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In vitro evaluation of the remineralizing effect of two different bioactive principles on bovine tooth enamel using X-Ray fluorescence

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Dental erosion, in general, can be interpreted as a situation of a chronic loss of dental hard tissue that is chemically etched away from the tooth surface by acid and/or chelation without bacterial involvement. Thus, if the erosion process takes place for a period of time, a cavity can be formed (caries lesion). A procedure for inhibiting the formation of erosion and enhance the remineralization process is to use chemical agents. However, the aim of this *in vitro* study was to evaluate the effects of remineralization on bovine enamel using the of X-ray fluorescence technique (XRF). We obtained 15 specimens (CP) whose were randomly divided into three groups with different treatments of the remineralization: Group 1 - Saliva, Group 2 - Fluoride and Group 3 – CG Tooth Mousse. All measurements of X-ray fluorescence were obtained using commercial equipment (Artax™ 200). The evaluated chemicals elements from the XRF spectra were P, Ca e Sr. The remineralization process was first evaluated using the Bonferroni test (α=0.05) for comparison of parametric average loss of P, Ca and Sr in each stage of treatment in the groups. Besides that, it was also used the t Student test (α=0.05) for comparison between means of different treatment groups. The results show that GC Tooth Mousse group was not effective in remineralization of bovine enamel subjected to erosive challenge in this study. Nevertheles, its performance was similar to saliva, and therefore inferior to fluoride.

Keywords: X-Ray fluorescence, tooth erosion, tooth remineralization

1. INTRODUCTION

The quantitative analysis of chemical elements plays an important role in the biomedical area, since some elements may be essential to the physiology of the biological tissue. Several diseases are associated with a deficiency or excess of those elements, interfering with the metabolism of human beings [1].

The teeth are composed by three tissues: enamel, dentin and pulp. The first is composed mainly of mineral, mainly hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. This mineral whenever submitted to acid reactions (pH<4) is cleaved in $10Ca_2^{+}.6H_2PO_4$ and $2H_2O$. This constant dissolution can generate dental erosion. The dental erosion is defined as irreversible loss of tooth structure due to a chemical process, not involving microorganisms, triggered by acid of intrinsic origin (associated with xerostomia) or extrinsic origin (acids from diet, chronic drug use, illicit drug use, water ponds with inappropriate pH due to industrial fumes). In recent years, studies have investigated the effect of fluoride in preventing dental erosion [2, 3]. The conventional dentifrices that contain fluoride do not seem able to effectively protect against erosive challenges. However, there seems to be a growing market for new product development in order to prevent and/or treat tissue loss by mineral dental erosion [4].

The energy dispersive X-ray fluorescence spectrometry (EDXRF) is a multi-elemental and non-destructive analytical technique. EDXRF is a well-established technique that can be applied to many areas of science and technology [5,9]. Teeth analysis have been carried out in many different studies on the influence of environmental pollution on the human body and rarely in the study of dental erosion/remineralization. Thus, EDXRF can be used to determine changes in elemental composition for both eroded surfaces and cross sections, as can also be applied for the detection deposition of active agents in therapeutic treatments on the surface of teeth. The X-ray fluorescence (XRF) is a physical phenomenon that occurs in the process of transition between electron orbitals. The analysis by X-ray Fluorescence is a based on the measurement of the intensities of the characteristic X-rays emitted by elements that constitute one sample (the number of X-rays detected by unit of time). The X-ray beam striking the sample promotes the process of interaction of X-rays with the sample causing the release of the electrons bounded to the atom, consequently less bound electrons in the outermost orbital migrate to these orbital with "holes" left by the ejected electrons. The result of this process is the release of energy as electromagnetic radiation called characteristic X-rays. The energy of the emitted radiation represents the difference of the energies of electrons in the outermost orbital and the ejected electron orbital. This energy difference is characteristic of each element, which can be used as an elemental XRF signature. Thus, by detecting the radiation that is emitted in the process the signatures of the elements in the sample can be identified in the XRF spectrum [8,9].

The objective of this study was to evaluate *in vitro* remineralizing effect of different bioactive materials contained in GC Tooth Mousse (CPP-ACP casein phosphopeptides and amorphous calcium phosphate) products, artificial saliva and neutral sodium fluoride gel on bovine enamel subjected to an erosive challenge using X-ray fluorescence.

2. MATERIALS AND METHODS

2.1. Sample Preparation

In order to achieve the objective, it was used 15 bovine incisors, shown in Figure 1a. The samples were stored in 0.05% thymol for up to one week before the tests. The teeth were sectioned at the cementum-enamel line, discarding the root portion, and stored in ultra-pure water at a temperature of 4°C. Then the teeth were fixed with epoxy resin with the aid of a delineator so that the buccal stay parallel to the horizontal plane of the bench and were contained in PVC rings for 24 hours for the polymerization of the resin, shown in Figure 1b. After that, the tooth was taken to a metallographic polishing, employing the silicon carbide abrasives (abrasives water) abrasive 600 and copious irrigation, thus, made by standardizing planning area. After inclusion and planning, the specimens were immersed in an ultrasonic bath for 5 minutes and then insulated with tape and white enamel so that only six small round areas were exposed to the external environment as shown in Figure 1c. 15 specimens (CP) randomly divided into 3 groups, of 5CP were obtained. Each group was designated to a specific treatment: Group 1 (G1): Saliva (artificial saliva); Group 2 (G2): Fluor (Fluoride gel neutral) and Group 3 (G3): Recaldent (GC Tooth Mousse: CPP - ACP casein phosphopeptides and amorphous calcium phosphate).

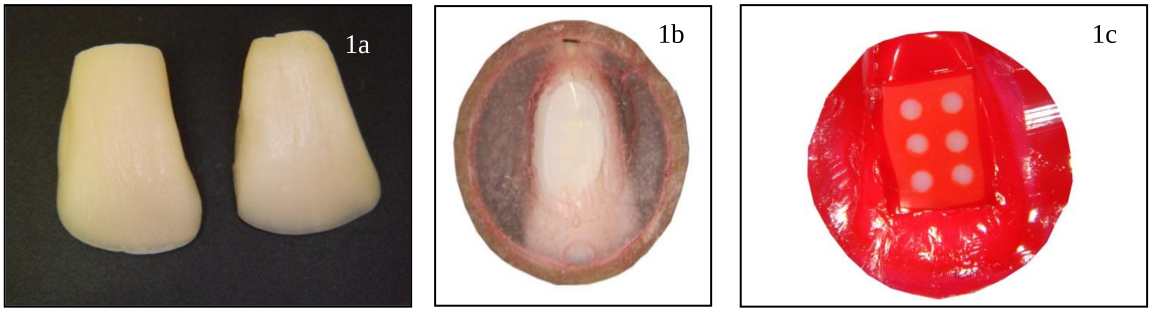

Figure 1: The samples used

2.2. X-ray measurement

All teeth of the three groups were irradiated by the X-ray beam in order to obtain the counts due to P, Ca and Sr from the X-ray spectra. The experimental conditions were: X-ray tube´s high voltage of 30 kV, anode current of 400 μA, molybdenum target and X-ray spectra acquisition time of 100 s. Figure 2a shows ARTAX and geometric set-up of the X-ray beam on the sample. In each CP was performed XRF measurement at predefined 6 small round areas and the X-ray beam is positioned in the central region of the points as shown in Figure 2b.

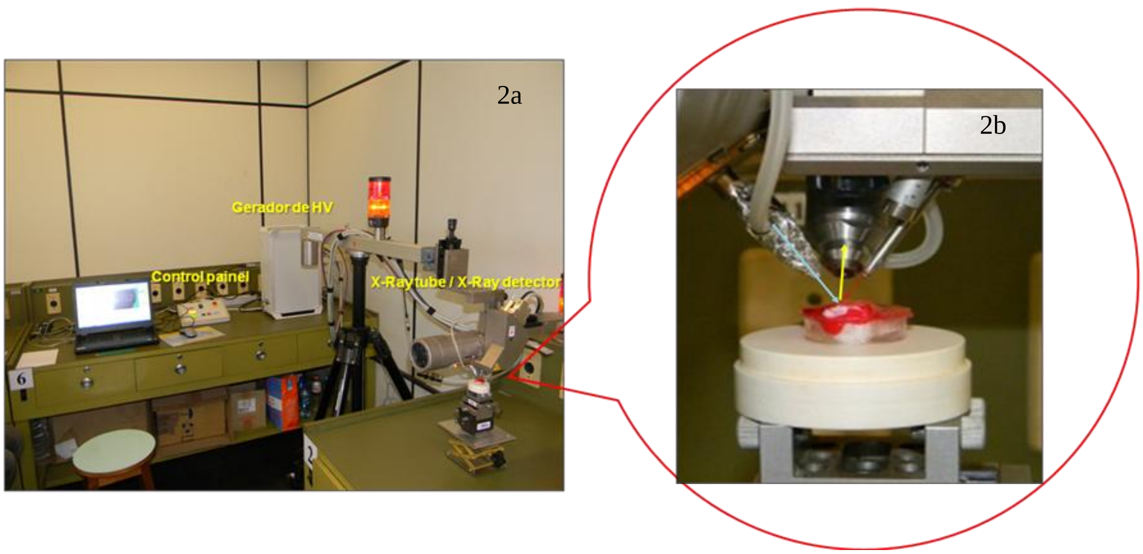

Figure 2: ARTAX with zoom of geometric set-up of the X-ray beam on the sample

After XRF measurements, all tooth samples, were stored for 7 days in Milli-Q water at a temperature of 4°C. After this period the specimens suffered erosive challenge. The erosion process was carried out with citric acid following the procedure: each group of samples was immersed in a beaker identified in a solution of 10 ml of 2% citric acid pH 2.6 for 90 minutes. After that, the teeth were washed with ultrapure water Milli-Q and dried. Subsequently, a new sequence of XRF measurements was performed at the same points. Immediately after obtaining the XRF spectra tooth samples were submitted to erosive challenge, each group received a corresponding treatment. The samples were stored in separate bottles and labeled in correspondence with the respective treatment. XRF measurements were performed seven days after the treatment of the samples and repeated for three consecutive weeks.

3. RESULTS AND ANALISYS

Figure 3 shows a typical XRF spectrum of the bovine enamel. This spectrum was obtained during analysis of a sample of bovine teeth using the Artax™ 200. In addition, it can be seen the presence of the chemical elements P, Ca and Sr. Phosphorus and calcium are major elements of the matrix of tooth enamel (hydroxyapatite). Moreover, it can be observed the presence of the element strontium in the XRF spectrum because of its chemical affinity with calcium. Table 1 shows the results obtained for the identified chemical elements using the XRF technique before any treatment and after three weeks of remineralization treatment.

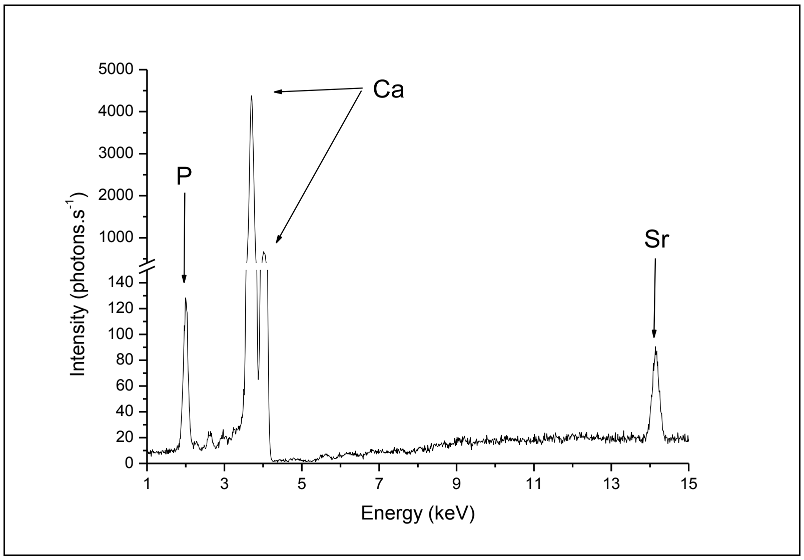

Figure 3: XRF spectrum of bovine tooth.

Table 1: Intensity of Phosphorus, Calcium and Strontium of each specimen before any treatment and after three weeks of remineralization treatment.

Status	Group	Phosphorus		Calcium		Strontium	
		Average	CV (%)	Average	CV (%)	Average	CV (%)
without any treatment.	Saliva	878±28	3.2	46589±891	1.9	1039±255	24.5
	Fluor	908±57	6.3	47331±2014	4.3	959±377	39.4
	Recaldent	844±29	3.5	45351±532	1.2	955±138	14.4
after 3 weeks of treatment	Saliva	806±46	5.7	46589±891	1.9	1078±267	24.7
	Fluor	844±21	2.5	47331±2014	4.3	994±364	36.6
	Recaldent	765±36	4.8	45351±532	1.2	931±157	16.9

Bonferroni test ($\alpha = 0.05$) was used for parametric comparison of means within groups of loss of P, Ca and Sr in each treatment. All statistical analyzes were performed using the software "SPSS for

Windows10.0.1". No difference has been verified between groups. Figures 4-6 show the relative intensity of phosphorus, calcium and strontium, respectively, in all groups.

The relative intensity was obtained by normalizing all mean results in relation to the results obtained without treatment as citric acid (erosive challenge). Figures 3-4 show that the element strontium, despite having chemical affinity with calcium, did not present changes regarding the treatment with citric acid (erosive challenge).

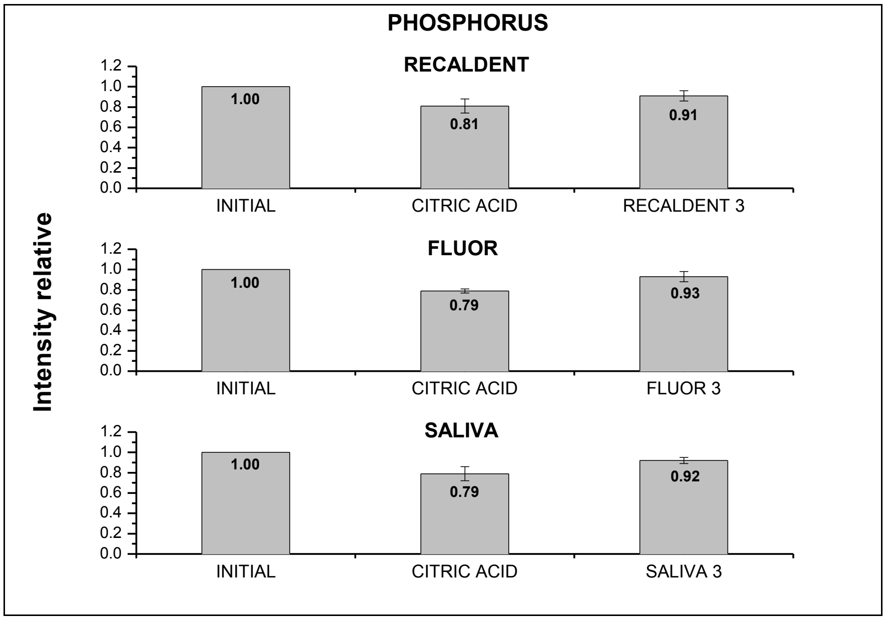

Figure 4: Relative intensity of phosphorus in all groups

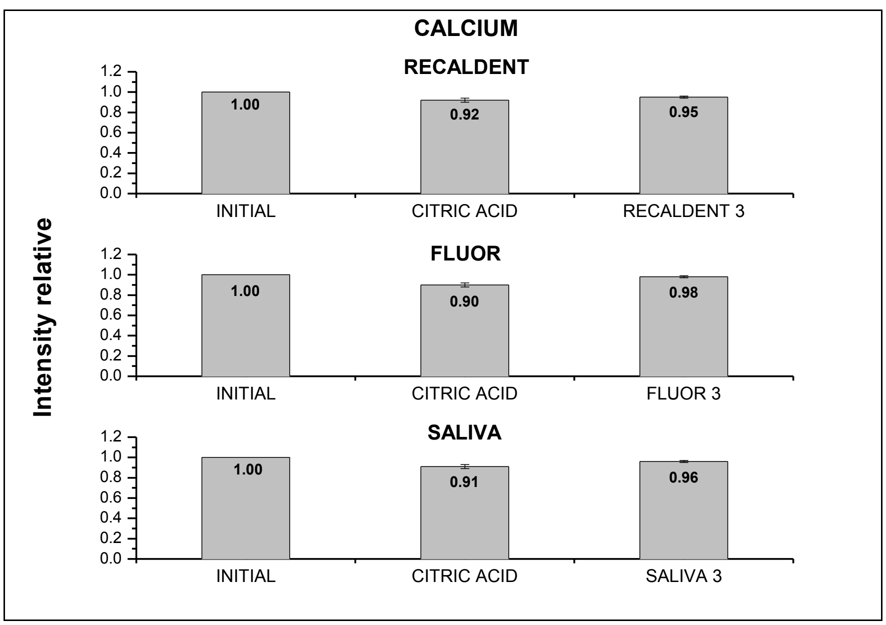

Figure 5: Relative intensity of calcium in all groups

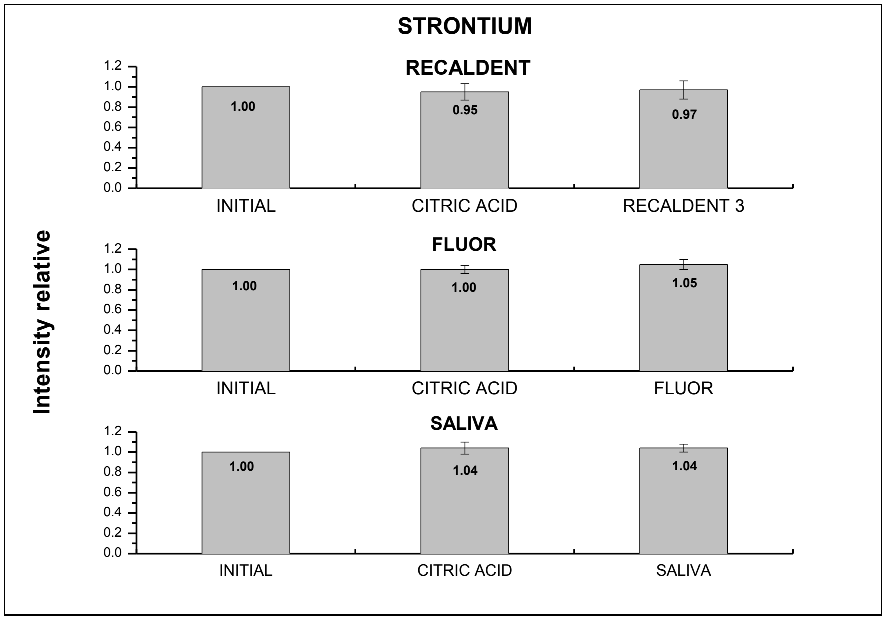

Figure 6: Relative intensity of strontium in all groups

Regarding the treatment with fluorides, some authors [4] state that it does not efficiently protect the growing process of erosive challenge, while others conclude that there is significant improvement in the lesions caused by erosion [10,12]. These controversies may be due to the fact of using different products and methodologies, making it difficult to compare results.

In this current study, neutral sodium fluoride was found to be effective against gain of P at the third week of treatment, showing a statistical difference with the control. Therefore, there was partial efficacy; since Ca did not increase sufficiently to statistically match with the initial amount (control). Additionally, there was no statistically significant difference between the control group and the FLOUR group in the first and second week of treatment the experiment ($p > 0.05$). The FLOUR group only proved superior to saliva group (control) at the third week of treatment ($p < 0.05$). The acid exposure was performed only once and before the application of Fl, which may explain the difference in efficacy of Fl in this present study when compared with other studies exposing specimen to acid after treatment [10,12].

The bioactive compound of GC Tooth Mousse is CPP-ACP, casein phosphopeptides and amorphous calcium phosphate, which not effective in the remineralization of bovine enamel subjected to erosive challenge in this current study, in agreement with the results of other in vitro studies [13, 14]. Based on the mechanism of action of products based on milk proteins, the CPP-ACP complex needs the presence of plaque and acquired pellicle to be effective. Any erosive challenge causes loss of salivary organic substances, which cover the tooth surface, such as pellicle [15]. Therefore, there is a limitation to the reversal of the loss mineral structure due to erosion for these CPP-ACP products. Furthermore, this current in vitro study did not have the presence of plaque and / or biofilm, which may also influence the results.

The Sr appears in the spectrum of the tooth due the fact that is biologically and chemically similar to Ca. Thus, the organism may incorporate some proportion of that element in its structure. The Sr not showed statistically significant differences, in either group after acid attack and after three weeks of treatment

4. CONCLUSION

The methodology for evaluating the remineralization chosen for this study was the X-ray fluorescence (XRF), a method based on the measurement of the intensities (the number of X-rays detected per unit time) of the characteristic X-rays emitted by chemical elements present in a sample, allowing the evaluation of all phases of the study in the same teeth [8]. Not being a destructive method permits the use of the same specimen at different steps of the study, reducing, therefore, the variables.

It can be concluded that, under the current experimental conditions (in vitro), and when evaluating the effect of different remineralizing substances on bovine teeth subjected to erosive challenge that: GC Tooth Mousse, neutral sodium fluoride gel and saliva showed similar action after three weeks of treatment when comparing the average values of Phosphorus. GC Tooth Mousse and saliva showed similar action after three weeks of treatment when comparing the averages of Calcium. The neutral sodium fluoride gel showed greater efficacy after three weeks of treatment when compared to saliva, in terms of average Ca. The neutral sodium fluoride gel and GC Tooth Mousse were similar in terms of average Ca at the third week of treatment. Besides that, the element strontium showed an interesting behavior in relation to treatment erosive (erosive challenge). So, it would be important in future work to evaluate in more detail, the importance of this element in tooth structure in relation to its role in the mechanism of erosion.

5. ACKNOWLEDGEMENTS

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