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# Protective Effect of Phosphates and Fluoride on the Dissolution of Hydroxyapatite and Their Interactions with Saliva

Michele M. Manarelli[a] Juliano P. Pessan[a] Alberto C.B. Delbem[a]
Jackeline G. Amaral[a] Mayra F. Paiva[a] Michele E. Barbour[b]

[a]School of Dentistry, São Paulo State University (UNESP), Araçatuba, Brazil; [b]School of Oral and Dental Sciences, University of Bristol, Bristol, UK

**Keywords**

Fluoride · Hydroxyapatite · Pellicle · Polyphosphates · Saliva

**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. The 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; 1,100 ppm F; 1,100 ppm F/1% TMP; 1,100 ppm F/8% TMP; 1,100 ppm F/1% TRI; 1,100 ppm F/8% TRI. In the second phase, HA discs were immersed in pooled human saliva (37°C/2 h) and treated with PLA, 1,100 ppm F/1% TMP, 1,100 ppm F/8% TMP, 1,100 ppm F/1% TRI, and 1,100 ppm F/8% TRI. After treatments, final dissolution rates were measured from 3 consecutive 30-min assays. Statistical analyses were performed using 2-way ANOVA followed by the Fisher test ($\alpha = 0.05$). 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 (1,100 ppm F) significantly reduced HA dissolution by 20.7%. When fluoride and phosphates were associated, 1,100 ppm F/1% TMP, 1,100 ppm F/8% TMP, and 1,100 ppm F/8% TRI showed the highest percentage reductions of dissolution (40.3–46.1%). 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.



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.,

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Prof. Juliano P. Pessan
Department of Pediatric Dentistry and Public Health
School of Dentistry, São Paulo State University (UNESP)
Rua José Bonifácio 1193, Araçatuba, SP 16015-050 (Brazil)
E-Mail jpessan@foa.unesp.br

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, 2013] and in situ [Moretto et al., 2013]. Similarly, sodium tripolyphosphate and sodium pyrophosphate tetrabasic have also been shown to promote significant reductions on the dissolution of HA discs [Barbour et al., 2005] and HA crystals [Scaramucci et al., 2011, 2015] using a pH-stat approach, despite these phosphates not being 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 influence of the salivary pellicle on this process. 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, NY, USA. All products were obtained from Sigma-Aldrich (Poole, 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 1,100 ppm F, as sodium fluoride); 1,100 ppm F with TMP at 1 and 8% (1,100 ppm F/1% TMP and 1,100 ppm F/8% TMP, respectively); 1,100 ppm F with TRI at 1 and 8% (1,100 ppm F/1% TRI and 1,100 ppm F/8% TRI). Fluoride concentrations were chosen based on those present in conventional formulations (1,100 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, 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 ultrasonicated in deionised 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 backs 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 the 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 3 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; 1,100 ppm F; 1,100 ppm F/1% TMP; 1,100 ppm F/8% TMP; 1,100 ppm F/1% TRI; 1,100 ppm F/8% TRI) for 2 min with gentle stirring, followed by rinsing with deionised water. Following this, the posttreatment dissolution rate was determined in 3 consecutive periods of 30 min each. For this, 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 titrant (50 mM HCl) was recorded for 30 min using pH-stat, similarly 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 solution (PLA; 1,100 ppm F/1% TMP; 1,100 ppm F/8% TMP; 1,100 ppm F/1% TRI; 1,100 ppm F/8% TRI) 100 mL for 2 min with gentle stirring. After rinsing in water, a series of 3 posttreatment 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 without salivary pellicle) and time (baseline control, posttreatment 1, 2, and 3) were considered as variation factors. The baseline was determined for each specimen by averaging the 3 control runs before treatment. Thus, data were submitted to 2-way ANOVA, followed by the Fisher test ($p < 0.05$).

## Results

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 1,100 ppm F 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, 1,100 ppm F/1% TMP, 1,100 ppm F/8% TMP and 1,100 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 (Fig. 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.

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

| Groups | Post-treatment run 1 (30 min) | Post-treatment run 2 (60 min) | Post-treatment run 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) |
| 1,100 ppm F | $20.7^{c}$ (5.7)* | $12.3^{b}$ (1.7)* | $8.5^{b}$ (4.0) |
| 1,100 ppm F/1% TMP | $40.3^{d}$ (5.5)* | $17.2^{e}$ (4.7)* | $2.6^{d}$ (5.8) |
| 1,100 ppm F/8% TMP | $42.2^{d}$ (4.8)* | $13.1^{e}$ (6.5)* | $4.6^{d}$ (5.8) |
| 1,100 ppm F/1% TRI | $32.3^{c, d}$ (7.8)* | $11.5^{b, d}$ (8.1)* | $7.0^{b}$ (7.5) |
| 1,100 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 differences among treatment solutions. Asterisks indicate a significant difference from mean baseline control (Fisher test, $p < 0.05$, $n = 8$/group). PLA, placebo solution (deionised water); TMP, sodium trimetaphosphate; TRI, sodium tripolyphosphate; PYRO, sodium pyrophosphate.

## Discussion

In this study, the pH-stat method was employed to investigate the immediate and sustained effect of TMP, TRI and PYRO with or without fluoride on HA 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 HA dissolution as well as the duration of this effect was significantly influenced by the type of phosphate and the 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 1,100 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 posttreat-

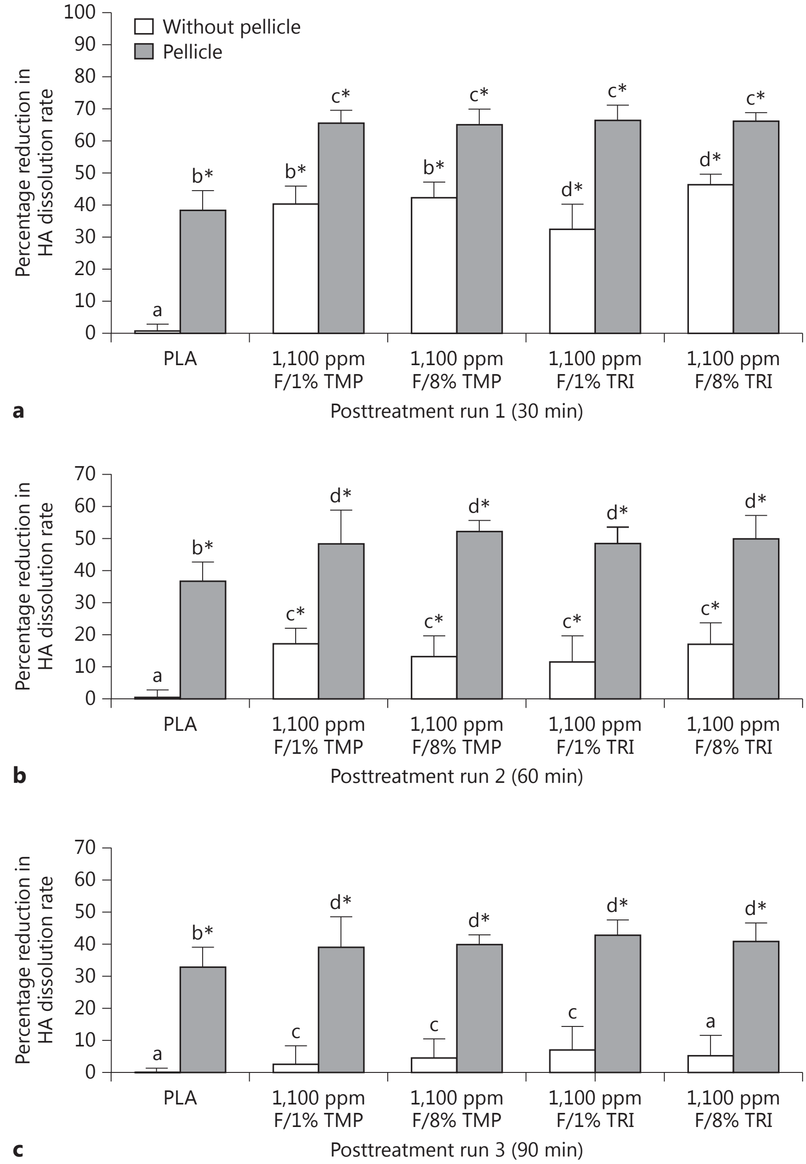


**Fig. 1.** Mean inhibition of HA dissolution for each treatment solution after exposure to citric acid in specimens with or without salivary coating. Bars indicate standard deviations. Asterisks indicate significant differences between each posttreatment run and the control (baseline) measurement. Different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

ment run, confirming that fluoride alone might have a limited action against enamel erosion.

Regarding the phosphates evaluated, the highest reduction of HA dissolution rate (approx. 37%) was observed for the solution containing 8% of TRI, and its effects were persistent up to the third posttreatment run (90 min). Despite the fact that 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%), which 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 (approx. 13%) than at 8%, and its effects were only significant for the first posttreatment 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 posttreatment runs. The modest effect of TMP is in agreement with previous findings showing that TMP alone produces 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 HA 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 a 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, although 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 remain 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%), 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 HA 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 HA dissolution (approx. 65% for all solutions together) was observed compared with discs not previously treated with saliva (approx. 40% for all solutions together) in the first posttreatment run, which 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 posttreatment run, and by 66% between the second and the third posttreatment run, 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 were not totally dissolved from the HA surface after the first exposure to the acid medium 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 such 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 pretreated with saliva as well as native surfaces.

To sum up, it can be concluded that TMP and TRI provided reduction of HA dissolution when an erosion-like model was used. In addition, 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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## Disclosure Statement

The authors declare no conflict of interest that might introduce bias or affect the manuscript's judgment.

## Author Contributions

Study idea and design: J.P.P., A.C.B.D., and M.E.B., Accomplishment of experiments: M.M.M., J.G.A., and M.E.B. Data analysis: J.G.A., M.F.P., J.P.P., and A.C.B.D. Manuscript preparation: M.M.M., J.P.P., A.C.B.D., J.G.A., M.F.P., and M.E.B.

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