Effect of Sodium Trimetaphosphate on Hydroxyapatite Solubility: An *In Vitro* Study

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This study evaluated the effect of different concentrations of sodium trimetaphosphate (TMP) with and without fluoride (F) on the concentration of calcium (Ca), phosphorus (P) and F in hydroxyapatite (HA). Synthetic HA powder (0.15 g) was suspended (n=6) in solutions (75 mL) of TMP at 0%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, 4.0%, 6.0%, 8.0% and 10% concentrations in the presence and absence of 100 ppm F and subjected to a pH-cycling process. The precipitates were filtrated, dried at 70° C for 24 h and ground onto a fine powder. The concentrations of F (KOH (CaF$_2$) and HCl (FA) soluble), Ca (Arsenazo III), and P (molybdate method) in HA were determined. The Ca P, and Ca/P ratio data were subjected to Tukey's test and the F data were subjected to Student-Newman-Keuls test (p<0.05). The addition of TMP to the samples reduced F deposition to 98% (p<0.001). The groups containing 100 ppm F and 0.4% or 0.6% TMP exhibited a higher Ca concentration than the group containing only 100 ppm F (p<0.05). Furthermore, the HA treated with 0.2% and 0.4% TMP and 100 ppm F showed a higher Ca/P ratio than the other groups (p<0.001). In conclusion, TMP at 0.2%, 0.4% and 0.6% concentrations combined with F seemed to be able to precipitate HA with low solubility. However, especially at high concentrations, TMP interferes with F deposition on HA.

Key Words: hydroxyapatite, trimetaphosphate, demineralization, solubility, fluoride.

Introduction

Fluoride (F) is the most important agent for preventing dental caries (1); however, at the same time, its extensive ingestion is related to dental fluorosis (2). Thus, F in toddlers must be used with caution. As a safety measure, oral health promotion programs for toddlers (baby clinics, 0-3 years) currently use topical solutions with low F concentrations in the dental office (0.02% NaF/day) instead of F-containing dentifrice (3,4). However, the 0.02% NaF solution shows lower anticaries efficacy (1,5,6). Addition of inorganic phosphate can be performed to improve the effectiveness of mouthrinses containing 0.02% NaF (7,8).

Polyphosphate has a strong affinity for the hydroxyapatite (HA) surface and resistance against successive acid attacks while reducing enamel dissolution (9). Sodium trimetaphosphate (TMP), a cyclic condensed phosphate, has lower capacity to bind to HA than polyphosphates, but it remains bound to enamel for a longer period (10). Studies have demonstrated the reduction of enamel solubility, suggesting an affinity of TMP to enamel surface and/or HA (7,8,10).

Manarelli et al. (7) investigated whether addition of TMP in a mouthrinse could improve its effectiveness against dental erosion. The solution containing 100 ppm F and 0.4% TMP presented a greater protective effect under erosive conditions than a solution containing 225 ppm F. In another study (8), the same mouthrinse (100 ppm F and 0.4% TMP)

was superior in inhibiting dental caries *in vitro* because it prevents acid diffusion into the enamel. It has also been observed a higher F content in the enamel after use of this mouthrinse, although the type of F deposits on the enamel has not been identified: loosely bound F (calcium fluoride - CaF$_2$) or firmly bound F (fluorapatite - FA) (11). Other studies demonstrated that an adequate TMP/F ratio can improve the effectiveness of F-based products even at low F concentrations (12-14). Nevertheless, the mechanism of TMP action is not yet completely clear. Using the study designs used so far, it has not been possible to define the mechanism of TMP action when associated with F at different concentrations. The analysis of the effect of TMP with and without F on HA allows the evaluation of the changes that occur by combining these two additives, as well as shed light on the effects of TMP on the deposition of CaF$_2$ and FA.

The aim of this study was to evaluate the effect of different sodium TMP concentrations (0%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, 4.0%, 6.0%, 8.0% and 10%) with and without F (100 ppm) on the concentration of calcium (Ca), phosphorus (P) and F in HA when subjected to demineralization and remineralization.

Material and Methods

Synthesis of HA

HA was synthesized according to Qu and Wei (15): 1

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mol L^{-1} (300 mL) calcium nitrate solution (Ca(NO$_3$)$_2$.H$_2$O; Sigma-Aldrich Corp. St. Louis, MO, USA) and 0.3 mol L^{-1} (600 mL) diammonium phosphate solution ((NH$_4$)$_2$HPO$_4$; Sigma-Aldrich Corp.) were prepared. The pH of each solution was raised to 10-12 by adding NH$_4$OH (29.5%). The diammonium phosphate solution was added slowly to the calcium nitrate solution (2-5 mL/min) under constant agitation at 37° C in order to precipitate the fully-crystallized HA. The precipitates were aged for 7 days at 37° C while the pH was maintained around 10 for the growth and formation of a single crystalline phase.

The precipitate was collected by filtration using a Buchner funnel attached to a vacuum system (-600 mmHg), washed repeatedly with deionized water (250 mL/0.15 g of HA), and dried at 70° C for 24 h. The precipitate was then ground onto a fine powder. The HA powder was heated at 1,000° C for 2 h to decrease the amount of impurities such as CO$_2$, CO$_3$$^{2-}$, HPO$_4$$^{2-}$ and water (16). Six samples of approximately 0.15 g were taken for F, Ca, and P analysis.

Demineralization and Treatment

Solutions (75 mL) of TMP (Na$_3$P$_3$O$_9$, Sigma-Aldrich Corp.) were prepared at 0.0%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, 4.0%, 6.0%, 8.0%, and 10% concentrations, associated with or without 100 ppm F (NaF, Merck, Darmstadt, Germany). Synthetic HA powder (0.15 g) was suspended (n=6) in each of the prepared solutions. Samples of the HA powder (n=6) were suspended in deionized water and used as negative control. After 15 min of stirring at pH 7.0, the pH of the suspensions was slowly reduced to 4.0 using 1 mol L^{-1} nitric acid (HNO$_3$, Merck) under agitation. After 30 min of equilibration, the pH of each solution was raised to 7.0 by the addition of 1 mol L^{-1} sodium hydroxide (NaOH, Merck). This pH-cycling process was repeated thrice, ending with pH 7.0 at 37° C (15). The precipitates were filtrated, dried for 24 h at 70° C and ground onto a fine powder. After completion of this process, the precipitates were separated by filtration using a Buchner funnel attached to a vacuum system (-600 mmHg), washed five times with deionized water (250 mL/0.15 g of HA) to eliminate sodium residues remaining in the solution, and subsequently dried at 70° C for 24 h. The precipitate was then ground into a fine powder using an agate mortar and pestle for further F , Ca, and P analysis (16).

Calcium and Phosphorus Analysis

For Ca and P determination, 1 mg of HA powder was weighed into pre-weighed micro-centrifuge tubes and 1.0 mL of 1 mol L^{-1} HCl was added. After agitation for 1 h (Shaker, SK-300; Lab. Companion, Kimpo City, Korea), Ca analysis was performed using a spectrophotometer (Powerwave 340; Biotek, St. Paul, MN, USA) with a wavelength of 650 nm by adopting the Arsenazo III colorimetric method (17). Aliquots of 5 µL were taken from the samples (diluted 1:10 and partly neutralized) and added to 50 µL of deionized water and arsenazo. For calibration, standards containing 40 to 200 µg Ca/mL were used. Phosphorus was measured by the molybdate method (colorimetric method) for an aliquot of 20 µL from the samples, which were subsequently added to a mixture of 50 µL molybdate solution and 20 µL of reactive reducer, as described by Fiske and Subbarow (18), at a wavelength of 660 nm; standards containing 1.5 to 24 µg P/mL were used.

Fluoride Analysis (CaF$_2$ and FA)

For F analysis (CaF$_2$ and FA), 1 mg of HA powder was weighed into pre-weighed micro-centrifuge tubes, and 1.0 mL of 1 mol L^{-1} KOH was added for CaF$_2$ extraction, according to Caslavska et al. (19). After 24 h under continuous agitation (Shaker, SK-300; Lab. Companion), the samples were centrifuged for 20 min at 2,900 *g*. A 0.5 mL aliquot of the supernatant was neutralized with 0.5 mL of TISAB II (total ionic strength adjustment buffer) modified with 1 mol L^{-1} HCl (8.2 mL HCl/L). CaF$_2$ was determined by using a specific electrode (Orion 9409; Orion Research, Inc., Boston, MA) and a reference electrode (Orion 900100; Orion Research, Inc.) connected to an ion analyzer (Orion 720^{plus}; Orion Research, Inc.).

For FA determination, the precipitate was washed thrice with deionized water and once with methanol. After methanol evaporation (overnight at 60° C), 1 mL of 1 mol L^{-1} HCl was added, and the samples were homogenized for 30 s by vortexing and agitated for 1 h at room temperature. Next, 0.5 mL aliquots of these samples were added to 0.5 mL of TISAB II modified with 20 g NaOH/L. The FA samples were analyzed in a similar manner as CaF$_2$.

Statistical Analysis

F (CaF$_2$ and FA), Ca, and P (mg/g) concentrations were subjected to a normality test (Shapiro-Wilk's test) and a homogeneity test (Cochran's test) to determine the most appropriate data analysis. Ca, P, and the Ca/P ratio Data showed a normal and homogeneous distribution and were subjected to two-way ANOVA followed by Tukey's test. F concentration data were subjected to logarithmic transformation and then to two-way ANOVA followed by Student-Newman-Keuls' test. The SigmaPlot 12.0 statistical software was used and a significance level of 5% was adopted.

Results

Synthetic HA powder was subjected to heating at 1,000° C for 2 h, and it showed a Ca/P proportion of 1.98

(0.05) (mean (SD)). Concentrations (mg/g) (mean (SD)) of Ca, P, FA, and CaF_2 in synthetic HA were 587.6 (91.5), 203.8 (17.1), 0.10 (0.01) and 0.06 (0.01), respectively. The Ca concentration showed a difference between the HA and the HA subjected to the pH-cycling process (without F and TMP) (p=0.002), but no significant difference was observed for P (p=0.228), FA (p=0.388), and CaF_2 (p=0.302) concentrations.

Increase in the percentage of TMP in the solutions resulted in a higher Ca content in HA. The samples with TMP concentration between 0.2% and 1% as well as 6% and 10% differed in their CA content from the group containing no TMP (p<0.001). When TMP was associated with F (100 ppm), the samples with 0.4% (p<0.001) and 0.6% (p=0.023) TMP showed higher Ca values than the group with no TMP (100 ppm of F and 0% TMP). TMP percentages higher than 1.0, in the presence of F, had lower Ca concentrations than the other groups (p<0.001) (Fig. 1A).

With respect to P concentration, for the group without F, only the samples containing 0.4%, 0.6%, and 2% TMP differed from the group with 10% TMP (p<0.001). In the presence of F, the samples containing 0%, 0.2%, 4.0%, 6.0%, 8.0%, and 10% TMP showed different P concentrations compared to the samples without F (Fig. 1B).

The groups containing F showed higher values of CaF_2 and FA than the groups containing no F (p<0.001). For both CaF_2 and FA, in the groups with no F, similar values were observed among the different evaluated concentrations (p>0.05). Samples with 100 ppm F containing 0% and 0.1% TMP showed the highest concentrations among the investigated samples (p<0.001). Further values for the variation of CaF_2 and FA with TMP concentration, associated with F are in Figures 2A and 2B.

Groups with TMP concentrations of 0.2% and 0.4% and associated with 100 ppm F showed higher and similar (p=0.998) Ca/P ratios when compared to the other groups (p<0.001). Furthermore, the group containing 0.6% TMP and 100 ppm F showed similar ratios as the groups containing 0.2% (p=0.571) and 0.4% (p=0.843) TMP as well as F. The Ca/P ratio in the group without F was lower than in the group containing F, especially within the range from 0% to 0.8% TMP (p<0.001). In both groups (with and without 100 ppm F), with increase of TMP concentration, a higher Ca/P ratio was observed up to a concentration of 2% TMP.

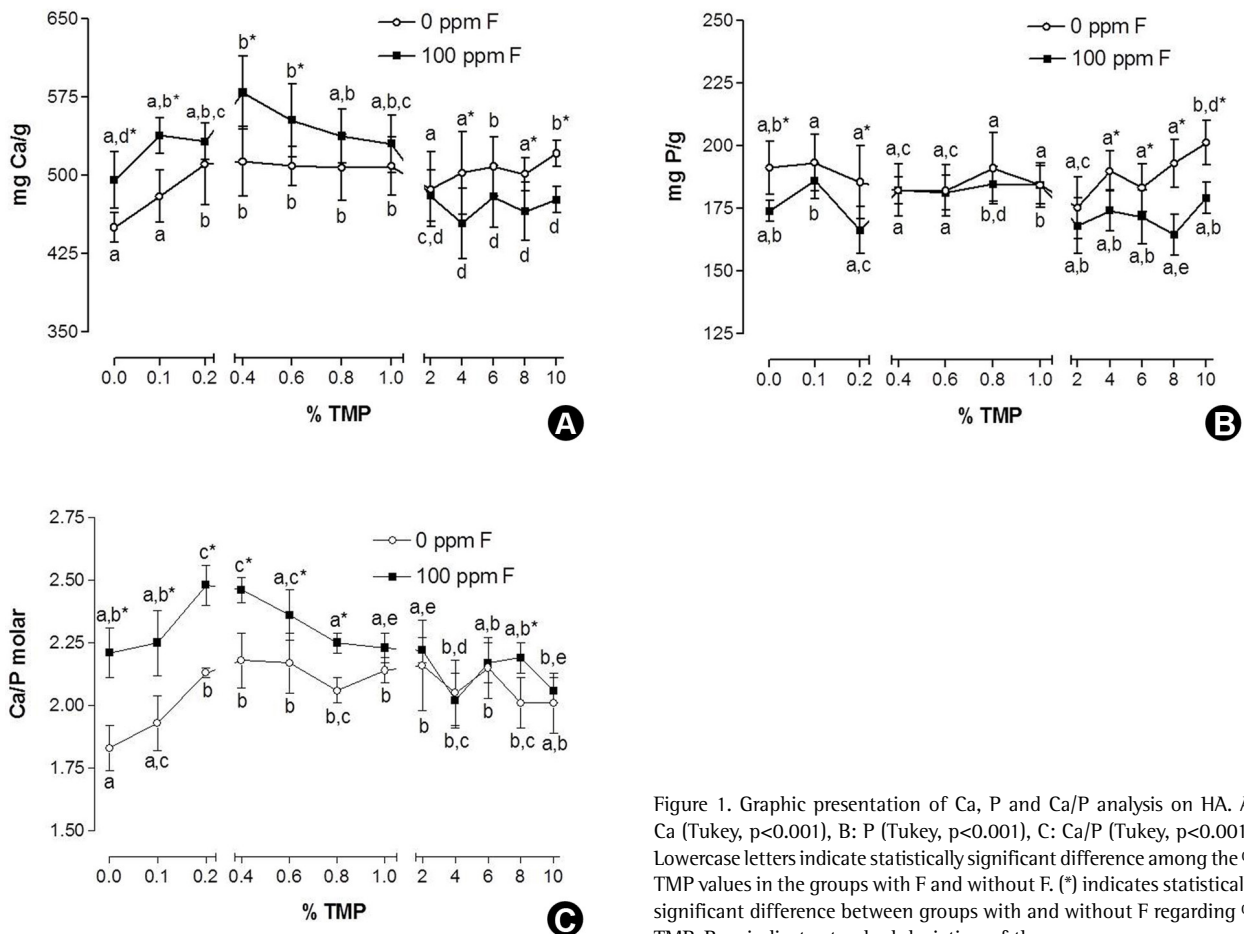*Sodium trimetaphosphate on hydroxyapatite*

Figure 1. Graphic presentation of Ca, P and Ca/P analysis on HA. A: Ca (Tukey, p<0.001), B: P (Tukey, p<0.001), C: Ca/P (Tukey, p<0.001). Lowercase letters indicate statistically significant difference among the % TMP values in the groups with F and without F. (*) indicates statistically significant difference between groups with and without F regarding % TMP. Bars indicate standard deviation of the mean.

Other comparisons can be observed in Figure 1C.

Discussion

Several studies have suggested that TMP reduces the demineralization process, and that an ideal TMP/F ratio allows an enhancement of the effects of F-containing products (7,8,10-13). When TMP and F are co-administered, the adsorption of TMP on the enamel surface can change the selective permeability and facilitate the diffusion of ions such as Ca and F (12) into the enamel (20,21). In order to determine which TMP concentration would produce the optimum effects for mouthrinses, this study evaluated the supplementation of different TMP concentrations (between 0 and 10%) with and without 100 ppm F.

An *in vitro* model and a heated HA powder was used to simulate dissolution and precipitation for the evaluation of the effect of TMP and/or F on HA. However this is a chemical model and therefore it presents limitations inherent to all *in vitro* protocols: they are not able to reproduce the complex intraoral conditions and to mimic solid surface area/solution ratios. Thus, the data obtained using this model (or any other *in vitro* protocol) should be considered carefully. This statement is supported by data from this study that observed reduction in F concentration in the hydroxyapatite with the addition of TMP, while in the study by Favretto et al. (8) there was an increase of F in the enamel with 0.4% TMP. The adsorption of TMP on enamel surface can modify the selective permeability of the enamel by facilitating the diffusion of ions (12,14,20). In the present study, there was not an organized solid enamel surface and the hydroxyapatite crystals were free to react promptly with the TMP and F.

According to Elliott (22), in addition the stoichiometric

HA, there are non-stoichiometric apatites in the form of Ca-deficient apatites (with a Ca/P molar ratio from 1.5 to 1.667) and Ca-rich apatites (with a Ca/P molar ratio greater than 1.667). The HA was precipitated by a wet method and subsequently heated to reduce the amount of impurities, resulting in a HA with a Ca/P ratio of over 1.66 (15,23,24). The pH-cycling model used showed a reduction in the Ca/P ratio of the control group (no TMP or F), which was mainly caused by loss of Ca from HA (Fig. 1A and 1B). The loss of Ca was lower when TMP was present in the pH-cycling solution, resulting in an exponential reduction according to the concentration of TMP, and stabilizing at 0.2% or higher TMP concentrations. The loss of P from the crystal showed little variation, which resulted in a higher Ca/P ratio, confirming the hypothesis that TMP reduces enamel demineralization by reducing the loss of Ca (10). In the presence of 100 ppm F, the Ca/P ratio showed a peak at 0.4% TMP and a subsequent gradual reduction until it intersects at a concentration of 2% TMP, with the curve for HA treated with TMP only. Despite the suggestions that TMP and F have different mechanisms of action or different sites of action (14), the TMP/NaF ratio is important for optimizing the anti-caries effect (7,12). According to the TMP/F ratio, this association influences the loss of Ca from HA (Fig. 1A).

An increase in TMP concentration led to the exponential reduction of F (FA and CaF_2) on HA, reaching a loss of 99% at 0.2% TMP concentration. The adsorption of TMP to HA seems to involve the same binding sites as those of F and can therefore interfere with its action depending on the TMP concentration. This may be related to the low F concentration used in this study, which is too low to compete with the adsorption capacity of the TMP to HA.
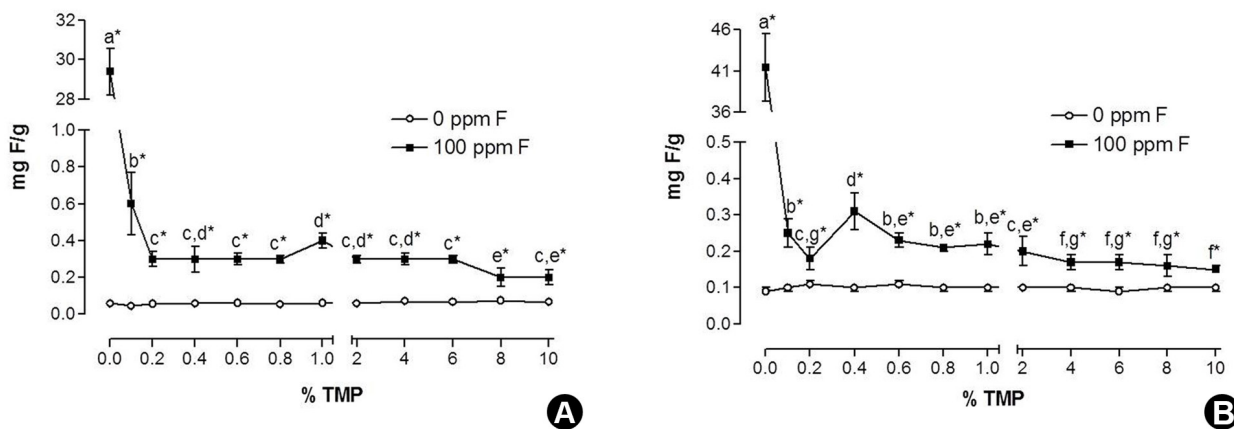

Figure 2. Graphic presentation of F analysis on HA. A: CaF_2 (Student-Newman-Keuls, p<0.001). B: FA (Student-Newman-Keuls, p<0.001). Lowercase letters indicate statistically significant difference among the % TMP values in the groups with F and without F. (*) indicates statistically significant difference between groups with and without F regarding % TMP. Bars indicate standard deviation of the mean.

On the other hand, Favretto et al. (8) reported that the addition of 0.4% TMP increased F concentration in enamel. In this case, the adsorption of TMP occurs on the enamel surface and it acts facilitating the diffusion of Ca and F (12,14,20). Yet, even though the deposition of the two F forms on HA is reduced, the presence of a relatively low amount of TMP (between 0.2% and 0.6%) improved the Ca/P ratio. The synergistic effect of TMP and F increased the Ca/P ratio with 16% to 9% compared to the groups treated without F. However, above 1% TMP, the combination of TMP and F does seem to increase the loss of Ca, probably by interfering with the remineralization of HA. Thus, other F concentrations should be evaluated in order to optimize the synergy between these components.

The outcomes of the present study may explain those of Manarelli et al. (7) and Favretto et al. (8), who observed a reduction in enamel demineralization mainly at the concentration of 0.4% TMP in the presence of 100 ppm F. Van den Hoek (25) found that F incorporation into the apatite structure increases its crystallinity and the Ca/P ratio, which was also observed in this study for several TMP concentrations in the presence of F. Ca/P ratios were high compared to the samples without F and this can be explained by a lower loss in Ca and P content. The present study also showed that the 1.2:1 and 3.7:1 TMP/NaF ratios, which are similar to the ratios used by Manarelli et al. (7) and Favretto et al. (8), were able to precipitate the HA with high Ca/P ratio and low solubility. However, TMP interfered with the deposition of F on HA.

Based on the obtained results, it may be concluded that TMP at 0.2%, 0.4% and 0.6% concentrations combined with F seemed to be able to precipitate HA with low solubility. However, especially at high concentrations, TMP interferes with F deposition on HA.

Resumo

Este estudo avaliou o efeito de diferentes concentrações de trimetafosfato de sódio (TMP) com e sem fluoreto (F) nas concentrações de cálcio (Ca), fósforo (P) e F na hidroxiapatita (HA). Pó de HA sintético (0,15 g) foi suspenso (n=6) em soluções (75 mL) de TMP com concentrações de 0%, 0,1%, 0,2%, 0,4%, 0,6%, 0,8%, 1,0%, 2,0%, 4,0%, 6,0%, 8,0% e 10% na presença ou na ausência de 100 ppm F e foram submetidas ao processo de ciclagem de pH. O precipitado foi filtrado seco a 70°C por 24 h e triturado em um pó fino. As concentrações de F (solúvel em KOH: CaF_2, e em HCl: FA), Ca (Arsenazo III) e P (método do molibdato) foram determinadas na HA. Os dados de Ca, P e de proporção Ca/P foram submetidos ao teste de Tukey e os dados de F ao teste Student-Newman-Keuls (p<0,05). A adição de TMP reduziu a deposição de F em 98% (p<0,001). Os grupos contendo 100 ppm F e TMP 0,4% e 0,6% apresentaram maiores concentrações de Ca do que o grupo contendo somente 100 ppm F (p<0,05). Além disso, a HA tratada com 0,2% e 0,4% de TMP e 100 ppm F apresentou maiores proporções Ca/P em relação aos demais grupos (p<0,001). Em conclusão, TMP nas concentrações de 0,2%, 0,4% e 0,6% quando associado ao F é capaz de precipitar uma HA com menor solubilidade. Entretanto, especialmente em altas concentrações, TMP interfere com a deposição de F na HA.

Acknowledgements

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