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DENTAL ENAMEL SUBMMITED TO GAMMA RADIATION AND Er,CR: YSGG LASER ASSOCIATED TO FLUORIDE

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ABSTRACT

The *in situ* caries model can use bovine dental samples in human volunteers. Gamma radiation is a very efficient sterilization method that is not expected to alter the mineral content of the hard tissues, avoiding biases in the results. Samples (n=40) were irradiated through a source of ^{60}Co multipurpose irradiator aiming complete sterilization (25 kGy/h) with the purpose of accumulating the native plaque on them at an in situ study. An Er,Cr:YSGG laser was used alone and in combination with the topical applications of: 1- dentifrice (1,100 µg F-/g) or 2-APF (12,300 µg F^-/g). Morphological and chemical analyses were performed by scanning electron microscopy (SEM), determination of alkali-soluble fluoride concentration by specific ion electrode and surface microhardness determination. Then, the 15 volunteers used palatal devices containing previously treated samples and remained using F dentifrice. The effects of Fformation, on the reduction of demineralization were correlated. The biochemical analysis for quantification of alkaline soluble F^- determined the groups in which the laser was used after the topical application of the two types of fluoride products of different concentrations (dentifrice and APF) to be statistically different ($p \leq 0.05$), suggesting a prolonged effect of the synergy of the treatments in the reduction of the demineralization. Electronic Microscopy Scanning analysis has not shown thermal damage neither interprismatics changes from hydroxyapatite crystals, at dental enamel outside the buccal environment, after 25-kGy gamma irradiation, established that gamma radiation could be used aiming dental enamel sterilization.
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1. INTRODUCTION

Due to the prevalence of carious lesions, there is an effort to study this disease, either in vitro, *in situ* or *in vivo*. In view of the ethical restrictions for the use of human teeth in research and its difficulty of obtaining, considering that fewer and fewer teeth are extracted, alternatives are necessary for studies, such as, for example, bovine dental enamel replacing the human. According to Turssi *et.al*.[1] there are many justifications for the use of bovine enamel, among them: Availability; More uniform composition compared to human; Orientation of crystallite, percentage of calcium and protein compasciation are compatible with those of human enamel. However, unlike human enamel, it has arrangement of the prisms in ungulates; The crystallinity is almost twice as thick and has the widest interprismatic region [1].
In situ studies was proposed by Kolourides and Volker[2] and later modified by Zero[3]. This model allows the conditions of the experiment to be similar to the conditions *in vivo*, but without changing the dentition of the volunteer. It is performed through a device that is taken to the oral environment containing the samples, such as dentin or enamel, and these can have a process of demineralization and remineralization in the oral cavity against the cariogenic

challenge, undergoing normal interferences to this environment, such as food, the fluorine ion and the bacterial plaque [4].

Thinking about an in situ environment using human teeth, in addition to the problems already mentioned, Ghaeth *et. al.*[5] still cite the risk of cross-infection of the individuals of the research, which, together with other factors, justify the use of non-human teeth, such as primate, bovine, swine, equine and shark teeth. Considering also the ease of obtaining and uniform composition, bovine teeth have been widely used. However, although bovine samples are smaller, there is still a risk of disease transmission to humans, where Bovine Spongiform Encephalopathy (BSE)[6] is the most worrying. It is caused by the prion, which although its mechanism has not been clarified, is lethal and extremely resistant to conventional methods of chemical and mechanical disinfection that do not alter the structure to be analyzed. Thus, for bovine osseous grafts, gamma radiation with a dose between 15 and 25 kGy has been used to maintain biosafety and biological properties of the graft[7]. Using the same principle for the sterilization of biological samples, it is of extreme value as it minimizes the risks to the research individuals and the operators, besides, sterilization / disinfection processes alter the structure of the material as the hypochlorite can alter microhardness analyzes superficial[8], chlorhexidine gluconate can decrease the amount of microorganisms in the biofilm[9] and autoclave sterilization changes the crystalline pattern of hydroxyapatite due to pressure[10].

Sterilization by gamma radiation has proved to be useful because, in addition to the reduction of microorganisms, it does not alter the microhardness of the surface [11,12,13]. As Co^{60}, doses up to 25 kGy have only the chromatic alteration as a result of the deterioration of the organic material of the dental enamel and there are no indications that doses up to this limit can alter the formation of dental caries[13].

Considering the multifactorial etiology of dental caries, is the need for a susceptible host, a cariogenic diet and the presence of specific microorganisms, the role of saliva is fundamental and is where the importance of the in situ study is found. The imbalance of the dynamic process of demineralization and remineralization is the cause of dental caries and this imbalance is caused by microorganisms of the oral cavity that make the fermentation of carbohydrates generating an acid environment, thus having a predominance in the demineralization. In the condition of demineralization, the mineral components, being mostly hydroxyapatite, that are soluble in water, tend to dissociate in the oral environment seeking the saturation with the saliva. Saliva helps in the balance of the carious process because it has calcium and phosphate in its composition, so besides the removal of the substrate it still allows the neutralization of the acid environment and consequently the replacement of lost calcium and phosphate ions. In this way, the in situ study allows the cariogenic challenge to be performed extra-oral so that it does not interfere with the dentition of the volunteer, but allows to evaluate the buffer capacity of saliva and other events in the oral environment.

A preventive intervention for dental caries should consider all etiological factors ranging from hygiene guidelines to individual measures of protection, such as the topical application of fluoride. The fluorine present in the saliva has the main effect of reducing the solubility of the mineral by the formation of fluorapatite, thus reducing the diffusion of the mineral ion due to the concentration of calcium and phosphate decrease because they are combined with fluoride[15]. Like fluoride, lasers have been used for preventive purposes in dentistry as it allows for modifications in hard dental tissues through absorption of light and conversion to heat, altering its composition or solubility.[16] Laser irradiation causes a melting and resolation of the hydroxyapatite, causing the water to evaporate and increase the mineral amount proportionally, which leads to higher acid resistance[17]. When in combination with fluoride products, such as fluoridated dentifrice and topical application of fluoride, it allows an increase in fluoridation and incoporation of the enamel, enhancing its effect [18,19]. In vitro studies show reduced demineralization of enamel irradiated with Er, Cr: YsGG (Erbium-Chromium: Yttrium, Scandium, Gallium, Garnet) laser (8.5 J/cm²) with fluoridated dentifrice (NaF – Sodium Fluoride), increasing acid resistance [20,21,22].

2. OBJECTIVES

This work aims to evaluate the morphological alterations of gamma irradiation in dental enamel and the preventive effects of irradiation with Er, Cr: YSGG laser isolated or associated with fluoride dentifrice (1,100 μg F^- / g), as well as associated with application topical fluoride (12,300 μg F^- / g).

1- To evaluate the in vitro effects of gamma irradiation for sterilization purposes for in situ study;
2- Evaluate the synergistic effect of the parameters used;
3- Evaluate and quantify the formation of alkali soluble products (weakly bound fluorine) because of the proposed treatments;
4- Evaluate the preventive potential in enamel demineralization in the presence of biofilm *in situ*.

3. METHODS

The study was divided into two parts: an in vitro experiment to evaluate the enamel morphology alterations resulting from gamma radiation and an in situ experiment of Er, Cr: YSGG laser irradiation evaluation.
Bovine teeth (n=40) were used, after approval in the Committee on Ethics in the Use of Animals (106 CUEA-IPEN / SP), from which it was possible to obtain 45 blocks of enamel. Prior to irradiation, the surface microhardness of each sample (Knoop number) was recorded and pre-irradiation analysis was performed on Scanning Electron Microscopy (SEM). Two dosimeters (Perspective Dosimeter, Harwell, UK) and a biological marker for sterilization were added. A multipurpose irradiator Co^{60} by means of the irradiation system of the superimposed source of the product. The irradiation had approximately 2 hours of duration, being in two cycles of irradiation of 12,5 kGy / cycle. After this cycle, some of the samples were submitted to sterility tests by counting Colony Forming Units (CFU) for bacteria, yeasts and fungi. The other sterilized samples were submitted to structural integrity analysis and evaluated morphologically by scanning electron microscopy (SEM). The mineral content was also analyzed by calculating the percentage of surface microhardness loss (% PDS).
In the second stage, it was necessary to approve the Ethics in Research Committee (34338 FO-USP), the teeth previously sterilized with radiation were randomized into 3 groups: 1-Er, Cr: YSGG; 2-Er, Cr: YSGG with dentifrice (1100 μg F^- / g); 3-Er, Cr: YSGG with APF (12,300 μg F- / g). They had microhardness of determined surface and received the proposed treatise. 15 palatal devices were made with one sample from each group. Each device was used by a volunteer for two weeks and extra orally a sucrose solution was dripped 8 times daily. After that period, they were removed from the devices and morphological analyzes were performed by SEM (TM 3000 Tabletop Microscope) determination of alkali-soluble fluoride concentration by specific ion electrode and microhardness determination.

3.1. Experiment in vitro

The study teeth were kept in distilled and deionized water and kept under refrigeration at 4°C. 45 blocks of 5 x 5 x 2 mm were obtained, which were planned and polished.

3.1. 1. Surface Microhardness and %SMHL

The initial surface microhardness of each sample was evaluated for the future calculation of the percentage of surface microhardness loss (% PDS) after sterilization by gamma radiation. The principle since testing consists of applying a load compatible with the material in question is making a deformation, which is subsequently measured and converted to Knoop hardness number. A 50 gf load was used for 5 seconds on a microdurometer (Shimadzu HMV-2000, Japan) with 11 indentations and finally averaging.

3.1.2 Gamma Irradiation

The samples were supplied with water in a way that maintains moisture, avoiding dehydration and still prevents heating during irradiation. A biomarker for sterilization and two dosimeters for gamma radiation (Perspex Dosimeter, Harwell, UK), one operand in the range of 1-30 kGy (Batch S. Harwell Amber) and the other in the range of 5-50 kGy (Batch KZ, Harwell Red). The gamma radiation was used of Cobalt-60 (Co^{60}) by means of a Co^{60} multipurpose irradiator with a total capacity of 37 Pbq (1 million Curies), with the initial activity of 3, 7 Pbq (100 kCi) through superimposed source of the product of the irradiation system. The irradiation had approximately 2 hours duration in two cycles of irradiation of 12.5 kGy / cycle, totaling 25 kGy. This technique allowed carrying out microbiological control by means of the reduction of living organisms or by complete sterilization, varying according to the dose used. The marker showed sterility due to discoloration and both dosimeters were sensitized.

3.1.3. Biological Essays

To confirm the effectiveness of the sterilization method a batch of samples underwent biological tests. Before the sterilization was carried out the counting of bacteria, molds and yeasts were performed by three culture media, respectively: TSA (Tryptic Soy Agar), SAB (Sabouraud Dextrose Agar), TSB (Tryptic Soy Broth). The total bacterial count was performed after 72 hours at 35øC and mold and yeast counts after 120 hours at 25øC. After gamma irradiation, sterilization was performed by membrane filtration using two culture media: TSB (Tryptic Soy Broth) at 20-25 ° C and THIO (Fluid Thioglycolate) at 30-35 ° C for 14 days.

3.1.4 Morphological Analysis

A batch of samples underwent morphological analysis in Scanning Electron Microscopy (SEM). The initial fixation in 2% glutaraldehyde solution for 2 hours was performed previously, 3 5 minute washes were performed with the 1 M phosphate buffer and the samples were kept in osmium tetroxide buffer for 20 minutes. The samples were washed three times with 0.1 M phosphate buffer solution and subsequently progressively dehydrated in increasing alcohol solutions (25%, 50%, 70%, 90% and 100%), immersed for 15 minutes in each solution. After this procedure, the samples were fixed in hexamethyldisilazane (HMDS) for 20 minutes and oven dried for 2 hours and kept dry in a desiccator for at least 24 hours before being analyzed. Samples were examined under scanning microscope TM 3000 (Hitachi, Japan) at a voltage of 5 kV, which does not require metallization. For each sample analyzed, at least four images were obtained in different predetermined increments: the first image was taken with the 50X magnification to verify the surface of the sample, followed by images of increase of 700 X, 2000 X and 4000 X or more. The structural and morphological aspects of enamel resulting from gamma irradiation were observed. The analysis of the images obtained was done through specific software.

3.2 Experiment *in situ*

3.2.1 Treatment of samples

After gamma irradiation, the enamel blocks were randomized into groups and treated:

G1 - Samples irradiated with Er, Cr laser: YSGG 8.5 J / cm²
G2 - Samples treated with 1,100 µg F- / g dentifrice and irradiated with Er, Cr: YSGG 8.5 J / cm² laser
G3 - Samples treated with topical application of Fluor Phosphate Acid 12,300 µg F- / g and irradiated with Er, Cr: YSGG 8.5 J / cm² laser.

Laser irradiation Er, Cr: YSGG (millennium, Biolase, San Clemente, USA): a power density of 8.5 J / cm² was used, scanning power of 1.25 W over the whole sample surface using part hand and sapphire tip, using automated XYZ step micrometer. In each 12 samples, the power was measured by power meter (Coherent FieldMaster GS and Detector LM-P101, Coherent, CA, USA), maintaining a mean of 33.60 mJ / pulse (Figure 1).
Immersion into a 1: 3 (w / w) suspension of dentifrice containing 1100 µg F^- / g as NaF under stirring for 10 minutes. They were applied every 4 hours, in 3 brushings. Topical application of Fluoride Phosphate Acid Gel with a concentration of 12,300 µg F^- / g (dentsply, Petropolis, Brazil) was maintained for 4 minutes.

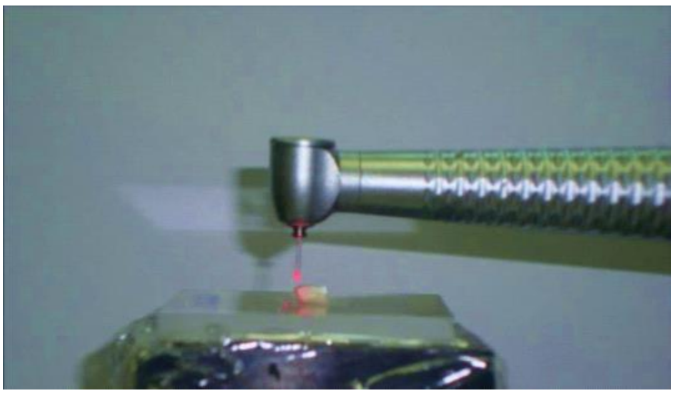


Figure 1: Laser irradiation Er,Cr: YSGG

3.2. 2 Volunteers and on-site experiment

The 15 volunteers selected had normal salivary flow, absence of caries lesions or periodontal disease. All were healthy and without systemic changes. All of them used fluoridated water supply (0.6-0.8 μg F / ml). The devices were made with a sample of each group inside, with face facing the oral environment and protected by plastic sterile fabric that allowed biofilm accumulation. The volunteers used the device (Figure 2) for 14 days and all brushed the teeth with the same dentifrice used in the treatment of the samples. They received 20% sucrose solution 8 times a day. After the determined period the samples were taken from the devices and analyzed.

Figure 2: Arrow indicates intraoral device

3.2.3 Experiment *in situ*

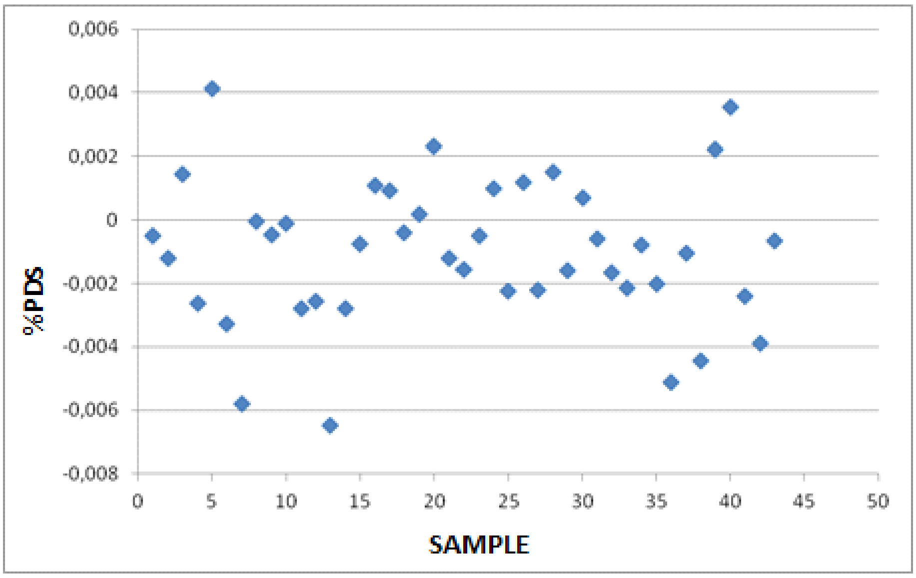
Approximately 50% of the samples from each group (n = 7) were submitted to qualitative analysis by means of the SEM study to evaluate the formation and retention of products on the enamel surface, following the aforementioned standards. In the other 50% of samples, a determination of alkali-soluble fluoride concentration by specific ion electrode was performed to evaluate the concentration of alkali soluble products formed on the enamel surface, for which the samples were placed in a tube containing 0.5 ml solution (pH 13-14) for 24 hours under continuous stirring at room temperature, after 24 hours the alkaline solution was neutralized and buffered with 0.5 ml of TISAB II solution containing 1 M HCl. The solutions were analyzed by an electrode (Orion 96098NW, Thermo Scientific, USA), previously calibrated and the millivoltage converted to μg F- / g calculated.

4. RESULTS

4.1 Experiment *in vitro*

4.1.1 % of surface microhardness loss

The average initial MDS was performed and the% PDS determined (Graph 1). There was no significant change after gamma radiation. Maintaining the homogeneity of the group. The confidence interval was 95% for% PDS (-0,0005), allowing to affirm that it is compatible with zero.



Graphic 1: Dispersion of %PDS of the samples subjected to sterillization by gamma radiation.

4.1.2 Sterility testes

Total bacterial count after 72 hours at 35 ° C and mold and yeast counts after 120 hours at 25 ° C x 105 CFU / g and <10 CFU / g, respectively. After sterilization, sterilization was performed by membrane filtration using two culture media: TSB (Tryptic Soy Broth) at 20-25øC and THIO (Fluid Thioglycollate) at 30-35øC for 14 days, with no bacterial growth total on every day. Although the pre-irradiation analyzes provided a satisfactory opinion, post-irradiation samples were considered sterile.

4.1.2 Morphological analysis

The morphological analysis by scanning electron microscopy was used to evaluate if the dental surface was heated in the proposed irradiation parameters (Figure 3). All the samples present an integrated and polished surface, with a prismatic hydrodroxyapatite pattern. No change from thermal origin change was observed. Some non-irradiated samples had impurities on their surface, evidencing the need for sterilization.

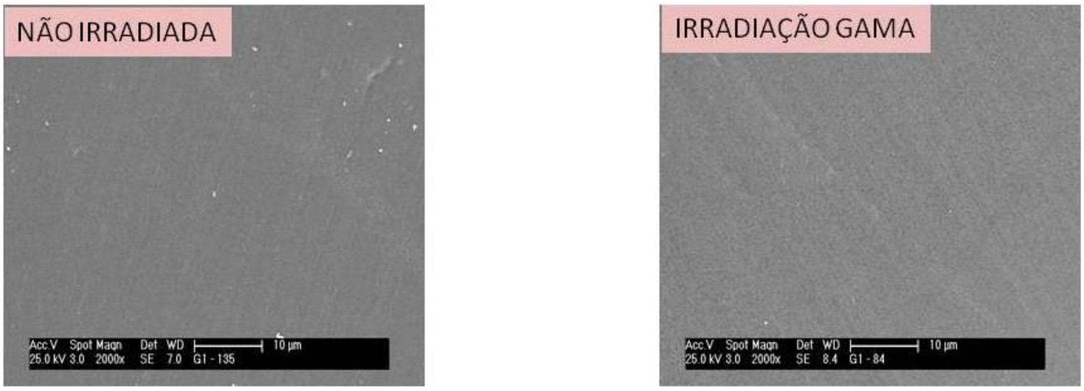


Figure 3: Surface of the human dental enamel sample before (left) and after gamma irradiation (25 kGy).

4.2 Experiment *in situ*

4.2.1 Morphological analysis

Images obtained by SEM after the use of the device showed biofilm formation and it was not possible to observe more details (Figure 4).

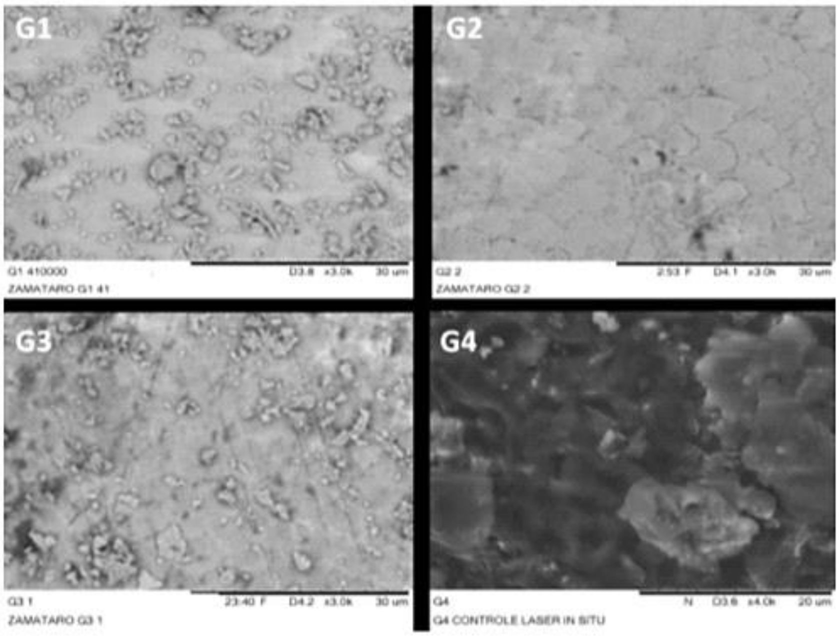


Figure 4: Sample after use in situ. Original increase of 3.000x

4.2.2 Alkali-soluble fluoride concentration by specific ion electrode

In the group treated with FFA 12,300 µg F- / g + Er, Cr: YSGG presented the highest concentration of fluoride after in situ use, considering that all groups received fluoride from the oral cavity of the volunteers who performed toothbrushing with fluoridated dentifrice.

The Er, Cr: YSGG group indicates a higher incorporation of the fluorine ion when compared to the Er, Cr: YSGG + Dentifrice group (Table 1).

	Fluoride Concentration (μg F^-)		
Treatment	Er,Cr:YSGG	Er,Cr:YSGG + Dentifrice	Er,Cr:YSGG + APF
Average	0.393	0.131	4.5
Standard deviation	0.129	0.039	2.5
Standard error	0.049	0.015	0.53

Table 1: Average values, standard deviation and standard error of fluorine concentration.

4.2.2 % of surface microhardness loss

The final values of the microhardness values found were reduced from the initial microhardness average, obtaining the percentage of hardness loss (Table 2).

5. DISCUSSION

This study was divided into two stages: *in vitro* experiment and *in situ* experiment, but both are inseparable. The first step consisted in the sterilization of the bovine teeth for use in situ. In contrast to other studies, it was hypothesized that radiotherapy irradiation would alter the microhardness and micromorphology of the enamel, but this *in vitro* study, even using higher doses than clinical protocols, did not confirm the hypothesis of structural modification.

	% PDS		
Treatment	Er,Cr:YSGG	Er,Cr:YSGG + Dentifrice	Er,Cr:YSGG + APF
Average	29,0	30	26,3
Standard deviation	8,4	5,5	7,4
Standard error	3,8	2,5	3,7

Table 2. Average values, standard deviation and standard error of % PDS

The bovine teeth showed patterns similar to the human ones, during manipulation and analysis of structures. The teeth used had a hardness pattern, and consequently a quantity of mineral, which increases the reliability due to the homogeneity of the group. In addition, the use of bovine teeth was more feasible in terms of availability and ethical conditions. Although the teeth are of different species from the human, it needs attention as it can transmit pathologies. Gamma radiation was proposed because of the protocols already used for graft biomaterials[23] and for this purpose, it did not have structural alterations of the material. By subjecting the human enamel to microhardness analysis using the sample prior to irradiation, which increases reliability, it was shown that the loss of hardness was not significant. Under analysis in scanning electron microscopy no thermal or structural damage was observed, maintaining the interprismatic pattern that it had before the irradiation. In view of the biological tests, we can consider that the bovine teeth were sterilized and presented safe for human use, which also increased the adhesion of the volunteers in step two of this work.

The *in situ* models had already demonstrated success in evaluating the anticariogenic potential in fluoridated dentifrices[24]. The weakly bound fluorine is what is absorbed on the surface of the enamel. Its action is in the reduction of the demineralization, because to the extent that the fluoride products precipitate in the enamel surface when the carbonated hydroxyapatite demineralizes. In the present study, the experimental treatments were intended to check if the formation of products on the surface of enamel in the form of weakly bound fluorine occurred, and which of the associations formed a greater quantity of these products, inferring a greater anticariogenic potential in the presence of dental plaque.

The fluoridated dentifrice due to its domestic application and self-care encompasses the population more than the application of Fluoride Phosphate Acid, which is of professional application, although this is a differential, both have a beneficial effect in the prevention of dental caries. The NaF of the dentifrice undergoes ionization and dissociation in contact with the water, releasing the fluoride so that it contacts the calcium of the enamel surface, forming CaF_2 globules, decreasing the pH allows a less demineralization and when it activates the remineration, at neutral pH, potentiates the remineralizing effect of saliva. Cruz et al. Cite that daily use of fluoridated dentifrices provides sufficient fluoride concentration to maintain appropriate levels in saliva and plaque during de-remiralization processes[25].

Fluoride applied in the form of a gel, in professional use, in this case was used Fluorine Phosphate Acid 12,300 μg F^- / g (FFA). It is used to prolong the anticariogenic effect of fluoride for a prolonged time. The residence time and the solubility of FFA in the oral cavity depend on high frequency of use in low concentration or a contribution of higher concentration and with a lower frequency of use, as in the case of APF.

The association of fluoride products with laser irradiation is intended to prolong the effect of fluoride. Both cited fluoride products used alone or associated with laser irradiation formed weakly bound fluorine on the surface of the enamel. When there is an increase in F concentration in the plate, after brushing with the dentifrices, the dissolution of CaF_2 providing F was not sufficient to prevent mineral loss. However, the deposition of this reservoir can be important when considering the daily frequency of use of the dentifrice, replacing these reservoirs with each brushing. The synergy with the Er, Cr: YSGG laser seems to be promising since the surface area increases by laser irradiation, providing new sites for the formation of microfluoride reservoirs to be released in the pH cycles.

For purposes of dental caries prevention, energy density should not promote morphological alterations detrimental to irradiated enamel, such as tissue ablation and carbonization. Previous work developed at the Laboratory of Biophotonics of the Center for Lasers and Applications of IPEN-CNEN / SP concluded that the energy density of 8.5 J/cm² promotes discrete ablations on the enamel surface and is safe for dental pulp. It also promotes crystallographic changes in the surface of the dental enamel which increases the cariostatic effect of CaF_2 formed by the application of fluoride products. This study was able to verify macroscopic morphological changes, perceptive to the naked eye as the reduction of polished enamel brightness, after being hydrated this alteration is no longer available. The energy density of 8.5 J/cm² was considered positive, considering the risks and benefits.

Considering the quantification of CaF_2 when compared between groups, it had higher results found in the group of topical application of FFA associated with irradiation. Expected result due to exposure to dentifrice received lower total fluoride concentration. This result is associated with the increase of the area of enamel surface roughness resulting in higher CaF_2 formation. Another significant finding was the possibility of maintaining the anti caries effect of the Er, Cr: YSGG synergy with FFA for a longer time even with a single application. This appears to be a promising protocol indicated for patients with high caries rates.

6. CONCLUSIONS

It is concluded that:

- The sterilization of dental enamel using gamma radiation is safe and minimal changes in the mineral content and in the morphological aspects of the enamel are not statistically significant. In addition, they contribute to minimize ethical concerns and offer safety to volunteers, which may increase the adherence of this model *in situ*.
- The association of laser irradiation with fluoride products increased the formation of CaF_2 on the enamel surface.
- The formation of products on the enamel surface (weakly bound fluoride) was higher when associated with laser irradiation with Fluorine Phosphate Acidulate.
- The parameters used for the Er, Cr: YSGG laser irradiation were efficient and potentiated the formation of CaF2 on the enamel surface, suggesting a greater reduction of demineralization in the presence of biofilm and a prolonged reduction effect.

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