from the University of Bristol, having received local ethical approval for this. When saliva was required, each volunteer was provided with a 50 ml polystyrene Universal tube, marked at the 25 ml level. Each volunteer chewed a square of Parafilm to stimulate salivary flow and expectorated saliva into the tube until the mark was reached. These samples were combined and centrifuged using a Centaur 1 (MSE, London, UK) at 4,000 *g* for 15 min at ambient temperature. The supernatant was used to treat HA specimens (2 mL/disc).

Experiments

Native HA. In the first series of experiments, inhibition was tested on non-saliva-treated HA [Barbour et al., 2005; Jones et al., 2013]. First, a sequence of three control measurements was performed. Next, the HA discs were individually immersed in 100 mL of the treatment solutions (PLA; 1% TMP; 8% TMP; 1% TRI; 8% TRI; 1% PYRO; 8% PYRO; 500 ppm F; 1100 ppm F; 1100 ppm F/1% TMP; 1100 ppm F/8% TMP; 1100 ppm F/1% TRI; 1100 ppm F/8% TRI) for ~~100mL/~~2 min with gentle stirring, followed by rinsing with deionised water. ~~Fluoride concentrations were chosen based on those present in conventional formulations (1100 ppm F), as well as in low-fluoride toothpastes (500 ppm F) available in some countries. Afterwards it was rinsed with deionized water and rinsing in water,~~ Following, the post-treatment dissolution rate was determined in 3 consecutive periods of 30 minutes each. ~~For this, each HA disc were immersed in 100 ml of the respective treatment solutions for 2 min with gentle stirring. Afterwards, it was rinsed with deionized water.~~ For this, ~~Next,~~ 30 mL of citric acid was added to the reaction vessel and the pH electrode and burette tip were fitted. After the system ~~had~~ reached equilibrium (pH 3.2 adjusted with KOH or HCl) the reaction was initiated by introducing the HA disc, and the addition of tritant (50 mM~~mmol 1^{-1}~~ HCl) was recorded for 30 minutes using pH-stat, similarly ~~for the measures did~~ as done for the control. Fresh citric acid solutions were added to the vessel for each 30-min assay. This sequence was performed on 8 separate HA discs for each treatment.

Saliva-Coated HA. After making control measurements of dissolution rate, discs were immersed in pooled mixed saliva supernatant for 2 h in Petri dishes in an incubator at 37 ° C. Next, the HA discs were immersed in treatment (PLA; 1100 ppmF/1% TMP; 1100 ppmF/8% TMP; 1100 ppmF/1% TRI; 1100ppmF/8% TRI) 100 ml/2 min with gentle stirring. After rinsing in water, a series of 3 post-treatment measurements of dissolution was performed. This sequence was performed on 8 separate HA discs for each treatment.

Statistical Analysis

Analyses were performed using the SigmaPlot (version 12.0) with 5% of statistical significance level. Data from native and saliva coated discs exhibited a normal (Kolmogorov-Smirnov) and homogeneous distribution. Treatment solutions (with or no salivary pellicle) and time (baseline control, post-treatment 1, 2 and 3) were considered as variation factors. The baseline was determined for each specimen by averaging the three control runs before treatment. Thus, data were submitted to 2-way ANOVA, followed by Fisher test ($p<0.05$).

Results

~~Eleven~~ Ten of the test solutions without pellicle effected a statistically significant reduction in the HA dissolution rate in citric acid solutions (Table 1). Among the phosphates, the highest percentage reduction of dissolution was seen for 8% TRI (36.9%), while 1100 ppm had the highest percentage reduction of dissolution (20.7%) among the solutions containing only NaF. For solutions containing NaF and a phosphate salt in combination, 1100 ppm F/1%TMP, 1100 ppm F/8%TMP e 1100 ppm F/8% TRI showed the highest percentage reductions of dissolution (Table 1), ranging from 40.3 to 46.1%.

Regarding the presence of salivary pellicle, all test solutions effected a statistically significant reduction in the HA dissolution rate in citric acid solution in specimens covered with salivary pellicle in comparison with their counterparts not treated with saliva (Figure 1). Salivary coating promoted a more sustained protective effect of the treatment solutions when compared to their counterparts not treated with saliva ($p<0.001$). Also, no significant differences were seen for the treatment groups in the presence of saliva regarding the reduction of dissolution rate and persistence of effect over time.

Discussion

In this study, the pH stat method was employed to investigate the immediate and sustained effect of sodium trimetaphosphate, sodium tripolyphosphate and sodium pyrophosphate tetrabasic with or without fluoride on hydroxyapatite dissolution. These phosphates have shown promising results against dental erosion, but most studies only evaluated their immediate effects. The present study showed that the reduction of hydroxyapatite dissolution as well as the duration of this effect was significantly influenced by type of phosphate and concentration tested, as well as by the salivary coating. Therefore, the study's hypothesis was accepted.

In the first set of experiments, all test solutions were evaluated without the presence of a salivary coating. It has been shown that fluoride can offer a protection against dental erosion and this effect is given by a formation of a layer of KOH-soluble calcium fluoride [Magalhães et al., 2011]. In the present study, a relationship between fluoride concentration and the protective effect was observed, since reductions in HA dissolution were 13% and 21% respectively for solutions containing 500 and 1100 ppm F. The results are in line with previous data showing a reduction of 12% in dissolution rate of native HA when 300 ppm F was administered [Jones et al., 2013]. However, these reductions did not persist beyond the first post-treatment run, confirming that fluoride alone might have a limited action against enamel erosion.

Regarding the phosphates evaluated, the highest reduction of HA dissolution rate (~37~~40~~%) was observed for the solution containing 8% of TRI, and its effects were persistent up to the third post-treatment run (90 min). Despite the present results are higher than data reported in previous investigations using the same methodology [Barbour *et al.*, 2005; Scaramucci *et al.*, 2011; 2015], it is noteworthy that the studies cited above used lower concentrations of this phosphate (ranging from 0.02 to 2%), what might help to explain the different results. This assumption is reinforced by the fact that 1% TRI promoted a much lower reduction in HA dissolution (~13%) than at 8%, and its effects were only significant for the first post-treatment run (30 min). As for the other phosphates tested, the reductions in HA dissolution were less pronounced for TMP and PYRO at 8%, and their effects did not persist over the 3 post-treatment runs. The modest effect of TMP is in agreement with previous findings showing that TMP alone produce negligible effects on bovine enamel de-/remineralisation when added to different topically applied dental products [Danelon et al., 2014; Manarelli et al., 2014]. The association of TMP and TRI with fluoride further reduced the rate of hydroxyapatite dissolution compared to these phosphates or fluoride alone. It is noticeable that while 1% TMP did not produce any significant effect on HA dissolution (1.2%), its association with 1100 ppm F led to maximum inhibition of 40.3%. Given that this value was 2-fold higher than that observed for 1100 ppm F alone, the results indicate that TMP and fluoride have a synergistic effect against HA dissolution, confirming previous observations using bovine enamel in a pH-cycling model [Castro et al., 2015]. In this sense, despite TMP has been shown to act like a partial barrier to CaF_2 deposition on enamel surface [Manarelli et al., 2014], CaF_2 and CaF^+ compounds are believed to retain on TMP molecules (adhered to HA) and to be released to saliva upon acidification of the oral environment, further reacting with salivary phosphates leading to the formation of more reactive calcium phosphates [Manarelli et al., 2014]. It is also noteworthy that the association with fluoride led to a significant increase in the protective effect of TMP at both concentrations tested (1 and 8%) tested, while for TRI this effect was only seen at 1%, indicating that each phosphate has an ideal molar ratio with fluoride in order to achieve the maximum additive or synergistic effect.

In the second set of experiments a salivary coating on hydroxyapatite was introduced, as it is known that saliva plays an important role on dental erosion. The acquired pellicle is composed of proteins and glycoproteins that act as a protective barrier, preventing the direct contact between the acid and the tooth surface [Buzalaf et al., 2012]. When compounds were tested with a salivary coating, a greater reduction of hydroxyapatite dissolution (~65% for all solutions together) was observed compared with discs not previously treated with saliva (~40% for all solutions together) in the first post-treatment run, what is also in line with previous data using a similar research protocol [Jones et al., 2013]. It is noteworthy that while the protective effect for native HA decreased by 63% between the first and the second post treatment run, and by 66% between the second and the third post-treatment, these decreases were much lower for HA previously exposed to saliva (15 and 20%, respectively). This indicates that the protective layer formed by salivary components, fluoride and TMP or TRI was not totally dissolved from the HA surface after the first exposure to the acid media, but occurred gradually from its external to the basal components [Joiner et al., 2008].

Although the present in vitro model does not fully reproduce acid challenges occurring in vivo, our data can be helpful in further investigations. In this sense, the results indicate that fluoride associated with phosphates as TRI and TMP could be an alternative to the development of oral products against dental erosion. It is important to highlight that an optimal ratio between phosphate and fluoride should be used in order to achieve optimum results, based on the present results and also on previous data from experiments performed with bovine enamel specimens [Takeshita et al., 2009; Castro et al., 2015]. Another important point is related to the presence of saliva in future studies, as the effect of the treatments was shown to be highly dependent on the salivary coating of the specimens [Buzalaf et al., 2012]. Thus, the screening of such agents should always include specimens that have been pre-treated with saliva as well as native surfaces.

To sum up, it can be concluded that TMP and TRI provided reduction of hydroxyapatite dissolution when an erosion-like model was used. In addition ~~to~~, the association of these phosphates with fluoride enhanced their effectiveness compared to fluoride or phosphates alone. Thus, these associations could be a potential alternative in future investigations in order to prevent dental erosion.

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Table 1. Mean (SD) percentage reduction of hydroxyapatite dissolution for each treatment solution after exposure to citric acid according to time following exposure to treatment solutions

Groups	Post-treatment 1 (30 min)	Post-treatment 2 (60 min)	Post-treatment 3 (90 min)
PLA	0.7^{a} (2.1)	0.6^{a} (2.1)	0.0^{a} (1.3)
1% TMP	1.2^{a} (5.8)	0.4^{a} (4.6)	0.0^{a,e} (3.8)
8% TMP	16.2^{b,c} (4.4)*	10.3^{b,f} (3.6)	5.2^{b} (5.4)
1% TRI	12.6^{b} (4.2)*	8.4^{b,f} (3.7)	2.0^{b,e} (3.8)
8% TRI	36.9^{c,d} (5.4)*	25.7^{c} (3.6)*	17.1^{c} (7.5)*
1% PYRO	7.7^{a,b} (6.7)	3.6^{b} (5.8)	0.0^{b} (5.2)
8% PYRO	18.2^{b,c} (4.9)*	10.3^{b,d} (2.8)*	4.6^{b} (2.5)
500 ppm F	13.1^{b} (3.3)*	9.0^{d,f} (4.2)*	4.8^{b} (6.1)
1100 ppm F	20.7^{c} (5.7)*	12.3^{b} (1.7)*	8.5^{b} (4.0)
1100 ppm F/1%TMP	40.3^{d} (5.5)*	17.2^{e} (4.7)*	2.6^{d} (5.8)
1100 ppm F/8%TMP	42.2^{d} (4.8)*	13.1^{e} (6.5)*	4.6^{d} (5.8)
1100 ppm F/1%TRI	32.3^{c,d} (7.8)*	11.5^{b,d} (8.1)*	7.0^{b} (7.5)
1100 ppm F/8%TRI	46.1^{d} (3.3)*	16.9^{f} (6.8)*	5.4^{b} (6.2)

Different superscript letters within each column show statistical difference among treatment solutions. Asterisks indicate significantly difference from mean baseline control (Fisher's test, $p<0.05$, $n=8$/group).

PLA: placebo solution (deionised water); **TMP**: sodium trimetaphosphate; **TRI**: sodium tripolyphosphate; **PYRO**: sodium pyrophosphate.

Figure captions

Figure 1. Mean inhibition of hydroxyapatite dissolution for each treatment solution after exposure to citric acid in specimens with or without salivary coating. Bars indicate SD. Asterisks indicate significant differences between each post-treatment run and the control (baseline) measurement. Different letters indicate significant differences among the groups (Fisher test, $p<0.05$, *n*=8/group).