Title: In situ evaluation of fluoride-, stannous- and polyphosphate-containing solutions against enamel erosion

Short title: fluoride, stannous and polyphosphates against erosion

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This is the author's manuscript of the article published in final edited form as:

João-Souza, S. H., Bezerra, S. J. C., de Freitas, P. M., de Lima, N. B., Aranha, A. C. C., Hara, A. T., & Scaramucci, T. (2017). In situ evaluation of fluoride-, stannous- and polyphosphate-containing solutions against enamel erosion. Journal of Dentistry. https://doi.org/10.1016/j.jdent.2017.05.014

Abstract

Objective: To evaluate the anti-erosive effect of solutions containing sodium fluoride (F: 225 ppm of fluoride), sodium fluoride + stannous chloride (F+Sn: 225 ppm of fluoride + 800 ppm of stannous), sodium fluoride + stannous chloride + sodium linear polyphosphate (F+Sn+LPP: 225 ppm of fluoride + 800 ppm of stannous + 2% of sodium linear polyphosphate), and deionized water (C: control), using a four-phase, single-blind, crossover in situ clinical trial.

Methods: In each phase, 12 volunteers wore appliances containing 4 enamel specimens, which were submitted to a 5-day erosion-remineralization phase that consisted of 2h of salivary pellicle formation with the appliance in situ, followed by 2min extra-oral immersion in 1% citric acid (pH 2.4), 6x/day, with 90min of exposure to saliva in situ between the challenges. Treatment with the test solutions was performed extra-orally for 2min, 2x/day. At the end of the experiment, surface loss (SL, in µm) was evaluated by optical profilometry. Data were analyzed using ANOVA and Tukey tests (α=0.05). The surface of additional specimens was evaluated by x-ray diffraction after treatments (n=3).

Results: C (mean SL ± standard-deviation: 5.97±1.70) and F (5.36±1.59) showed the highest SL, with no significant difference between them ($p>0.05$). F+Sn (2.68±1.62) and F+Sn+LPP (2.10±0.95) did not differ from each other ($p>0.05$), but presented lower SL than the other groups ($P<0.05$). Apatite and stannous deposits on specimen surfaces were identified in the x-ray analysis for F+Sn and F+Sn+LPP.

Conclusions: Sodium fluoride solution exhibited no significant anti-erosive effect. The combination between sodium fluoride and stannous chloride reduced enamel erosion, irrespective of the presence of linear sodium polyphosphate.

Clinical significance: Under highly erosive conditions, sodium fluoride rinse may not be a suitable alternative to prevent enamel erosion. A rinse containing sodium fluoride and stannous chloride was shown to be a better treatment option, which was not further improved by addition of the sodium linear polyphosphate.

Keywords: enamel; erosion; fluoride; stannous chloride; phosphate polymer

Introduction

Dental erosion is a complex condition that affects different age groups in populations worldwide [1]. The overall increase in its presence could be related to changes in lifestyles and nutritional habits, with a higher consumption of acidic foods and beverages [1,2]. In addition to avoiding exposure to erosive sources, the use of fluoridated products is highly

recommended for patients with erosion [3]. However, their effectiveness against erosion seems to be dependent on the type of fluoridated compound, F concentration and pH. Many studies have tested the anti-erosive ability of F solutions containing metal cations, such as stannous (Sn), with promising outcomes [4–7]. Sn can incorporate into enamel through a complex process of demineralization and reprecipitation; it can also induce the surface deposition of acid-resistant precipitates [8]. In situ investigations have shown that a solution containing 500 ppm F and 800 ppm Sn was able to reduce enamel and dentin loss in the range of 45-67% and 47-68%, respectively [4,5].

Despite these positive results, studies have demonstrated that the protection offered by F and Sn can be increased by combining them with some polymers. A dentifrice containing F, Sn and the biopolymer chitosan showed improved enamel erosion protection compared with a dentifrice containing F+Sn alone [9]. A previous in vitro investigation by our group demonstrated that the addition of a phosphate polymer (sodium linear polyphosphate - LPP) could increase the protection of a solution containing 225 ppm F and 800 ppm Sn by 11% under highly erosive conditions [6], irrespective of the presence of simulated salivary pellicle [7].

The salivary pellicle is important when evaluating film-forming agents such as LPP, due to the possibility of competition for binding sites on the enamel surface [10]. The salivary pellicle formed in vitro is known to differ from the in situ because, among other changes, the proteins of the saliva collected can undergo alteration or degradation [11,12]. Considering this fact, this study sought to evaluate the protective effect of the combination of F+Sn+LPP against erosion under more clinically relevant conditions, such as those achieved in in situ models. Our hypothesis was that LPP would improve the protective effects of F+Sn against enamel erosion.

Materials and methods

Experimental design

This study consisted of a four-phase, single-blind crossover *in situ* clinical trial, involving 12 volunteers who met the inclusion/exclusion criteria described in detail elsewhere [13]. Briefly, the volunteers were at least 18 years old, with good general and oral health, without any allergy or any other condition that could compromise their safety. Their unstimulated and stimulated salivary flow rate had to be ≥ 0.5 ml/min and ≥ 1 ml/min, respectively. The exclusion criteria were: pregnancy (or intention to become pregnant) during the study period, nursing, concomitant participation in another research study, and inability to comply with study procedures. In each study phase, the volunteers used removable mandibular devices containing 4 specimens of bovine enamel. The study followed a completely randomized experimental design, with test solution as the single experimental

factor, at 4 levels: C: Control (deionized water); F: Sodium fluoride solution (11.83 mM of NaF; 225 ppm of fluoride; pH 4.5); F+Sn: Sodium fluoride plus stannous chloride solution (11.83 mM of NaF + 10.75 mM of $SnCl_2$; 225 ppm F, 800 ppm Sn; pH 4.5); F+Sn+LPP: Sodium fluoride, stannous chloride and sodium linear polyphosphate solutions (11.83 mM of NaF + 10.75 mM of $SnCl_2$ + 2% of LPP; 225 ppm F, 800 ppm Sn; pH 4.5). The response variable was tooth surface loss, in µm, determined by optical profilometry at the end of the clinical phase. As an additional test, the surface of extra enamel specimens was evaluated by x-ray diffraction after treatments (n=3).

Ethical Aspects

This study was conducted in the Restorative Dentistry Department of School of Dentistry, University of São Paulo, São Paulo, SP, Brazil. The study protocol was reviewed and approved by the local Ethics Committee on Research with Humans (CAAE: 27621214.9.0000.0075). To participate in the study, all subjects had to sign a term of free and informed consent.

Sample size

For this *in situ* study, 12 subjects were recruited. This sample size was chosen based on a previous study [14] with a similar design, which showed significant difference between experimental groups using a sample size of 10 individuals.

Study population

The recruitment of the subjects was carried out in the Restorative Dentistry Department of School of Dentistry, University of São Paulo. First, the subjects were informed about the nature of the study, its possible risks and data confidentiality. After agreeing to participate, their medical and dental history was evaluated. Unstimulated and stimulated salivary flow rates were measured using established procedures, as previously described [13].

The subjects who met the inclusion/exclusion criteria received an oral hygiene kit containing a toothpaste (Colgate Cavity Protection, 1500 ppm F, Colgate-Palmolive, Osasco, SP, Brazil), a regular toothbrush (Colgate Twister Fresh, Colgate-Palmolive, Osasco, SP, Brazil) and dental floss, to be used on the 7 days before the study began (lead-in phase) and throughout the entire study period. They were not allowed to use any other oral hygiene products. Subjects were instructed to perform oral hygiene twice a day, with the oral appliance removed from the mouth. They were also advised not to brush their teeth with toothpaste in the 2 h prior the beginning of the experimental procedures, and also in the 30 min after eating.

All the eligible subjects were identified by a unique study number. In each week, they were randomly assigned to the treatment solutions according to a standard randomization table. Before the study began, the subjects were thoroughly trained in all experimental procedures, and they received a written protocol containing all the instructions. In each study phase, they also received a schedule and a digital timer to guide their conduct and recording of the experimental procedures.

Intraoral device

An impression of each subject's mandibular arch was taken with heavy consistency condensation silicone (Clonage®, DFL, Jacarepagua, RJ, Brazil). From the impressions, bi-lateral mandibular intraoral appliances were prepared with acrylic resin [15]. In these devices, four niches of approximately 4 mm x 4 mm x 2 mm were made on the buccal surfaces of the premolars and molars.

The intraoral devices were disinfected with 2% chlorhexidine solution for 10 min before and after each study phase, and rinsed with tap water. Before mounting the specimens in the appliances, they were sterilized with gamma radiation (Experimental irradiator Cobalt-60, Gamacell 220, IPEN, São Paulo, SP, Brazil). The day before each phase began, the sterilized specimens received adhesive unplasticized polyvinyl chloride (UPVC) tapes on their polished surface, leaving a central area of 3 mm x 1 mm exposed. The specimens were fixed in the 4 niches with sticky wax, so that their surfaces remained 1 mm below the appliance surface, to avoid abrasion of the buccal soft tissues.

Specimen preparation

Enamel specimens were prepared from bovine incisors that were firstly cleaned with periodontal curettes (Hu-Friedy, Chicago, USA), and subjected to prophylaxis with a mixture of pumice and water applied with rubber cup at low speed. The teeth were kept in 0.1% thymol solution (Sigma-Aldrich Co.), at 4°C, until the experiment began. The crowns were separated from the roots. Then enamel specimens measuring 3 mm x 3 mm x 1.5 mm were sectioned from the buccal sides of the crowns, by using a precision cutting machine (Isomet 1000, Buehler Ltd, Lake Buff, Illinois, USA). The pulp surfaces of the specimens were flattened with a polishing machine (Buehler Ltd, Lake Buff, Illinois, USA), fitted with a #600 grit abrasive disc (Buehler Ltd), under constant water cooling. Subsequently, the buccal surfaces were ground flat and polished using a sequence of abrasive discs with decreasing granulations: #600, #1200, #2400 and #4000 (Buehler Ltd), and polishing cloth sprayed with diamond suspension (1 µm, Buehler Ltd) for 3 min. At the end of the polishing procedures, the specimens were sonicated with distilled water for 3 min. Specimens without any cracks or structural defects were selected.

The surface microhardness (SMH) of the specimens was analyzed. Three indentations were made in the central area of the specimens with a Knoop indenter (Shimadzu Co, Tokyo, Japan), using a load of 50 g for 15 s, with a distance of 100 μm between each indentation [16]. The mean value of the 3 indentations was calculated, and specimens with similar SMH were then selected. These specimens were further analyzed with an optical profilometer (Proscan 2100, Scantron, Tauton, UK) to identify those with surface curvature below 0.3 μm, which were finally included in this study.

Experimental solutions

The experimental solutions are described in Table 1. The NaF (Sodium fluoride, Sigma Aldrich, St Louis MO, USA), LPP (Sodium polyphosphate, Merck Milipore, Darmstadt, Germany) and Sn (Stannous chloride, Sigma Aldrich Co.) concentrations were in accordance with those of previous studies [6,7,17]. Deionized water was used as control. All the solutions (except the water) had the pH adjusted to 4.5, with KOH solution or concentrated HCl. For the erosive challenge 1% citric acid (Sigma-Aldrich, St. Louis, MO, USA) was used, with natural pH of 2.4 [6].

Erosive challenge

At the beginning of each day, subjects wore the intraoral devices for 2 h to allow salivary pellicle formation. After this, the specimens were immersed in 20 ml of the acid solution extra-orally, for 2 min, 6x/day, with 90-min intervals between them, during which specimens were exposed to saliva in situ.

After each erosion challenge, the excess acid was removed with absorbent paper and the intraoral devices were returned to the mouth. For the treatments, the specimens were immersed in 10 ml of their respective experimental solution for 2 min, 2x/day, 45 min after the first and the last erosive challenges. The excess solution was also removed with absorbent paper after treatments. To avoid contact of the individual´s teeth with the acidic solution and with the experimental solutions, the intraoral devices containing the specimens were immersed in the solutions extra-orally. All solutions were renewed for each exposure. Figure. 1 shows a flowchart of the experimental procedures.

The intraoral devices were used during the day, except during the meals and oral hygiene procedures, when they were stored in containers with gauze moistened with distilled water. The intraoral devices were also stored in these containers during the overnight period, under refrigeration. Between the study phases, a wash out period of 7 days was incorporated into the study design.

Surface loss

At the end of the experimental procedures, the specimens were removed from the intraoral devices and had the adhesive tapes removed from their surfaces. Subsequently, a central area of 2 mm x 1 mm of the specimen surface was scanned with an optical profilometer, according to the following parameters: 200 steps of 0.01 mm in the X axis and 20 steps of 0.05 mm in the Y axis. This analysis covered both reference surfaces and treated surface. For the surface loss assessment, specific software was used (Proscan Application Software version 2.0.1.7).

Additional test

As an additional test, 12 extra specimens (3 for each group) were prepared as described before, and treated with the test solutions for 2 min. Afterwards, they were rinsed in distilled water and evaluated by x-ray diffraction, using a Rigaku diffractometer (Rigaku Americas, The Woodlands, Texas, USA), model DMAX-2000, equipped with a chrome tube and a vanadium filter. A Grazing incidence angle of 2.5°, 2θ varying from 380 a 1300 was used. All measurements were performed on the specimen surface plane.

Data analysis

Solutions were compared for differences in mean surface loss using an ANOVA suitable for a crossover study, which included a random effect for subject and fixed effects for treatment sequence, study phase and solution. Pair-wise comparisons were made using the Tukey multiple comparisons procedure. The level of significance was set at 5%. The diffraction patterns obtained were compared with the data from the International Centre for Diffraction Data (ICDD).

Results

The selected specimens presented a mean SMH value (standard-deviation) of 335 (25) and a mean (SD) surface curvature of 0.21 (0.07). All volunteers completed the study.

ANOVA showed a significant difference in surface loss among the solutions ($p<0.001$). Figure 2 shows the means and standard deviations (SD) of the profilometry analysis for each experimental group.

Control and F showed the highest surface loss, with no significant difference between them. F+Sn and F+Sn+LPP did not differ significantly and presented significantly lower surface loss than C and F.

The patterns obtained in the x-ray diffraction analysis for the 4 experimental groups are shown in Figure 3. The matrix was identified as potassium calcium hydrogen carbonate phosphate hydrate as ICDD file No. 47-260. The arrows in the patterns of groups F+Sn and

F+Sn+LPP suggested the appearance of the phase of $Sn_3F_3PO_4$, as ICDD file No. 76-2280, in very low concentration and only at the surface.

Discussion

In this study, LPP was unable to improve the protection of F+Sn against enamel erosion, leading to rejection of our hypothesis. This result was unexpected and contrasts with our previous laboratory findings [6,7]. We speculated that the lack of additional protection by the LPP may be explained by the presence of the naturally-formed acquired salivary pellicle in the present study. The salivary pellicle is an organic layer, mainly composed of adsorbed salivary proteins, which covers the dental structures in the natural oral environment [18,19]. Many of the pellicle proteins contain calcium-binding domains [20], presenting high affinity to the enamel surface, which could have occupied the potential sites for LPP interaction, thereby reducing its effectiveness. Although the salivary pellicle was simulated with clarified human saliva in our previous in vitro study [7], we speculated that the naturally formed pellicle presented different structural and maturation levels, having a different influence on the LPP binding to enamel and subsequent protective effect. To allow the pellicle formation and relative maturation in the present in situ study, the volunteers wore the oral devices for 2h before the experimental procedures [21].

Another possible explanation for the lack of LPP effect would be the reduction in the frequency of application, which was previously used 3 times a day in vitro [6,7] versus the 2 times used in the present study. The frequency of application was reduced to resemble the clinical scenario more faithfully, as mouth rinses are frequently used only once or twice a day [15]. It could be suggested that higher frequency would allow prolonged protection, which would be translated into lower enamel surface loss. However, in a preliminary in vitro test (unpublished data) we observed no additional protection when F+Sn+LPP solution was applied three times a day. Therefore, it is unlikely that exposure to additional LPP rinses would lead to increased protection.

Although extensive in vitro investigations were performed to determine the optimal concentration of LPP used in this study, we suggest that values higher than 2% may be needed to lead to enamel protection in situ. Condensed inorganic phosphates, such as LPP, also have the ability to complex with polymers, especially proteins [22]. Therefore, in the right concentration, it could adsorb to the salivary pellicle potentially increasing its anti-demineralization ability. Corroborating this idea, a previous in situ study revealed that the addition of 9% sodium hexametaphosphate to a 1% NaF gel reduced enamel surface loss more than the 1% NaF gel did without it [23]. Another investigation reported that the addition of 5% trimetaphosphate to a 2.5% NaF varnish increased its protection against erosion and erosion-abrasion [24]. Nevertheless, it has to be born in mind that higher concentrations of

phosphate polymers influence the properties of the solution, such as viscosity, and may interfere in the interaction between F/Sn and the enamel surface. This may be a concern especially when dealing with long chain length polymers. Further studies are needed to verify the feasibility of using higher LPP concentrations.

In line with previous reports, the combination between F and Sn reduced enamel erosion [4,6,17]. This could be attributed to the formation of less soluble precipitates, such as Sn_2OHPO_4, $Sn_3F_3PO_4$ and $Ca(SnF_3)_2$, as described in a study using x-ray diffraction to identify these crystalline compounds [25]. In the cited study, a SnF_2 solution with a higher concentration was used, and the pattern of diffraction was obtained by analyzing hydroxyapatite powder and not the enamel surface per se. In the present investigation, the x-ray diffraction analysis of enamel samples treated once with the Sn-containing solutions yielded the presence of $Sn_3F_3PO_4$, at very low concentration. It can be suggested that the low concentrations of F and Sn present at the solutions did not allow for a detectable amount of precipitates by this method, which is 2% of the component in the sample. In addition, the evaluation was performed in enamel specimens and not in hydroxyapatite powder, which has a higher surface area for the precipitation of deposits. This result may strengthen the theory that in the context of erosion, Sn incorporation into enamel would be more relevant for surface protection than the formation of less soluble precipitates [8].

In situ studies have shown positive results with sodium fluoride rinses in the prevention of enamel erosion, with fluoride concentrations ranging from 500 to 950 ppm F [4,26,27]. However, the present study failed to show a difference between the control and the fluoride (F) groups. This could be related to the low concentration of F used (225 ppm F), which was chosen in attempt to simulate the concentration usually found in commercially available oral rinse products. Probably, at the low pH (4.5) and concentration used, only little CaF_2-like deposits were formed. The pH of 4.5 was chosen for the F solution to avoid having a confounding factor, because the Sn-containing solutions are not stable at higher pH levels [7]. It has to be considered that the protocol of some in situ studies required the erosive challenge and treatments to be performed intra-orally, in contrast to the present study, in which they were used extra-orally, because of the experimental nature of the testing solutions. Intraoral exposure to the fluoridated rinses may allow more sites to be found for F retention, such as the soft oral tissues, which could potentially increase F availability [28]. Moreover, when rinsing is performed intra-orally, it may lead to F interaction with the calcium from the saliva, allowing the formation of more CaF_2-like material. Finally, the in situ model used simulated highly erosive conditions, with successive episodes of exposure to acid and no exposure to saliva overnight. This experimental condition could have minimized the remineralization enhancing action of the test solutions, which could also explain the reduced protection observed by the F solution [29].

Conclusions

In conclusion, sodium fluoride associated with stannous chloride was capable of reducing enamel erosion; however, this effect was not improved by the presence of the sodium linear polyphosphate. Further studies are needed to verify the effectiveness of LPP in improving F+Sn protection by using a higher LPP concentration.

Acknowledgements

The authors would like to thank São Paulo Research Foundation (FAPESP) for the grant received (#2014/14055-4) and George J. Eckert for conducting the statistical analyses.

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Legend of the Figures

Figure 1. Flowchart showing the sequence of the experimental procedures.

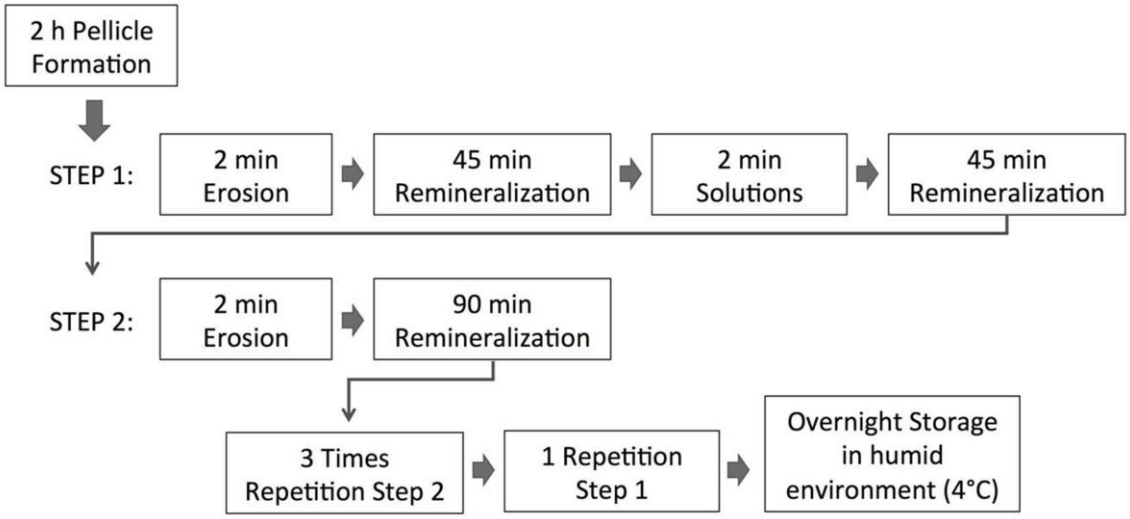

Figure 2. Mean (SD) of surface loss, in µm, for the experimental groups. Different letters indicate significant difference among groups ($p<0.05$).

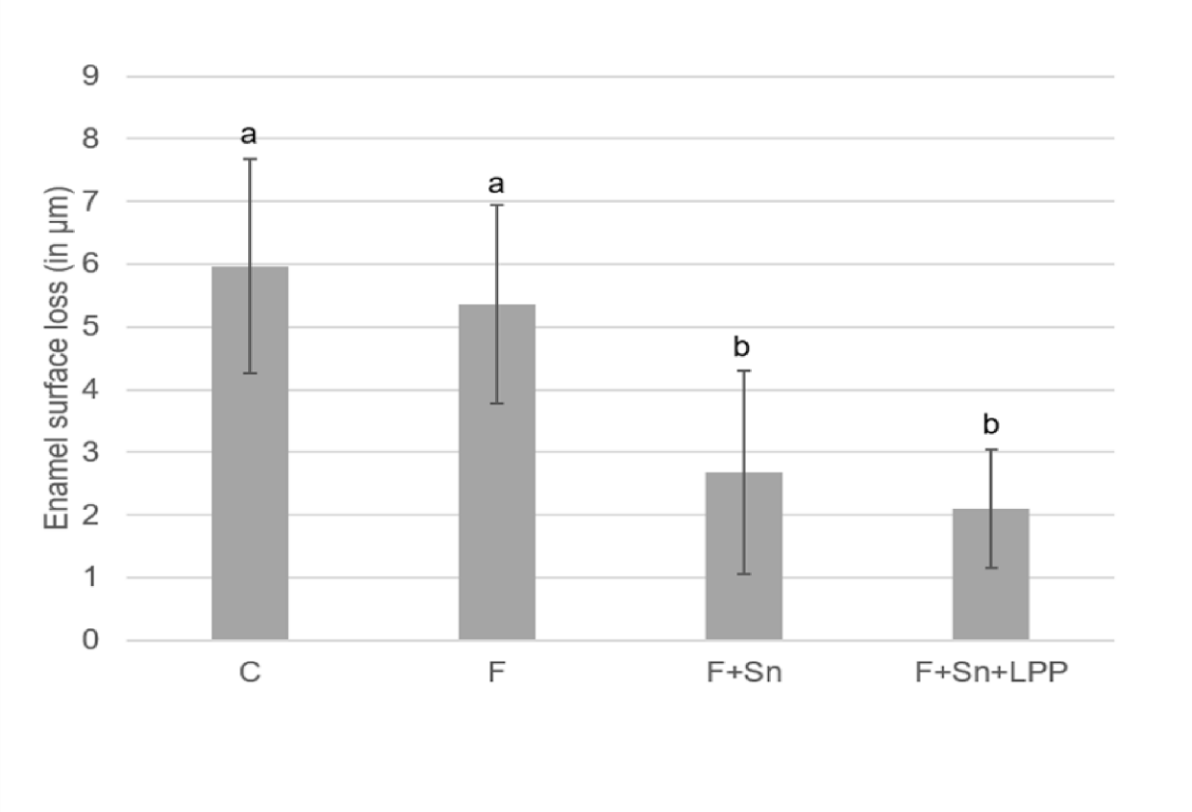

Figure 3. Diffraction Patterns of the experimental groups, indicating the presence of apatite. The arrows suggest the presence of the phase $Sn_3F_3PO_4$ in groups F+Sn and F+Sn+LPP.

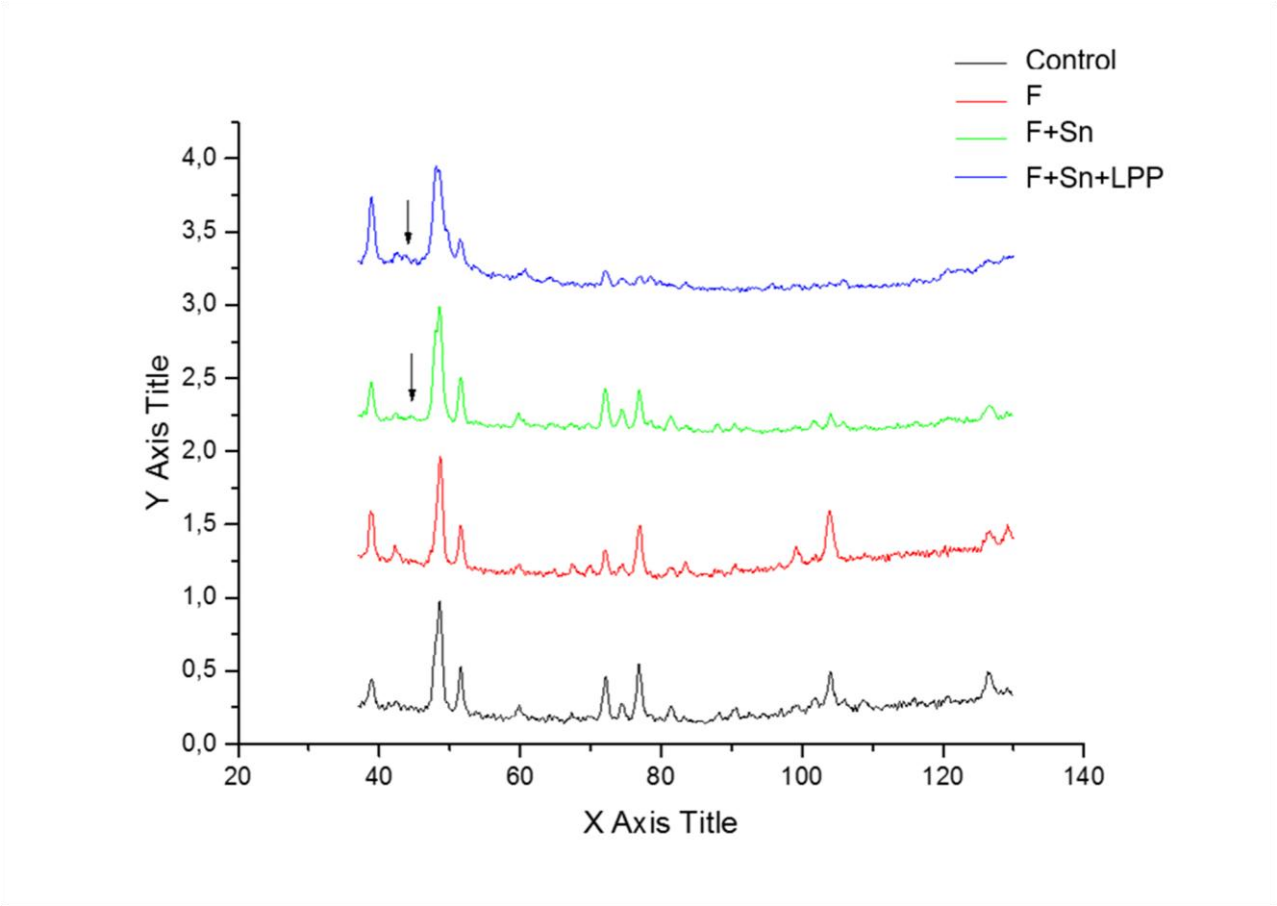

Table 1. Experimental solutions, reagents, concentrations and pH values.

Experimental solutions	Reagents	Concentration (g/l)	pH
C – Control (distilled water)	N/A	N/A	5.70
F – Sodium fluoride solution	NaF	0.497	4.50[1]
F+Sn - Sodium fluoride and stannous chloride solution	NaF + $SnCl_2$	0.497+1.277	4.50[1,2]
F+Sn+LPP - Sodium fluoride, stannous chloride solution and sodium linear polyphosphate	NaF + $SnCl_2$ + LPP	0.497+1.277+20	4.50[1,2]

[1] *The pH was adjusted to 4.5 with 1M of concentrated KOH or HCl*

[2] *2,3 g/l of sodium d-gluconic acid was added to the solution for stabilization purposes* [30]