UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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In vitro and *in situ* evaluation of the effects of high-fluoride and arginine-based toothpastes to control root caries

Avaliação *in vitro e in situ* do efeito de dentifrícios fluoretados de alta concentração de fluoreto e a base de arginina no controle da cárie radicular

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Tese apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Dentística.

Orientadora: Profa. Dra. Linda Wang
Co-orientadora: Profa. Dra. Ana Carolina Magalhães

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Joseph Addison

ABSTRACT

In vitro and _in situ_ evaluation of the effects of high-fluoride and arginine-based toothpastes to control root caries

The focus of our studies has been the root substrate dentin, once root caries prevalence has been reported as increasing in part due to people living longer and maintaining their natural teeth into old age. Furthermore, this condition is highly prevalent in patients undergoing head-and-neck radiotherapy (HNR). To better understand the effect of different toothpastes in sound and non-irradiated root dentin, this research was developed in 3 parts with specific objectives, involving both in non-irradiated and irradiated substrates. In study 1, high-F toothpaste (5,000 μg F/g) associated or not with *f* tri-calcium phosphate (*f* TCP) were compared *in vitro* with conventional one (1,450 μg F/g) to control bovine root caries development. The study 2 was conducted to evaluate if the *in vitro* performance would be similar in a closer clinical situation. An *in situ* design was performed and the effect of high-F toothpaste combined or not with *f* TCP comparing to 1,450 μg F/g combined or not with arginine-based toothpastes in reducing the net demineralization of sound root dentin and on the remineralization in initial artificial caries lesions was verified. In study 3, the effects of radiation exposure on human root dentin composition, structure and mechanical properties were evaluated. In the first study, our findings highlight the importance of using high-F toothpastes to prevent root caries development. In the second one, the results showed great performance of high-F toothpastes and arginine-based toothpastes, in clinical situations. The results of study 3 showed that radiation exposure changed the composition and structure of human root dentin, which may detrimentally affect its mechanical properties. Overall, the studies suggest that at high-risk population, such as elderly people and patients undergoing HNR, it is important to develop protocols to minimize damages caused by carious lesions, inhibiting the net demineralization of root caries. The current results can clarify the effects of radiation on root dentin to help further studies in this area. We also could observe that conventional toothpaste is not as effective as high-F toothpastes to prevent this condition, in non-irradiated root dentin. This knowledge is of special interest to determine the quality of life of high-risk population to dental caries presenting available tools that can be of at-home use with beneficial effects on demineralization protection and reversion.

Key words: Root caries. Toothpastes. Radiotherapy.

RESUMO

O foco de nossos estudos tem sido o substrato dentinário radicular, uma vez que há relatos do aumento da prevalência de cárie radicular, devido principalmente ao aumento da expectativa de vida da população e a manutenção dos dentes naturais nos idosos. Além disso, essa condição é altamente prevalente em pacientes submetidos à radioterapia de cabeça e pescoço (RCP). Para melhor entendimento do efeito de diferentes dentifrícios em dentina radicular não-irradiada e irradiada, essa pesquisa foi desenvolvida em 3 partes, com objetivos específicos. No estudo 1, dentifrícios de alta concentração de F (5000 µg F/g) associado ou não com tri cálcio fosfato (*f* TCP) foram comparados *in vitro* com dentifrícios convencionais (1450 µg F/g) no controle do desenvolvimento de cárie radicular bovina. O estudo 2 foi conduzido para avaliar se os resultados do *in vitro* seria o mesmo diante de uma situação mais próxima da clínica. Um desenho *in situ* foi realizado e o efeito de dentifrício de alta concentração de F combinado ou não com *f* TCP e comparado com dentifrícios convencionais 1450 µg F/g combinado ou não com dentifrícios a base de arginina na redução da desmineralização de dentina radicular hígida e na remineralização de lesões cariosas previamente desenvolvidas foi avaliado. No estudo 3, os efeitos da exposição da dentina humana à radiação na sua composição, estrutura e propriedades mecânicas foram avaliados. No primeiro estudo, os resultados destacam a importância do uso de dentifrícios de alta concentração de F para prevenir o desenvolvimento de cárie radicular. No segundo, os resultados mostraram boa performance clínica dos dentifrícios de alta concentração de F e a base de arginina. O estudo 3 mostrou que a exposição à radiação altera a composição e estrutura da dentina radicular humana. De modo geral, os estudos sugerem que em população de alto risco, como os idosos e pacientes submetidos à RCP, é importante desenvolver protocolos para minimizar danos causados pelas lesões de cárie, inibindo a desmineralização líquida da cárie radicular. Os presentes resultados podem clarificar os efeitos da radiação na dentina radicular e ajudar em estudos posteriores nessa área. Também é possível observar que dentifrícios convencionais não são tão efetivos como os de alta concentração para prevenir essa condição, em dentina radicular não irradiada. Tal conhecimento é de especial interesse para garantir a qualidade de vida da população de alto risco à cárie, apresentando ferramentas disponíveis que podem ser usadas em casa com efeito benéfico na proteção da desmineralização.

Palavras chave: Cárie radicular. Dentifrícios. Radioterapia.

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

As the life expectancy has been longer overtime (PETERSEN et al., 2005), the presence of retained natural teeth it is frequent and the prevalence of gingival recessions increases with age (WIERICHS; MEYER-LUECKEL, 2015). All exposed root surfaces run a risk of developing root caries, once they are sites of biofilm retention (HEASMAN et al., 2017). Therefore, root caries, which refers to tooth decay on this surface (BANTING, 2001) has significantly increasing in prevalence over the last years (TAKABASHI; NYVAD, 2016).

This condition is also highly prevalent in patients undergoing radiotherapy of head-and-neck (KIELBASSA et al., 2006). As post radiation lesions develop mainly in the cervical and incisal area of the tooth, as a “cervical ring” aspect (KIELBASSA et al., 2006), dentin root caries rapidly progress, which may lead to severe tooth destruction. Thus, the efforts are mainly related in preventing root caries development in these patients at high-risk.

Root caries is a preventable disease (GALAN, 1994), which can be arrested at any stage. Accordingly, the recommendation of fluoridated toothpaste is the most cost-effective caries- preventive tool (ROLLA, 1999; MARINHO et al., 2003), because they combine the mechanical disruption of tooth biofilm and F delivery (TENUTA; CURY, 2013), which could interfere positively to reach the balance between demineralization and remineralization. However, its effects can be influenced by certain factors, such as the F concentration (WALSH et al., 2010).

Laboratory and clinical trials have been shown that the use of high-F concentration toothpastes (5,000 µg F/g) have significant better performance to active remineralization and arrest root caries than conventional one (1,000-1,450 µg F/g) (BAYSAN et al., 2001; EKSTRAND et al., 2013; GARCIA-GODOY et al., 2014). However, the evidences regarding it are limited and inconclusive, once there are very few well-conducted randomized controlled trials in this field (WIERICHS; MEYER-LUECKEL, 2015). In addition, the studies have focused mainly on root caries remineralization (BAYSAN et al., 2001; EKSTRAND et al., 2013) and not on prevent caries initiation (WIERICHS; MEYER-LUECKEL, 2015).

Moreover, there are a trend to introduction of new biofunctional materials containing Ca and P ions as a strategy for aided remineralization. Toothpastes containing tricalcium phosphate (*f* TCP) technology indicates several benefits of adding *f* TCP to them, which may improve the mineralization (KARLINSEY et al., 2010). However, we still have limited information if the combination of F and *f* TCP would really be beneficial to the population on preventing tooth demineralization and its effect on dentin was not investigated yet.

Furthermore, whilst these approaches are focused on the host tissue as a means of damage control, once the caries process has been initiated and is in progress (CUMMINS, 2013), new technologies have been also developed to prevent caries development at an earlier stage, by specifically targeting the residual biofilm. These new technologies include an innovative toothpaste based upon 1.5% arginine and 1,450 µg F/g, in an insoluble Ca base and has been tested with promising results (SOUZA et al., 2013; CUMMINS, 2013). However, there is insufficient evidence in support of a caries-preventive effect for the inclusion of arginine in toothpastes and more rigorous studies are still required (ÁSTVALDSDÓTTIR et al., 2016).

Thus, the aim of this thesis was to evaluate the *in vitro* and *in situ* the effect of these toothpastes to control the development and progression of root caries in non-irradiated teeth. We have also evaluated the effects of radiation in root dentin, to better understand it and test the effect of these toothpastes in irradiated teeth in further studies.

2 ARTICLES

2 ARTICLES

- ARTICLE 1 - High-fluoride toothpastes combined or not with *f* tri-calcium phosphate reduce initial root dentin demineralization *in vitro*
- ARTICLE 2 - Caries lesions inhibition and repair using high-fluoride toothpastes with or without tri-calcium phosphate and conventional toothpaste containing 1.5% arginine: an *in situ* investigation
- ARTICLE 3 - Radiotherapy alters the composition, structure and mechanical properties of root dentin

2.1 ARTICLE 1

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High-fluoride toothpastes combined or not with *f* tri-calcium phosphate reduce initial root dentin demineralization *in vitro*

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Short Title: High-fluoride toothpastes aid reducing dentin root caries
Keywords: Demineralization. Fluoride. pH cycling. Root dentin. Toothpastes.

ABSTRACT

Objectives: This *in vitro* study was conducted in two phases hypothesizing that high-fluoride (F) toothpaste (5,000 μg F/g) associated or not with *f* tri-calcium phosphate (*f* TCP) would provide additional protection against root caries development (1) and progression (2). For both phases, bovine root dentin specimens were selected by surface hardness (SH). **Methods:** In phase 1, sixty specimens were daily subjected to alternate immersion between demineralizing and remineralizing solutions (7 days). The specimens were randomly distributed into four treatments groups (n=15): non-F toothpaste, CT: Colgate Total 12® (1,450 μg F/g); CP: Colgate Prevident® (5,000 μg F/g) and CL: Clinpro 5,000® (5,000 μg F/g) associated with (*f* TCP), and treated with toothpastes/water slurries (1: 3 w/w) (5 min), twice a day. In phase 2, the efficacy of the toothpastes to inhibit the progression of the developed lesions was assessed, using the previously described pH-cycling model without additional treatments. The response variables were based on the percentage of surface hardness change, cross-sectional hardness, mineral content/ lesion depth (TMR) and the alkali-soluble F uptake. **Results:** (1) Both high-F toothpastes were efficient to reduce dentin demineralization and increase the F uptake compared to non-F and conventional toothpastes ($p<0.05$). (2) All F toothpastes were able to control the caries lesion progression in subsequent de-remineralization episodes, but the effect of the both high-F toothpastes (especially CL) was reduced. **Conclusions:** We can state that high-F toothpastes, regardless of the presence of *f* TCP, were more effective than conventional F toothpaste in inhibiting initial root dentin demineralization.

Clinical significance: With the trend to increase dentin root caries, mainly in the elderly, our findings highlight the importance of using high-F toothpastes to prevent it. This strategy is less expensive and can be conducted without monthly hygienist applications such as F varnish or other products, improving the patients' quality of life.

Keywords: Demineralization. Fluoride. pH cycling. Root dentin. Toothpastes.

INTRODUCTION

Root caries has become a new clinical challenge, boosting for extensive research about appropriate approaches for its control [1,2]. The establishment of root caries is determined by the presence of a cariogenic biofilm and fermentable carbohydrates [3], exacerbated by particular constitution of dentin. Apatite crystals in dentin present higher carbonate content and its demineralization process also involves collagen degradation, which transform their components more water-soluble [2] and more susceptible to acidic dissolution than enamel apatite [4].

Root caries can affect young adults, but its occurrence is more often in elderly population [5]. This scenario is resultant of greater life expectancy overtime [6], with favorable conditions to retain natural teeth but associated with more prevalent gingival recessions [7]. Due their roughness, root surfaces are more susceptible to dental biofilm accumulation and their anatomy interferes in the access for cleaning, which potentially increases the risk for the development and progression of root caries [8].

So far, few defined management strategies with evidence-based prevention guidelines for root caries have been produced [9,10]. Nonetheless, the use of fluoride (F) is still the main effective non-invasive treatment to control dental caries [7] highlighting the efficacy of fluoridated toothpastes [11,12], because they combine the mechanical disruption of dental biofilm with F delivery. Since the introduction of high-F toothpastes, their application for controlling root caries has been compared to the conventional F toothpastes with inconclusive evidences [7]. F plays a major role both 'preventive effect' (reducing demineralization and the progression of lesions) and 'therapeutic effect' (reversal of lesions) [13]. Our *in vitro* study brings new knowledge on this field since previous *in vitro* and few well conducted clinical trials only showed a beneficial effect of 5,000 μg F/g over the conventional toothpastes (1,100 – 1,450 μg F/g) to reversal root carious lesions ('therapeutic effect') [14,15,16]. Residual effect of 5,000 μg F/g, in preventing root caries development and progression is unknown.

On the other hand, new biofunctional materials have been introduced [7,17], especially those containing calcium and phosphate ions, as a strategy for aided remineralization [17,18], improving the F uptake on dental substrates. Some studies have showed that toothpastes containing a functionalized tri-calcium phosphate (*f*

TCP) technology can bioavailable calcium and phosphate ions, which are provided to the teeth and work in synergy with F, enhancing their effect [17,18]. However, limited information is available regarding its combination with F, and until now, *f* TCP was not tested in root dentin.

Thus, the aims of this *in vitro* study were to compare high F toothpastes (5,000 µg F/g) (associated or not with *f* TCP) with a non-F and conventional F toothpaste (1,450 µg F/g) in two different pH-cycling phases to test the effect of frequent use of the products (phase 1) in inhibiting root dentin demineralization and their residual effect against further demineralization (phase 2). The null hypotheses tested were that high-F toothpastes, regardless of the presence of *f* TCP, are as effective as conventional F toothpaste in inhibiting demineralization (1) and the progression (2) of the root caries lesions.

MATERIALS AND METHODS

Experimental design

This *in vitro* study involved two independent variables: toothpastes treatments [in four levels: non-F toothpaste (negative control group); 1,450 µg F/g (positive control group); 5,000 µg F/g and 5,000 µg F/g + *f* TCP] and depth [in seven levels: 10, 30, 50, 70, 90, 100 and 220 µm]. As a third variable was assessed in the same specimen in different moments, the different phases of analysis was determined as repeated measurements [the first pH-cycling regime with treatments (pH-cycling 1) and the second pH-cycling regime without any treatment (pH-cycling 2)]. The experimental units were root dentin specimens obtained from bovine incisors and selected by surface hardness (SH). The response variables were based on the percentage of surface hardness change (%SHC), cross-sectional hardness (CSH), mineral content and lesion depth (TMR) and the uptake of alkali-soluble F.

Preparation and selection of root dentin specimens

Polished dentin specimens (4 x 6 x 3 mm) were obtained from the labial cervical roots of bovine incisor teeth, which were freshly extracted and stored in 0.1% thymol solution (pH 7.0) for 30 days. Baseline SH was determined by three indentations, using a Knoop diamond indenter, spaced 100 µm from each other. Assessments were made under 10-g load for 10 s, using a Buehler Ltd, MicroMet 6040, Lake Bluff, IL, USA. Before performing SH measurements, dentin specimens were allowed to dry spontaneously for 30 minutes to standardize the measurements [19]. Dentin specimens presenting baseline SH mean 35±0.66 kg/mm^2 were selected for this study. The selected specimens were laterally coated with an acid-resistant varnish to allow only the exposition of the prepared surface (Figure 1).

pH-cycling regimes

The study was conducted in two-phases pH-cycling (pH-cycling 1 and pH-cycling 2), using the same specimens. Both phases (1 and 2) were conducted to simulate two different conditions, respectively, where: (i) the specimens were treated with the respective toothpastes, 2x/daily, during dynamic cycles of demineralization and remineralization. Topical efficacy was evaluated in respect to the ability to reduce hardness loss and mineral loss; (ii) the specimens were not further treated, but they were submitted to a new cariogenic challenge, with new cycles of demineralization and remineralization. The capacity of the toothpastes in reducing the progression of hardness loss and mineral loss was evaluated.

Before the pH-cycling 1, 1/3 dentin surface was protected with acid-resistant varnish (8 mm^2) allowing the exposition of a surface area of 16 mm^2 (Figure 1). After the pH-cycling 1 and before the pH-cycling 2, another 1/3 of the polished surface were coated with acid-resistant varnish, allowing a surface area of 8 mm^2 was exposed (Figure 1).

In both pH-cycling regimes, we used the model adapted from Vieira et al. [20]. Sixty specimens were subjected to a pH-cycling model for seven days. In the pH-cycling 1, the specimens were randomly distributed according to their SH into four groups (n=15) and subjected to one of the following treatments: non-F toothpaste, CT: toothpaste with conventional concentration of F (1,450 µg F/g) – Colgate Total 12®, New York, NY, USA; CP: high-F toothpaste (5,000 µg F/g) – Colgate Prevident®, New York, NY, USA and CL: high-F toothpaste (5,000 µg F/g) associated with *f* TCP – Clinpro 5,000®, 3M ESPE, St. Paul, MN, USA. All toothpastes tested were NaF/SiO_2-based. The non-F toothpastes, without NaF, was formulated by Pharmacia Specifica, Bauru, SP, Brazil.

The specimens were daily subjected to alternated immersion in demineralizing solution (1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 50 mmol/L lactic acid buffer with pH 5.0) (30 mL/specimen) for 8 h [21] and in remineralizing solution (1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl, 20 mmol/L HEPES buffer, 5 mmol/L NaN_3 with pH 7.0) (30 mL/specimen) for 16 h [22]. This pH-cycling regimen lasted for 7 days at 37°C. Both solutions were daily renewed before the beginning of a new complete cycle.

Twice a day (before and after demineralization), the specimens were treated with toothpastes/water slurries (1: 3 w/w) [23] for 5 min [24], under agitation and at room temperature. The specimens were washed in purified water for 5 s before being immersed in the de- or remineralizing solution.

The pH-cycling 2 was conducted similarly to number 1, but in this case no treatment was done.

Hardness analysis

At the end of each pH-cycling regime (1 and 2), SH (n=15) was again determined. The mean values from the three indentations spaced 100 µm from each other was calculated and compared to the baseline means. The percentage of surface hardness change was calculated as follows: %SHC = {(SH final - SH lesion) / (SH baseline - SH lesion)] x 100, where SH final = SH after pH-cycling 2; SH lesion = SH after pH-cycling 1 and SH baseline = initial SH (sound).

After SH analysis, all specimens were perpendicularly sectioned to the orientation of the protective nail varnish, allowing all areas to be included in the analysis (sound, pH-cycling 1 and pH-cycling 2). The CSH analysis was determined in one of the halves, while the other half was used for TMR analysis. Half of each specimen was embedded in acrylic resin and polished. The indentation was conducted using Knoop Diamond under a 10-g load for 15 s and three rows of 7 indentations spaced 100 µm from each were made on each area (sound, pH-cycling 1 and pH-cycling 2). The indentations were made at 10, 30, 50, 70, 90, 110 and 220 µm from the outer dentin surface. The mean hardness values (kg/mm^2) at all measuring points of each distance from the surface were compared.

TMR analysis

The other half of the specimens was then handily polished to obtain a specimen of an approximate thickness of 110 µm using water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler, USA). The final thickness of each specimen was checked with a micrometer and they were immersed in ethylene glycol

(Sigma-Aldrich, Steinheim, Germany) for 24 h before the analysis to avoid shrinkage during X-ray exposure due to dehydration [25].

The fragments were fixed with adhesive tape in a sample-holder together with an aluminum calibration step wedge with 14 steps (99.9% Al) for calibration, using glass plates that were exposed to X-ray Cu Ka (20 kV and 20 mA) for 13 min. The X-ray was directed perpendicularly to the lesion at a distance of 30 cm. After each exposure, the glass plate was prepared (4 min 30 s, 20°C), fixed (7 min, 20°C) and washed with running water (10 min). The plate was analyzed using a transmitted light microscope fitted with a 20x objective (Axioplan, Zeiss, Oberkochen, Germany) and a CCD camera (XC-77 CE, Sony, Tokyo, Japan) coupled to a computer with software for calculations (TMR 2012 and TMR 2006 software, Inspector Research BV, Amsterdam, The Netherlands). The microradiographs were saved as images with a 640 x 480 pixel resolution and 256 grey scales [25,26]. The mineral content was calculated from the specimen grey levels using the formula of Angmar et al. [27], assuming the density of the mineral to be 3.15 kg/l. The mineral content of sound dentin was assumed to be 50 vol% [27]. The lesion depth (LD), the integrated mineral loss (ΔZ) and the average mineral loss (R) were calculated for both pH-cycling models.

For the comparison among the treatments, the difference were calculated as follows: ΔΔZ= ΔZ lesion (after pH-cycling 1) - ΔZ final (after pH-cycling 2), ΔLD = LD lesion – LD final.

F alkali-soluble in dentin

Sixty additional specimens (n=15) were equally prepared and a 3.14 mm^2 surface area was exposed to determine F concentration, after the first pH-cycling phase. The specimens were individually immersed in 1.0 mL of 1 M KOH solution for F-alkali soluble extraction [28]. After 24 h under agitation (100 rpm), the extract was buffered with 1.0 mL TISAB II (1 M acetate buffer pH 5.0, 1 M NaCl and 0.4% CDTA) containing 1 M HCl. The calibration curve ranged from 0.125 to 2 µg F/mL. F measurements were performed using an ion selective electrode (Thermo Orion 96-09) and an ion analyzer (Thermo Orion 720A+, USA), previously calibrated. The data were expressed as µg F/cm^2.

Statistical analyzes

Data were calculated and statistically analyzed with Statistica 10.0 software (Statsoft®, Tulsa, Oklahoma, USA). Normal distribution and equality of variances were checked for all the variables using Kolmogorov-Smirnov and Levene test, respectively. SH, CSH and TMR (ΔZ, LD, R) were analyzed using three way repeated measures ANOVA/Tukey's test. The %SHC, F content and ΔΔZ were analyzed using Kruskal-Wallis/Dunn tests. ΔLD and ΔR were analyzed by One way ANOVA, followed by Tukey Test ($p<0.05$). The level of significant was set at 5%.

RESULTS

The pH-cycling model created a narrow initial subsurface lesion as shown in Figure 2. Table 1 show that CT, CP and CL did not avoid initial dentin demineralization. In respect of %SHC, all treatments with toothpastes had significant effect on the reduction of progression of hardness loss when compared with non-F toothpaste (Table 1) ($p<0.05$). However, after pH-cycling 1 (with frequent use of fluoridated toothpastes) only high-F toothpastes (CP and CL) showed a protective effect to significantly increase uptake of alkali-soluble F, with no difference between them (Table 2).

Figure 3 shows the mean hardness profile over the depth, after both pH-cycling phases. For both pH-cycling regimes, all toothpastes significantly reduced the dentin hardness loss compared to non-fluoridated toothpaste up to 50 µm depth. In pH-cycling 1, at 10 and 30 µm, the hardness values were significantly higher for CL and CP compared to CT. At 50 µm, the hardness value was still higher for CL compared to CT, but no difference was found between CP and CT. After the pH-cycling 2, the hardness values were significantly higher for CL > CP > CT at 10 µm depth. At 30 and 50 µm, the hardness value was still higher for CL compared to CT, but no difference was found between CP and CT (Figure 3).

In respect to the integrated mineral loss and lesion depth after the pH-cycling 1, non-F toothpaste and CT groups presented the highest values with no difference between them, while CP and CL presented the lowest values at the limit of detection (Table 3). Figure 4 shows pictures of representative specimens after pH-cycling 1 and pH-cycling 2. Figures 4 c) and d) showed that the specimens treated with CP and CL did not developed initial caries-like lesions. In respect to the mean mineral loss over the depth, CT significantly reduced the R-values compared to non-F toothpaste. After the pH-cycling 2, CT was able to reduce the progression of root dentin lesions compared to non-F toothpaste except for the parameter R. The lesion depth did not increase for CT-treated samples after pH-cycling 2 compared to pH-cycling 1.

Reduction in the integrated mineral loss and lesion depth was significantly higher for CT compared to CL and CP (only for ΔLD). CP presented similar results to CL, which in turn was unable to reduce the lesion progression compared to non-F toothpaste. No difference were found among the treatments in respect to R values (Table 4).

DISCUSSION

Robust evidences support that fluoridated toothpastes should contain at least 1,000 µg F/g to control dental caries in permanent [29] or primary dental enamel [30,31]. For dentin, however, it is possible that a conventional concentration might not be high enough to control root caries and the regular use of high-F toothpastes could be indicated. The results of our study support this idea.

The pH-cycling model chosen was adapted from previous studies using dentin as substrate [21,22]. The model was able to simulate the demineralizing and remineralizing episodes that occur in oral cavity and create a narrow subsurface mineral loss without erosion (Figure 2). Regarding the time of F treatment with the toothpastes, even though 5 min may be considered a long period of brushing, it is appropriate to simulate the effective retention time for elevated F levels in the mouth after toothbrushing with F toothpastes [24].

Our results showed that a concentration of 1,450 µg F/g was not enough to avoid dentin demineralization as we can see in Tables 1 and 3 and Figure 3, when compared to high-F toothpastes. These results are supported by the F uptake that was significantly higher for the high-F toothpastes-treated dentin (Table 2). This incorporation has occurred even after the pH-cycling using the toothpastes treatment. CaF_2-like particles behave as a mineral reservoir releasing F for biofilm and tooth surface. In the present study, F released from this reservoir might have been incorporated into dentin [32], avoiding demineralization for the high-F toothpastes-treated dentin. The current results showed that despite a conventional F concentration could not avoid initial dentin demineralization when compared to high-F toothpastes, it was able to control the progression of the lesions over time (after pH-cycling 2).

Conventional toothpaste was similar to the high-F toothpastes in reducing the hardness loss (Table 1) and more effective than the high-F toothpastes (especially CL) in respect to mineral loss and lesion depth after the pH-cycling 2 (Table 4, pH-cycling difference). This performance has to be carefully interpreted since the effect of conventional toothpaste was more pronounced after the pH-cycling 2 likely due to the fact that highly demineralized and deep lesions demineralized less than shallow lesions (as the case of lesions from CP and CL groups) under further cariogenic challenges [33,34].

When both high-F toothpastes were compared, there was no relevant difference between them in respect to the development and the progression of root caries lesions. We expected to find better effect for CL (with *f* TCP) than for CP (this was seen only at 10 μm depth), due to the presence of compatible F functionalized with calcium phosphate ingredient (*f* TCP), which would act mainly on the subsurface lesion [35]. Fluoride, on the other hand, acts mainly at the surface layer (as shown in the present study where F acts up to 50 μm depth), which is incorporated into the crystal structure, forming fluorapatite [32]. Previous study showed beneficial effect of *f* TCP in enamel [17], but in the present results, the association of F with *f* TCP did not show be more beneficial to control root caries than F alone. This is an important data, since the introduction of biofunctional materials as *f* TCP on toothpaste might not a cost benefit strategy for dentin.

However, despite our results showed similarity, we speculate that if the treatments have been applied over the pH-cycling 2, some difference could have been detected between CP and CL, once the effect of *f* TCP could be associated with its frequency of use, but it need to be tested yet. The aim of the experimental design was to check the preventive and residual effect of the treatments on the root caries progression, despite we know that toothpaste is continually used in the toothbrushing routine.

The CSH applied in our study was very important in differentiating the treatments up to 50 μm depth, especially after the first pH-cycling. Hardness reflects the mechanical resilience of the demineralized dentin and it is indicated for shallow lesions. On the other hand, TMR provides a quantitative measurement of the amount of mineral loss and lesion depth; however, its limit of detection is about 400%vol.um. Accordingly, shallow lesions are not detectable by TMR [36] as happened for dentin samples treated with both high-F toothpastes after the pH-cycling 1. Therefore, it is important to combine more than one method to better understand the lesions behavior [37]. No previous study has applied TMR associated with other variables conducted herein to compare the response of root dentin to non-F, conventional and high-F toothpastes, which turns our results even more relevant.

An important knowledge from this study is that the residual effect of F toothpastes (especially for the high-F toothpastes) is not as good as their constant presence in the oral environment, which can indicate that part of the F reservoir found after pH-cycling 1 (CaF_2) might have not been incorporated to the tooth after ending the treatment, but lost to the demineralizing solution during the pH-cycling 2 (Table 3 and Figure 3). Finally, the progression of the lesion is dependent on the baseline profile as discussed elsewhere [33,34]; highly demineralized lesions progressed lower than shallow lesions when submitted to further cariogenic challenges. This might be the reason for the lower residual \ effect of CL and CP compared to CT after pH-cycling 2.

Based on the present data, the null hypotheses were rejected, as high-F toothpastes, regardless of the presence of *f* TCP, were more effective than conventional F toothpaste in inhibiting root dentin demineralization, but they were similar to conventional toothpaste on the progression of the root lesions. The results allow inferring that high-F toothpastes may be more indicated than conventional F toothpastes in the prevention of root caries. However, we need further *in situ* or clinical trials to confirm this statement. So far, there are some clinical studies showing benefits of 5,000 µg F/g toothpaste in root caries arrestment [14,15,38] but the evidences are weak [7] and none pH cycling model was conducted in root caries prevention. Furthermore, there is limited information regarding the 5,000 µg F/g toothpaste modified by the inclusion of *f* TCP, especially in dentin. In root dentin, the use of *f* TCP associated with F was tested for the first time.

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LEGENDS

Table 1. Surface hardness (SH) measurements at baseline (SH baseline), SH after the pH-cycling 1, SH after the pH-cycling 2, and the surface hardness change [expressed as percentage surface hardness change (%SHC)], for the pre-demineralized root dentin specimens treated with different toothpastes (n = 15).

Table 2. Uptake of alkali-soluble F by root dentin (Median ± Interquartile range)

Table 3. Mean and standard deviation or *median and interquartile range of the values obtained in the TMR analysis for the specimens treated with different toothpastes (n=11 for all groups, except for CP with n=13).

Figure 1 (1, 2 and 3). Scheme showing the specimens protection according to the acid challenge.

Figure 2. TMR image evidence the production of artificial caries-like subsurface lesion in root dentin by the dynamic pH-cycling regime (Demineralization/Remineralization). Arrows show the narrow subsurface lesion.

Figure 3. Means of dentin Knoop hardness (kg/mm^2) according to treatments and the distance (µm) from the surface, after the pH-cycling 1 and pH-cycling 2 (Bars denote standard deviations; n = 15). Sound dentin values were plotted as reference.

Figure 4. Microradiograph pictures of representative specimens after pH-cycling 1 and pH-cycling 2. (a) Treatment with non-F toothpaste after pH-cycling 1 shows a significant subsurface caries-like lesion. (b) Treatment with CT toothpaste after pH-cycling 1 did not show a significant reduction of the lesion. (c) Root dentin treated with CP after pH-cycling 1; note that there was not lesion development. (d) Root dentin treated with CL after pH-cycling 1; note that there was not lesion development. (e) After pH-cycling 2, the progression of caries-like lesion treated with non-F toothpaste increased significantly. (f) CT toothpaste was able to reduce the progression of the lesion. (g) and (h) After pH-cycling 2, there was the development of caries-like lesion when treated with CP and CL, respectively.

Table 1.

	non-F	CT	CP	CL
SH baseline	35.52 ± 2.38^{A}	36.03 ± 2.82^{A}	35.17 ± 1.78^{A}	34.53 ± 3.27^{A}
SH after pH-cycling 1	10.59 ± 0.6^{Aa}	12.17 ± 1.21^{Aa}	24.06 ± 0.88^{Bc}	23.21 ± 1.25^{Bc}
SH after pH-cycling 2	4.77 ± 0.49^{Ab}	11.68 ± 1.7^{Ba}	22.92 ± 2.63^{Cc}	23.18 ± 2.23^{Cc}
%SHC*	-24.53 (-28.52/20.42)^{A}	-1.51 (-5.86/4.28)^{B}	1.20 (-13.62/11.94)^{B}	0.36 (-16.01/14.37)^{B}

Mean and standard deviation or *median and interquartile range (minimum/maximum)

For SH after pH-cycling 1 and 2 (2-way ANOVA): Values in the same line with different superscript uppercase letters significantly differ from each other ($p<0.05$). Values in the same column with different superscript lower case letters significantly differ from each other ($p<0.05$), excluding SH baseline and %SCH.

* Kruskall-Wallis, $p<0.05$. Different letters show significant difference.

Table 2.

Treatments groups	Alkali-soluble F in dentin (μg F/cm^2)
non-F	0.002 (0/0.00)^A (n=11)
CT	0.005(0.02/0.08)^A (n=14)
CP	0.267 (0.1/0.363)^B (n=13)
CL	0.369 (0.16/0.61)^B (n=12)

Distinct uppercase letters indicate difference statistically significant (Kruskall-Wallis, $p<0.05$) between the treatments.

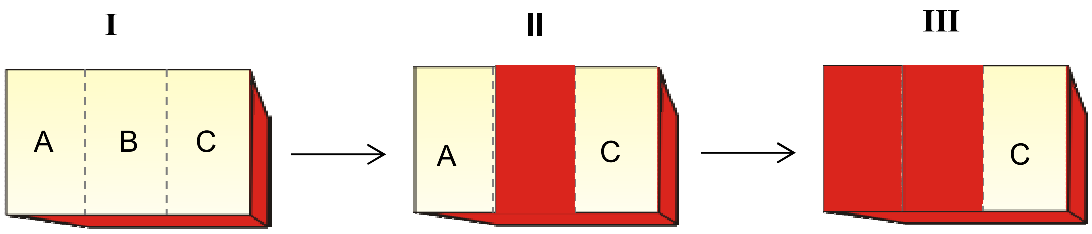
Table 3.

Treatment groups	pH-cycling 1			pH-cycling 2			pH-cycling difference**		
	ΔZ, % min. vol × μm	LD, μm	R	ΔZ, % min. vol × μm	LD, μm	R	*ΔΔZ, %min. vol × μm	ΔLD, μm	ΔR
non-F	1646.36± 195.97^{Aa}	77.17± 13.49^{Aa}	21.85± 3.96^{Ab}	2827.27± 413.55^{Ba}	151.85± 29.38^{Aa}	19.85± 3.88^{Aa}	-1290.00 (-540/900)^{A}	-74.7± 37.9^{A}	2.0± 6.5^{A}
Colgate Total 12®	1321.82± 304.49^{Aa}	85.62± 16.35^{Aa}	15.9± 4.6^{Aa}	1671.82± 165.82^{Bb}	93.49± 14.29^{Bb}	18.37± 3.89^{Ab}	-440.000 (-640/0)^{C}	-7.9± 24.0^{C}	2.5± 6.8^{A}
Prevident®	303.85± 82.31	23.31± 13.28	15.14± 5.25	975.38± 121.83	66.95± 14.26	14.94± 2.64	-690.000 (-725/625)^{BC}	-43.6± 18.1^{B}	0.2± 6.1^{A}
Clinpro 5,000®	170.00± 74.57	15.57± 8.01	11.91± 5.45	1144.55± 144.46	78.53± 19.57	15.48± 4.89	-940.000 (-1040/-890)^{AB}	-63.0± 21.0^{AB}	-3.6± 7.8^{A}

Mean and standard deviation or *median and interquartile range

For all TMR parameters after pH-cycling 1 and 2 : Values in the same line with different superscript uppercase letters significantly differ from each other ($p<0.05$). Values in the same column with different superscript lower case letters significantly differ from each other, excluding CP and CL ($p<0.05$). CP and CL were not included in the statistical analyses, as their specimens presented ΔZ values less than 400 (after the pH-cycling 1), which mean that a caries-lesion did not development in these groups after the pH-cycling 1.

** Kruskall-Wallis, $p<0.05$. Different letters show significant difference for each parameter.



I: sound prepared specimens

II: specimens treated with the respective toothpastes (A and C)

III: new pH-cycling DES/RE, without treatments (C)

Figure 1.

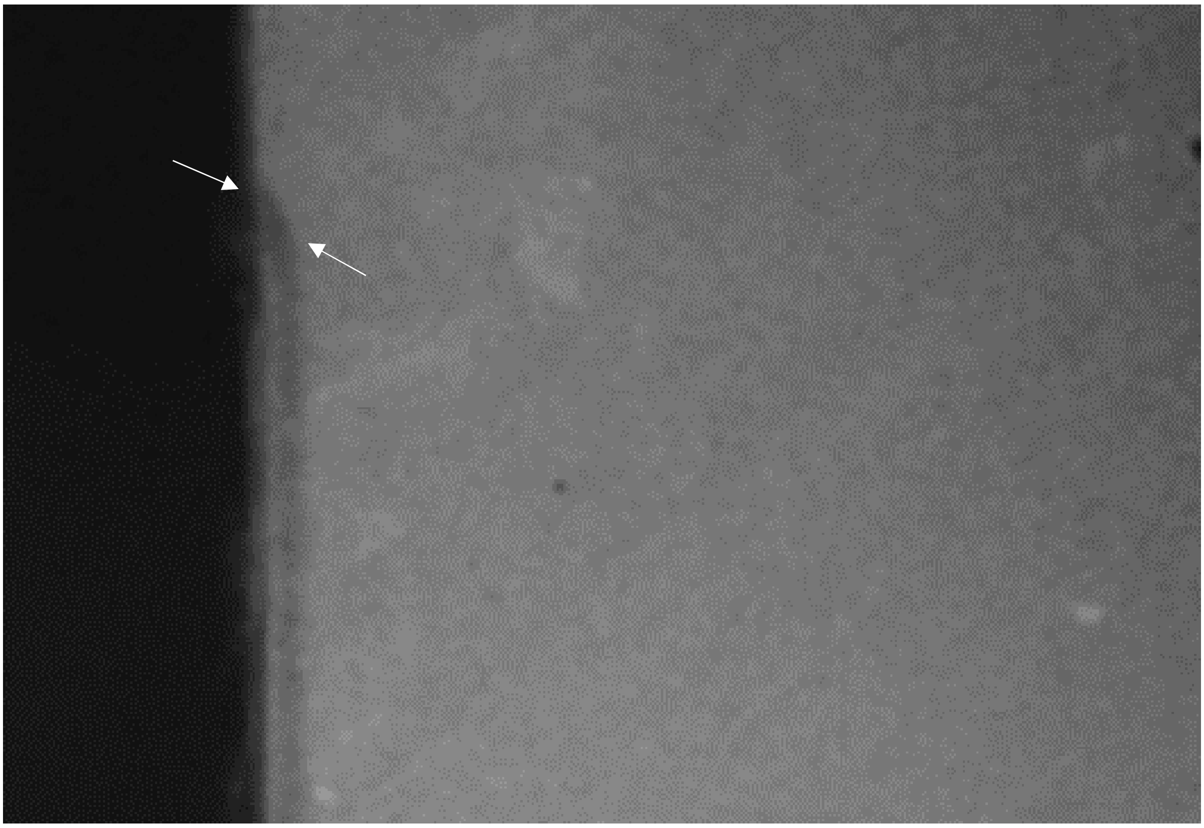


Figure 2.

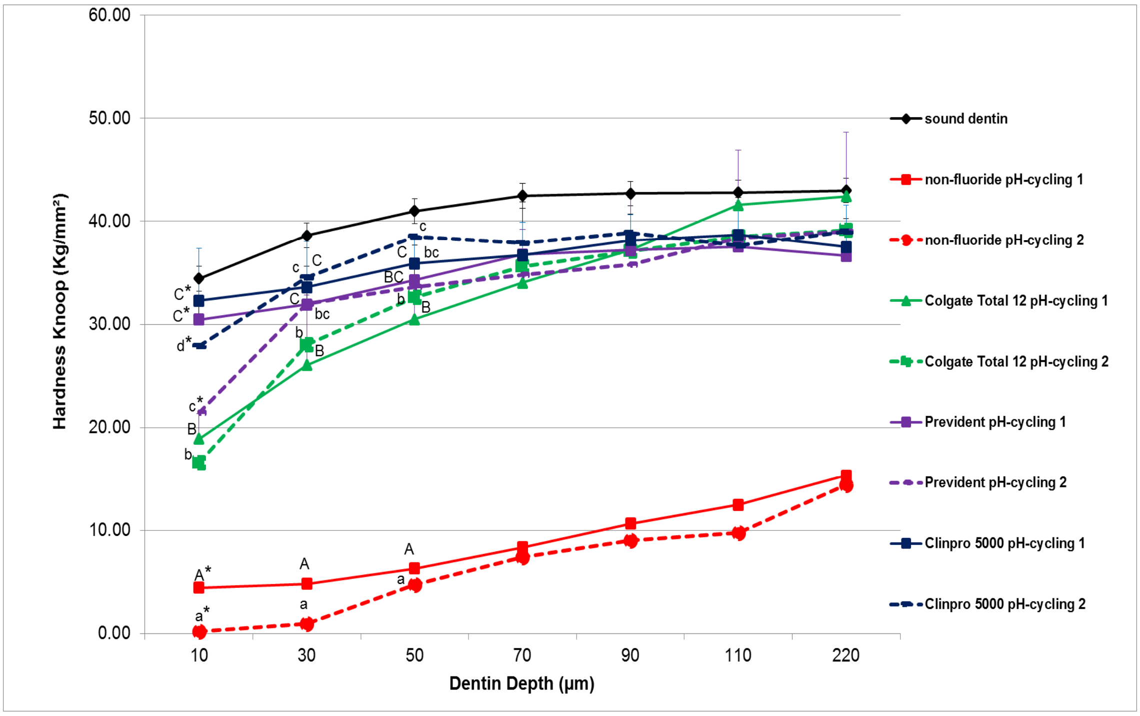


Figure 3.

Distinct capital and lower cases letters indicate difference statistically significant by ANOVA 3 ($p \leq 0.05$) among the treatments within each pH-cycling (capital letter – pH cycling 1, lower case letter – pH cycling 2)

*Asterisk indicate difference statistically significant ($p<0.05$) between the pH-cycling within each treatment. No statistically significant difference was observed among the groups from 70µm onwards

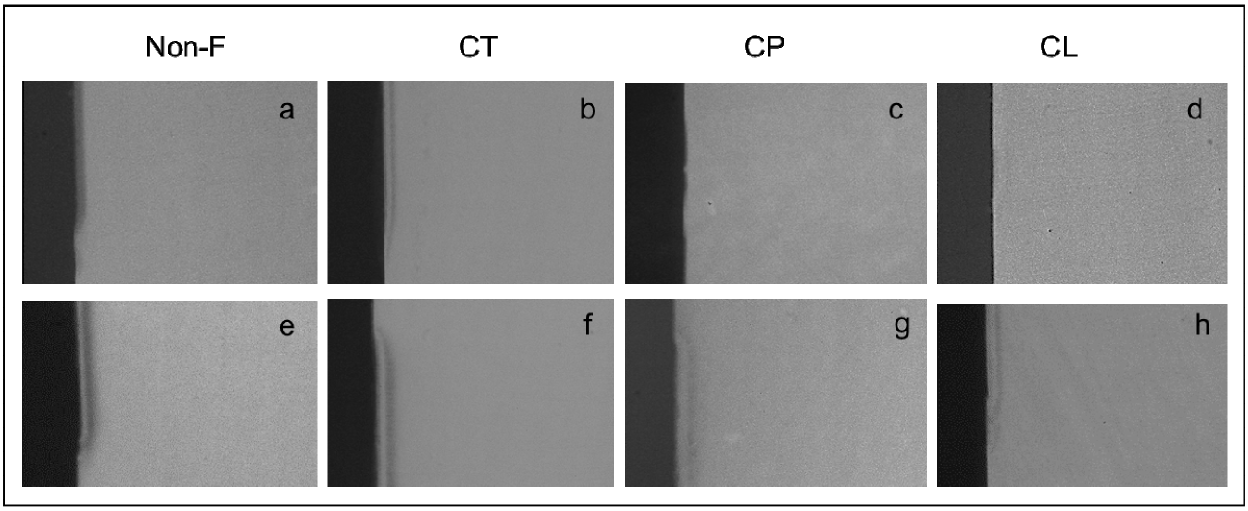


Figure 4.

2.2 ARTICLE 2

This article will be submitted to Caries Research

Caries lesions inhibition and repair using high-fluoride toothpastes with or without tri-calcium phosphate and conventional toothpaste containing 1.5% arginine: an *in situ* investigation

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Running head: High-fluoride toothpastes aid reducing root dentin caries

Keywords: Demineralization. Fluoride. Root dentin. Toothpaste.

ABSTRACT

High-fluoride (F) toothpaste has been recommended to remineralize root dentin lesions and, when associated with tri-calcium phosphate (*f* TCP), it has been used to control enamel caries, similary to conventional fluoridated toothpaste combined with 1.5% arginine. The aim of this double-blind, crossover, *in situ* study, which was conducted in 4 phases of 14 days each, was to evaluate the effect of high-F toothpaste with or without *f* TCP and conventional arginine-based toothpaste in preventing and remineralizing root caries. Twelve volunteers wore palatal appliances with sound (*S*) [subjected to cariogenic challenge] and pre-demineralized [(*PD*) not subjected to cariogenic challenge] root dentin blocks, to assess their performance to inhibit demineralization and activate remineralization, respectively. In each experimental phase, the volunteers used fluoridated toothpastes, 3x/day, exposing the dental blocks to one of the following treatments: (i) 1,450 µg F/g; (ii) 1,450 µg F/g + 1.5% arginine- $CaCO_3$; (iii) 5,000 µg F/g and (iv) 5,000 µg F/g + *f* TCP. Placebo toothpastes was used during washout periods (7 days). Biofilm accumulation was allowed and 20% sucrose solution was dropped 8x/day only on the *S* blocks. After the *in situ* phases, percentage of surface hardness changes and cross-sectional hardness were evaluated. Statistical analysis was performed by ANOVA, followed by Tukey’s test ($p<0.05$). High-F toothpastes showed similar effect to prevent caries initiation and they promoted higher hardness values compared to CN, at 10 µm ($p<0.05$). The results indicate promissory action of high-F for both prevention and remineralization action, but arginine-based toothpaste can be also a great option to control root caries.

INTRODUCTION

With the trend to increase the older population in the next years [Who, 2011; Takabashi and Nyvad, 2016], who are potentially at high-risk of developing root caries, it is expected an increase demand for the prevention and treatment of these lesions [Baysan et al., 2001]. As with coronal caries, the initiation and developing of root caries occurs mainly due to the association of a cariogenic biofilm and high consumption of carbohydrates [Ravald, 1986]. However, root caries is also characterized by the presence of gingival recession and exposure of the root dentin, which is more susceptible to dental biofilm accumulation, potentially increasing the risk for the development and progression of caries lesions [Clarkson, 1995].

Root caries is a preventable disease [Galan, 1994], which can be arrested at any stage through changes in the oral cavity environment, such as the reduction of dietary carbohydrates, the modification and reduction of cariogenic dental biofilm or the application of chemical agents. Accordingly, the recommendation of fluoridated toothpaste is the most cost-effective caries- preventive tool [Rolla, 1999; Marinho et al., 2003], because they combine the mechanical disruption of tooth biofilm and F delivery, favoring the balance between demineralization and remineralization. However, its effects can be influenced by certain factors, such as the F concentration [Walsh et al.,2010].

Although there is reasonable evidence showing high-F toothpastes benefits, their evidences are still inconclusive, once there are very few well-conducted randomized controlled trials in this field [Wierichs and Meyer-Lueckel, 2015]. Moreover, available clinical trials have focused mainly on the role of these toothpastes on root caries reversal, but not on their prevention [Wierichs and Meyer-Lueckel, 2015].

Despite of the widespread use of fluoridated toothpastes, as the main topical strategy, an inadequate amount of available calcium (Ca) and phosphate (P) can limit tooth remineralization. Therefore, new biofunctional materials have been introduced to positively interfere in dental caries development and progression, bringing new perspectives for high-risk patients [Souza et al., 2015]. The functionalized tri-calcium phosphate (*f* TCP) can prevent Ca from prematurely interacting with ionic F, thus delivering F and Ca ions more effectively [Karlinsey, 2010]. The *f* TCP has been shown to have remineralizing effects on enamel surface [Karlinsey, 2010], but no information is available for root dentin.

Another active ingredient that has been added to fluoride dentifrices is arginine. This new technology has been introduced to prevent caries development at an earlier stage, by specifically targeting the residual biofilm. An innovative toothpaste based upon 1.5% arginine and 1450 µg F/g in an insoluble Ca base has been tested with promising results [Souza et al., 2013; Cummins, 2013]. However, up to know, there is insufficient evidence to support a caries-preventive effect for the inclusion of arginine in toothpastes and more rigorous studies are required before any definitive recommendations can be made [Ástvaldsdóttir et al., 2016].

Thus, the aim this *in situ* study was to evaluate the effect of high-F toothpaste combined or not with *f* TCP comparing to 1,450 µg F/g combined or not with 1.5% arginine in reducing the net demineralization of sound root dentin and on the remineralization of initial dentin caries lesions.

MATERIALS AND METHODS

Ethical Aspects and Volunteers

This study was conducted according to the guidelines of good clinical practice and following the Declaration of Helsinki and approved by the local Ethics Committee (No. 44955115.9.0000.5417); Ethics Committee of the Bauru School of Dentistry, University of Sao Paulo, Brazil). Twelve volunteers (2 male and 10 female), aged 20-32 years, fulfilled the inclusion criteria: good oral health with no active caries or periodontal treatment needs; stimulated physiological salivary flow rate of >1 mL/min, and non-stimulated physiological salivary flow rate of >0.25 mL/min and no antibiotic used during the 2 months prior to the study. The exclusion criteria included: systemic illness; pregnancy or breastfeeding; use of a fixed or removable orthodontic device, professional F application in the last 2 months and smoking. All volunteers signed a written informed consent to participate of the study, received written instructions, including schedules, and were trained for all procedures required during the study.

Experimental Design

This *in situ* study followed a double-blind, randomized and crossover protocol, comprising 4 phases of 14 days each, with an interval of 7 days between them. It involved the factors: 1- surface beginning condition (in two levels); 2- toothpaste (in 4 levels); 3- depth from surface [(in 7 levels) this factor was considered only for cross sectional hardness]. The experimental unit was the volunteer (n=12). The main responsible variable were surface hardness difference and cross sectional measurements.

During each phase, the volunteers used palatal appliances containing four bovine blocks with known surface hardness (SH), consisting of two sound (*S*) root dentin in one side of the appliance and two pre-demineralized (*PD*) root dentin in the other side of the appliance, to assess preventive (demineralization inhibition) and reversal (remineralization activation) effect, respectively. The distribution of dentin blocks condition was randomized for each volunteer to be located on right or left side. In the side of the appliance with the *S* blocks, the specimens were exposed to 20% sucrose solution 8 times per day [Aires et al., 2006] allowing biofilm formation, simulating situations of high cariogenic challenge [Cury et al., 1997]. On the other side with *PD* blocks no sucrose solution was applied. In each experimental phase, groups of volunteers used fluoridated toothpastes, 3 times per day, exposing the dental blocks to one of the following treatments: (i) 1,450 µg F/g (Colgate Sorriso®, CS, New York, NY, USA, control); (ii) 1,450 µg F/g + 1.5% arginine- $CaCO_3$ (Colgate Neutraçucar®, CN, New York, NY, USA); (iii) 5,000 µg F/g (Colgate Prevident®, CP, New York, NY, USA) and (iv) 5,000 µg F/g + *f* TCP (Clinpro®, 3M ESPE, CL, 3M ESPE, St. Paul, MN, USA). Placebo toothpastes (mint flavor) was used during the lead-in and washout periods for at least 7 days.

The demineralization which occurred in the originally *S* blocks and the remineralization in the originally *PD* ones was estimated by % of SH difference (%SH-*S*) and % of SH recovery (%SHR-*PD*), respectively. Cross Sectional Hardness (CHS) was also determined in the blocks.

Specimens and Palatal Appliance Preparation

Dentin blocks (4 x 4 x 2 mm) obtained from bovine incisor roots were prepared from the labial surfaces of the cervical portion of bovine roots. The surfaces of the specimens were ground flat with water-cooled silicon-carbide discs (320, 600 and 1,200 grades of $Al_2 O_3$ papers; Buehler Ltd.) and polished with felt paper wetted with diamond spray (1 µm; Buehler). The SH was measured at the beginning of the study

(SH baseline) by the mean of three indentations (Knoop diamond, 10 g/10 s), using a microhardness tester Buehler Ltd, MicroMet 6040, Lake Bluff, IL, USA. Blocks that showed variability higher or lower than 10% among the indentations (intrablock variability) and blocks with SH variability higher or lower than 10% of the average SH calculated for all slabs (interblock variability) were excluded [Nóbrega et al., 2016].

A total of 192 root dentin blocks (30.78 ± 0.78 kg/mm^2) were selected for this study. They were covered with nail varnish in order to create control areas (two sound surfaces) on either side of the central band of the surface. For the *PD* blocks (n=96), they were submitted to a solution containing 3 mM $CaCl_2$. 2H $_2$O (Labsynth, Sao Paulo, SP, Brazil), 3 mM KH_2 PO_4 (Sigma-Aldrich, St. Louis, Mo., USA), 50 mM lactic acid (Sigma-Aldrich), 6 µM tetraethyl methylhydroxydiphosphonate (Sigma-Aldrich), and traces of thymol (Sigma-Aldrich), pH 5.0 (10 M KOH to adjust pH, 30 ml per specimen), for 7 days, at 37°C. The SH for the *PD* blocks was measured immediately after demineralization. Microradiography analysis was conducted to verify whereas a subsurface lesion was developed in the *PD* blocks, without surface erosion. SH baseline (for *S*) and SH-*PD* means were used for the computerized random allocation sequence of the specimens in the experimental phases, volunteers and position in the appliance.

Acrylic resin intraoral palatal appliances, containing four cavities measuring 5 x 5 x 4 mm (two at each side of the appliance) were made for each volunteer. One side of the device contained the sound blocks and the other side the pre-demineralized ones. Before the specimens were inserted into the palatal appliances, they were sterilized by ethylene oxide (Acecil Central de Esterilização Com. Ind. Ltda, Campinas, SP, Brasil) [Amaechi et al., 1998]. The specimens were randomly accommodated into the cavities and fixed in place with wax, totalizing 2 sound and 2 pre-demineralized specimens per appliance (in each phase). A space was created in the acrylic appliance, leaving a 1.0 mm space for biofilm accumulation. In order to enhance the growth of the dental biofilm, the specimens were protected from mechanical abrasion by plastic mesh (pore area of 1 mm^2, Sanremo, São Paulo, SP, Brazil) fixed with wax and acrylic resin on the acrylic surface. Different color waxes were used to identify the side of the appliance in which the sucrose would be used (in the sound specimens).

Treatments

In each phase of the study, 3 volunteers were randomly assigned to one of the four treatments. The toothpastes were packed in blank tubes, labeled with colors to identify them for the investigator, however maintaining the volunteers and examiners blinded. During the *in situ* phase, volunteers dropped a 20% sucrose solution extraorally onto the sound specimens 8 times per day, 1 drop per specimen and after 5 min, the appliances were replaced. No sucrose solution was applied on the pre-demineralized specimens.

The volunteers were instructed to conduct their usual toothbrushing with the respective toothpastes using in each phase, using a toothbrush (Curaprox, CS1560 soft, Kriens, Switzerland) provided by the researchers in all phases of the study, 3 times a day, using a standard quantity of the respective toothpaste, except for the area containing the slabs, where only the toothpaste foam was slighted spread [Nóbrega et al., 2016]. During the *in situ* phases, the appliance was only removed for the main meals, which were kept with moist gauze. The volunteers were also instructed to do not use any other type of F or antibacterial product during the study.

Dentin Demineralization and Remineralization Assessment

At the end of each phase, the appliances of all subjects were collected and washed with purified water, and the SH was again determined. The mean values from the three indentations spaced 100 µm from each other was calculated and the percentage of surface difference (%SH-S) and %SH recovery (%SHR) were calculated for the originally *S* [Cury et al., 2003] and *PD* blocks [Cury et al., 2005], as follow: %SH-S = (initial SH – final SH/ initial SH) x 100 and %SHR-PD = [(SH final - SH lesion) / (SH baseline - SH lesion)] x 100, respectively.

After SH analysis, the blocks were longitudinally sectioned through the center and the CHS was determined in one of the halves. Three rows of indentations each were made at depths of 10, 30, 50, 70, 90, 110, 220 µm in relation to the surface [Moron et al., 2013].

Statistical Analysis

Statistical analysis was performed with Action Stat Pro 2.01 (2005). Normal distribution and equality of variances were checked for all the variables using

Kolmogorov-Smirnov. For all variables, one-way ANOVA was performed followed by Tukey test ($p<0.05$).

RESULTS

Figure 1 shows a microradiograph (TMR) image of the caries-like lesion in the blocks that were pre-demineralized. A subsurface lesion, without surface erosion can be observed, validating the artificial-caries produced lesions.

Table 1 shows data from the preventive effect of the toothpastes represented by %SH-*S* and their effect on root dentin reversal, represented by %SHR-*PD*. CS presented the lowest values of both variables when compared to the other treatments. When considering caries prevention (%SH-*S*), CP and CL showed similar effect, with better prevention ability compared to CN. The caries reversal data showed the highest remineralizing effect for CP, followed by CN and CL, which promoted similar degree of dentin hardness recovery.

Figure 2 shows the Cross Sectional Hardness results regarding the preventive action of tested toothpastes using *S* root dentin. CS toothpaste showed to be less effective in all evaluated depths ($p<0.05$). At 10 μm, the high-F toothpastes (CP and CL) promoted higher hardness values compared to conventional arginine toothpaste (CN). Up to 50 μm the effect of CP was similar to CN and CL, but CL showed higher prevention than CN. CL showed better performance compared to CP and CN at 70 μm and 110 μm. Figure 3 shows the CSH results of the remineralization action of tested toothpastes using *PD* root dentin. The control group showed the lowest values on all depths ($p<0.05$). In 10 μm, the remineralization effect followed the sequence of CP>CL>CN>CS ($p<0.05$). Up to 50 μm, CN was less effective than high-F toothpastes ($p<0.05$) (CP and CL), which resulted in similar dentin reversal ($p>0.05$)

DISCUSSION

Overall, the present results indicate promissory action of high-F toothpastes for both prevention and remineralization of caries lesion. Dental caries is a complex disease that causes progressive changes in the mineral content of teeth and the use of high-F toothpastes can reverse such lesions, mainly at earlier stages [Cummins, 2013]. The current results clearly show that conventional toothpaste is less effective to prevent initial caries development and to remineralize root caries compared to arginine-based and high-F toothpastes, in a highly cariogenic in situ challenge (Table 1, Figure 2 and Figure 3). Therefore, the benefits of conventional F toothpaste [Walsh et al., 2010] might be not enough for patients at high-risk to develop root caries.

The present findings, showing higher preventive effect and dentin remineralization by arginine-toothpastes than conventional one (Table 1 and Figure 2) are supported by the combination of arginine and an insoluble Ca compound. Arginine acts in non-pathogenic, arginolytic organisms, such as S. sanguis, who metabolizes arginine to ammonia which, in turn, can neutralize plaque acids after sugar challenge [Wijeyeweera et al., 1989]. The insoluble Ca compound acts as a reservoir of free Ca ions to improve the remineralization process [Cummins, 2013]. It is important to highlight that the study adopted an *in situ* protocol to simulate clinical conditions. Therefore, although CN contains sodium monofluorophosphate (MFP) in its composition and the MFP molecule is not active against caries, in a clinical situation, it is hydrolysed by non-specific bacterial phosphatases [Pearce and Dibdin, 2003], releasing F ion to act in the remineralizing process [Cury et al., 2003]. Thus, once F alone cannot completely prevent the caries process, because it does not act upon dental biofilm, toothpastes containing arginine can be a good choice to prevent root caries in the earlier stages.

When both high-F toothpastes were compared, CL (with *f* TCP) was expected to have better effect on caries progression than for CP, due to the presence of compatible F functionalized with calcium phosphate ingredient (*f* TCP), which would act mainly on the subsurface lesion. However, the effect of CP was better than CL in reversing caries lesion, when analyzed by SH-*PD* (Table 1) or by CSH-*PD* in 10 µm depth of the surface (Figure 3). Previous study conducted in enamel substrate showed that the *f* TCP can prevent Ca from prematurely interacting with ionic F and forming calcium fluoride (CaF_2), thus delivering more F and Ca ions to the surface [Karlinskey et al., 2012]. However, it is well known that when the F concentration in the oral environment is above 100 ppm, CaF_2 is formed and the F reservoir increases when F concentration is higher [Bruun and Givskov, 1991]. Once root dentin is less mineralized than enamel [Bignozzi, 2014], it seems that the effect of *f* TCP in preventing Ca from prematurely interacting with ionic F and forming CaF_2 was not enough to improve the effect of F alone, which could explain the better results presented by CP.

This study was designed to evaluate the anticaries potential of the toothpastes on root dentin, simulating highly cariogenic clinical conditions, which means that the results might be interpreted with caution. Overall, the present results showed great performance of high-F toothpastes, therefore patients at high-risk might benefit from higher F concentration to prevent and remineralize root caries lesions. Arginine-based toothpastes also showed great performance when compared to conventional one and might be a good option to prevent root caries, mainly by the action of arginine into the oral biofilm.

Acknowledgments

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Disclosure Statement

There are no conflicts of interest with respect to the authorship and/or publication of this work.

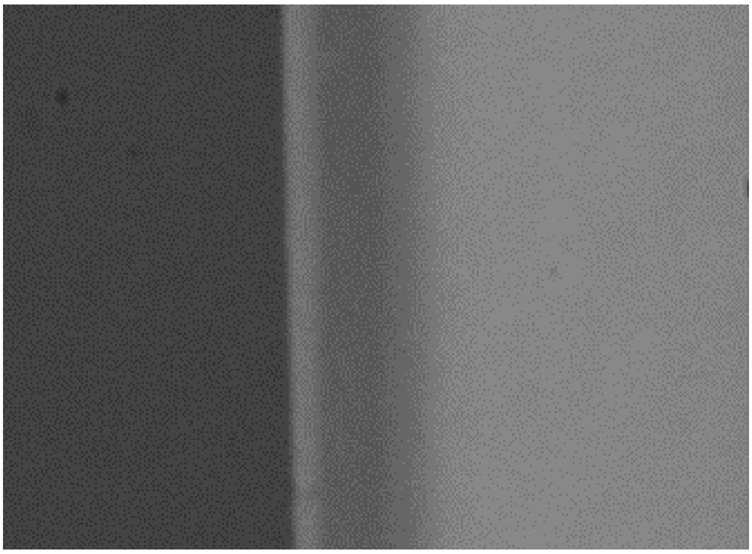
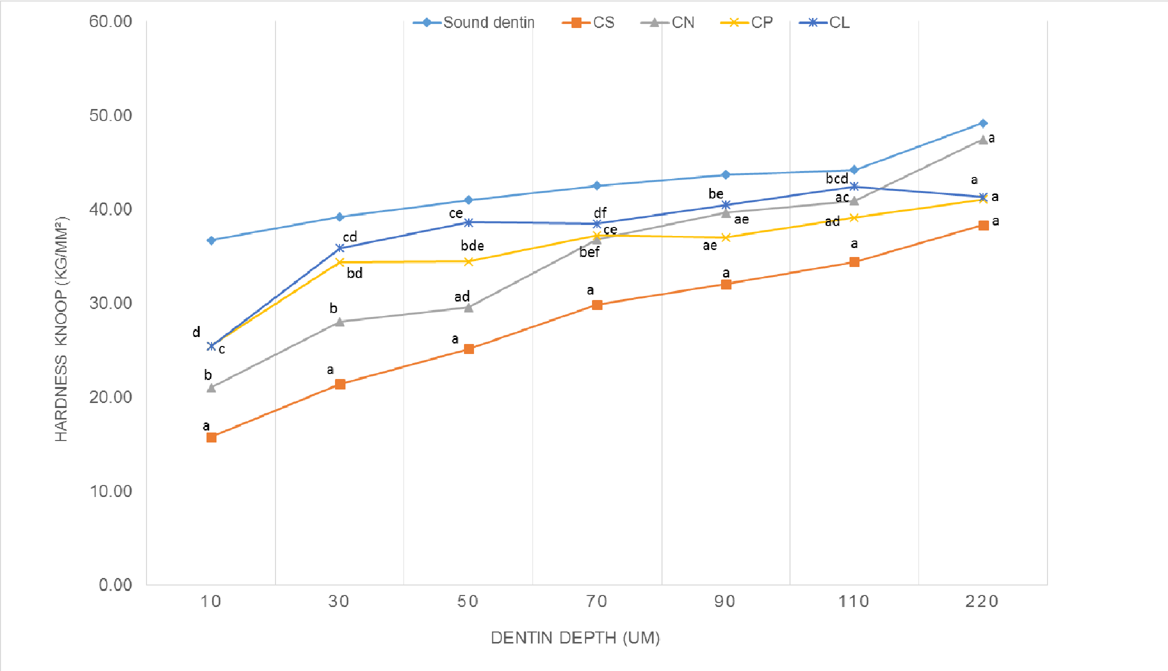


Figure 1. Root caries-like lesion after demineralizing process

Table 1. Effect of toothpastes to prevent and reversal root caries lesions (n=12)

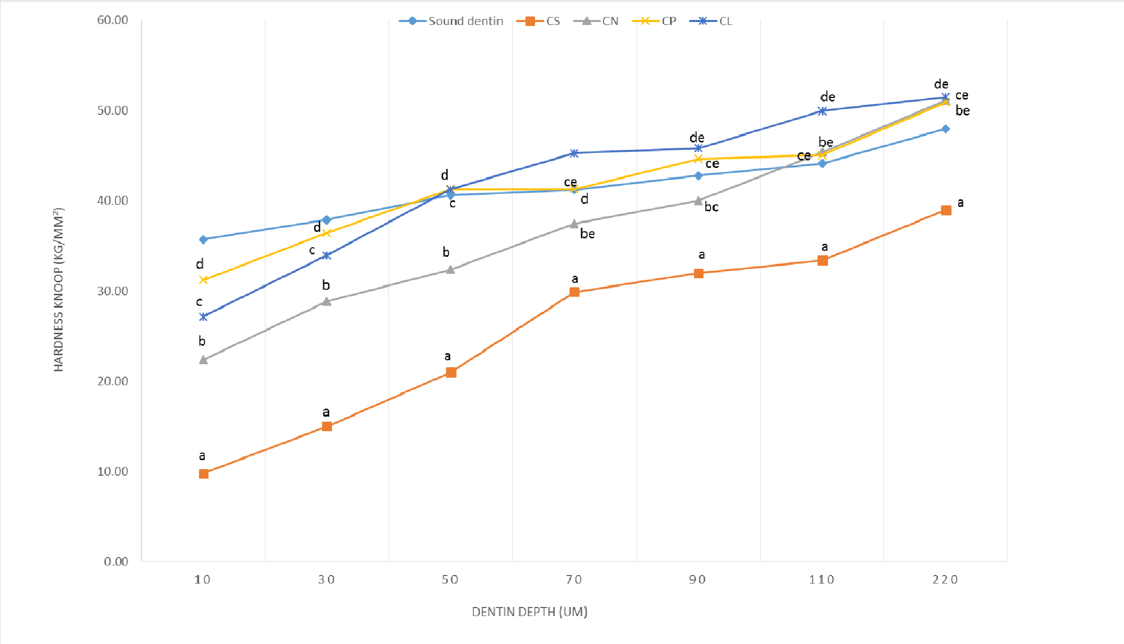
Treatments	Prevention	Reversal
	%SLH	%SHC
CS	80.10±5.32^{A}	0.19±7.58^{A}
CN	61.44±10.08^{B}	28.18±10.55^{B}
CP	35.96±9.04^{C}	49.32±6.2^{C}
CL	32.02±7.13^{C}	32.39±7.8^{B}

Distinct capital letters indicate difference statistically significant ($p \leq 0.05$) among the lines, in each condition. One-way ANOVA was performed followed by Tukey test ($p<0.05$).



Distinct lower cases letters indicate difference statistically significant ($p \leq 0.05$) among the treatments within each depth. One-way ANOVA was performed followed by Tukey test ($p<0.05$).

Figure 2. Means of dentin Knoop hardness (kg/mm^2) and the distance (μm) from the surface (bars denote standard deviations) to evaluate the prevention effective of the toothpastes. Sound dentin values were plotted as reference.



Distinct lower cases letters indicate difference statistically significant ($p \leq 0.05$) among the treatments within each depth. One-way ANOVA was performed followed by Tukey test ($p<0.05$).

Figure 3. Means of dentin Knoop hardness (kg/mm^2) and the distance (μm) from the surface (bars denote standard deviations) to evaluate the effect of the toothpastes on root caries progression. Sound dentin values were plotted as reference.

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2.3 ARTICLE 3

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Radiotherapy alters the composition, structure and mechanical properties of root dentin

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Abstract

Objectives: Post-radiation dental lesions affect mainly the cervical area of the tooth, which include dentin root caries as a notable clinical complication in patients undergoing radiotherapy. No studies have investigated the effects of radiation exposure on root dentin breakdown. To better understand this effect, we used human root dentin specimens obtained from third molars from similarly aged individuals. *Methods:* Twenty specimens were analyzed by the surface hardness (SH), energy-dispersive X-ray spectroscopy (EDX) and X-ray diffraction (XRD) to evaluate the baseline properties of their root dentin. Other six human teeth were prepared and analyzed by scanning electron microscopy (SEM). Then the specimens were randomly distributed between two groups (n = 13 per group) and irradiated with a total dose of 55 Gy or 70 Gy in a linear accelerator. The percentage of EDX and surface hardness loss (%SHL) were determined based on measurements before and after irradiation. The specimens were also analyzed after irradiation by SEM and XRD. The Ca/P weight ratio was calculated to assess changes in the inorganic and organic components. *Results:* Based on SEM analysis, radiation exposure induced dehydration of the dentin. The Ca/P weight ratio decreased ($p = 0.0045$). The %SHL of specimens irradiated with 70 Gy was higher than that of the 55-Gy group ($p < 0.05$), although even the lower dose induced root dentin breakdown. *Conclusions:* Overall, we can state that radiation exposure changes the composition and structure of human root dentin, which detrimentally affect its hardness.

Key-words: Head and neck. Oncology. Radiotherapy. Root caries.

Introduction

Radiotherapy is an ionizing radiation–based therapeutic approach that is widely used for cancer patients. When used for head and neck cancer treatment, it may cause adverse changes in the oral cavity [1], including mucositis, hyposalivation, osteoradionecrosis, dentition breakdown and radiation-related caries [2]. It was previously thought that radiation-induced hyposalivation was the main cause of radiation-related caries development [3]. In contrast, recent investigations have suggested that radiation has direct effects on tooth destruction and post-radiation dental caries [4,5,6].

Post-radiation dental lesions affect mainly the cervical area of the tooth [7,8], which include dentin root caries as a notable clinical complication. Human dentin is a complex tissue [9] that is highly soluble, possibly because of its mineral content and higher levels of carbonate and magnesium [10]. Therefore, dentin root caries rapidly progress, and this condition may lead to severe tooth destruction [8], which in turn also increases the risk of developing osteoradionecrosis [11] and negatively impacts the quality of life of the patient [12].

Whereas several studies have measured the mechanical properties of dental teeth after radiation exposure [5,15], there is a lack of knowledge concerning its effect on human dental structure and composition. In addition, most studies have focused on enamel [5,6] or coronal dentin [14,15] but not on root dentin. The structural pattern of coronal dentin differs from that of root dentin. The tubules run continuously from the dentin-enamel junction to the pulp in coronal dentin, and from the cementum-dentin junction to the pulp canal in the root [16]. This microstructure is related to the functional behavior of the tissue, as, for instance, the alignment of the tubules could affect its mechanical properties. Likewise, in the case of restorative procedures, changes in the substrate composition could potentially interfere with interactions with the restorative materials, especially during the adhesion process [17].

To date, no systematic study has been carried out relating the effect of radiation exposure on the chemical elements, structure and mechanical properties of root dentin. With the combined use of energy-dispersive X-ray spectroscopy (EDX), microhardness and X-ray diffraction (XRD) we evaluated these aspects of human root dentin before and after radiation exposure. In addition, scanning electron microscopy (SEM) was used to better understand the structure of radiated dentin. The null hypothesis was that radiation has no effect on root dentin.

Materials and methods

Experimental design

This *in vitro* study involved one factor: radiation exposure. Human root dentin specimens obtained from fresh sound third molars that had similar surface hardness (SH) and were from individuals of a similar age (18 to 25 years old) were randomly divided into two groups (n = 13 teeth per group) that were irradiated with 55 Gy or 70 Gy, in which 6 of them (n=3) were used to evaluate SEM analysis before and after radiation exposure. The percentage surface hardness loss (%SHL) and EDX were determined based on measurements before and after irradiation. The Ca/P weight ratio was determined by weight. In addition, the teeth were analyzed before and after irradiation by XRD and SEM (this response variable used different specimens).

Ethical considerations

Ethical approval for this study involving human teeth was granted by the local Ethics Committee, University of São Paulo, SP, Brazil (N^{o}. 49812515.1.0000.5417). Sound human third molars free of caries, from individuals 18 to 25 years old, were collected with informed donor consent.

Specimen preparation

Buccal flattened and polished dentin specimens (4 x 6 x 3 mm) were obtained from the cervical roots of sound freshly extracted human molar teeth. The crowns were removed at the cemento-enamel junction using an ISOMET Low Speed Saw cutting machine (Buehler Ltd., Lake Bluff, IL., USA). The surfaces were ground flat and polished in a metallographic polisher (Aropol 2V; Arotec, Cotia, SP, Brazil) using water-cooled carborundum discs (600 and 1200 grades of Al_2O_3 papers - CarbiMet paper discs; Buehler, Lake Bluff, IL, USA) and felt paper wet by diamond spray (1µm; Buehler). All the specimens were ultrasonically cleaned in distilled water for 10 min to remove the debris.

The specimens were randomly divided (Excel 15.0, Microsoft, Redmond, WA, USA) into two groups: Irradiated 55 Gy (55 Gy of radiation, n=10) and Irradiated 70 Gy (70 Gy of radiation, n=10). All the specimens were analyzed before and after irradiation using EDX, XRD and SH. The %SHL was calculated. In addition, other six human teeth were prepared for SEM. This analysis was conducted in different specimens before and after irradiation, due to the preparation of the specimens.

Gama irradiation exposure

The dentin specimens directly received a total of 55 or 70 Gy of radiation in a linear accelerator (Varian, Clinac 6EX, Palo Alto, CA, USA), to simulate clinical situations for the treatment for head-and-neck cancer patients [8,12]. During the radiation exposure, the samples were mounted on stubs and remained completely submerged in water with 5 mL deep per specimen.

EDX Analysis

For the analysis of the % component composition of the root dentin, EDX assessment was performed. The X-ray detector system was attached to a scanning electron microscope (FEI-Inspect S50, LNNano) operating at 20.0 kV, using a 5 nm spot size. This method allowed determining the relative amounts of calcium (Ca), phosphorus (P), oxygen (O), carbon (C) and magnesium (Mg) by volume percent.

SH tests

Baseline SH of the dentin specimens was determined by three indentations using a Knoop diamond indenter spaced 100 μm from each other. Assessments were made with 10-g load for 10 s, using a Buehler Ltd, MicroMet 6040 (Buehler, Lake Bluff, IL, USA). The specimens presenting baseline SH 60.07 ± 1.55 were selected for this study.

At the end of each radiation exposure, SH of the specimens was again determined at 100 μm distance each other. The mean values of the baseline values were also averaged, and the percentage of surface hardness loss [%SHL = 100 (SH after radiation - baseline SH)/baseline SH] was calculated.

SEM Analysis

SEM analysis was performed using the specimens from non-irradiated (control; n = 6) and irradiated teeth after the total dose of 55 Gy (n = 3) and 70 Gy (n = 3). The SEM prepared specimens were cleaned for 10 minutes in an ultrasonic bath with purified water. The specimens were fixed on stubs with a double-sided adhesive carbon tape (Electron Microscopy Sciences, Washington, PA, USA) and were sputter-coated with gold in a vacuum metallizing machine (SDC 050; Bal-Tec AG, Balzers, Germany). The specimens were examined with a scanning electron microscope (Philips XL30 FEG, Eindhoven, The Netherlands). The images were observed by SEM at an accelerating voltage of 15 kV, a working distance of 20 mm and with a magnification of 10000x and 20000x.

Control and post-irradiation tissue morphological changes were analyzed using two score systems, in the magnification of 1000x. For dentin tubules, scores were attributed as follows: (0) Regular, (1) Partially obliterated, (2) Totally obliterated. Presence of cracks and fissures was classified as (0) Absent or (1) Present.

XRD Analysis

XRD was carried out using a X-ray diffractometer powder system (Rigaku Geigerflex, Woodland, TX) with Ni-filtered CuKα radiation and a source operating at 40 kV and 25 mA. The data were collected in the 2θ range from 10–70 degrees.

Statistical Analysis

Statistical analyses were performed with the Statistica software (SSP) version 10.0 (Statsoft®, Tulsa, Oklahoma, USA). Control and post-irradiation values were analyzed for SH and EDX using two-way analysis of variance and Tukey's tests at a level of significance of 5%. Qualitative analyses were performed for SEM and XRD.

Results

EDX and SEM

The atomic percentages of Ca, P, O, C, Mg and the Ca/P weight ratio on the dentin were determined by EDX (Table 1 and Figure 1). The atomic percent of C tended to decrease in all of the groups after irradiation with statistically significant difference ($p=0.03$). O and Mg increased with significant difference in the groups ($p=0.000006$ and $p=0.00061$, respectively). Major changes in the chemical composition of dentin were observed in trace elements. Ca/P molar ratio decreased ($p=0.0045$).

The morphological changes were observed using SEM images, which are present in Figure 1. There were distinct difference between the morphologies of dentin before and after treatment in both irradiated specimens. SEM images suggested that the radiation induced a dehydration of the dentin indicated by the presence of cracks around of the tubules (Figure 1). Before radiation, the score attributed for dentin tubules was (0) Regular (Figure 1A). After 55 Gy, dentin tubules were partially obliterated (1) (Figure 1B) and after 70 Gy, they were totally obliterated (2) (Figure 1C). Presence of cracks and fissures was classified as Absent (0) for no-irradiated teeth (Figures 1 E and I) and Present (1) for irradiated teeth (Figures 1 F, J, G and K).

X-ray diffraction

Figure 2 shows the XRD results of the dentin specimens' surface, before and after radiation therapy with 55 and 70 Gy. The pattern of dentin produced peaks that were similar in their intensity and position in.the baseline and irradiated with 55 Gy. When teeth were irradiated with 70 Gy, there was a remarkable disorganization of dentin apatite crystals.

Mechanical properties of root dentin – SH

The changes in the hardness values of root dentin and the %SHL are presented in Table 2. There was no significant difference regarding SH among dentin specimens before irradiation ($p>0.05$). A significant reduction of SH mean was observed in all dentin specimens after irradiation ($p<0.05$). The %SLH of the specimens irradiated with 70 Gy was higher than the specimens irradiated with 55 Gy ($p<0.05$).

Discussion

The focus of our studies has been the root substrate dentin. Post-radiation dental lesions, which develop mainly in the cervical and incisal area, differ in their location and pattern when compared with caries lesions in patients who are not exposed to radiation [7,8]. This may be a particular concern mainly for elderly patients (frequently diagnosed with oral cancer), as their enamel may be worn away, mainly due to gingival recession and/or loss of the surrounding alveolar bone [18]. As a result, the root dentin of a patient undergoing radiotherapy might be directly exposed to radiation, which, in combination with reduced salivary flow because of radiotherapy or medication, might particularly predispose them to root caries. Based on the present results, radiation exposure affected the chemical composition, structure and mechanical property (surface hardness) of root dentin. Therefore, our null hypothesis was rejected.

Our results showed a decrease in the Ca/P weight ratio (Table 1, $p < 0.05$), which indicates that radiation exposure altered the inorganic and organic components of human root dentin [19]. The Ca/P weight ratio and Ca/P molar ratio determine the rate of hydroxyapatite mineralization [19], an important parameter, as both the mechanical properties of the tooth substrate and its rate of biodegradation strongly depend on it [19]. This ratio was calculated for stoichiometric hydroxyapatite (HA; Ca/P weight ratio = 2.151 and Ca/P molar ratio = 1.67) [19], which varies according to the level of tissue mineralization. With regard to the changes in the inorganic components shown herein, the lower values of the Ca/P weight after radiation exposure in both the 55 and 70 Gy groups ($p < 0.05$) indicate that the irradiated root dentin structure was less mineralized with respect to Ca content than the sound one. This alteration might have decreased the permeability and solubility of the substrate [20], which also explains the reduction in SH and the increase in %SHL after irradiation.

Another important factor to be considered is the high water content of dentin [9]. The interaction between radiation and water is high [21]. When radiolysis occurs, H^+ and OH^- are released and then it can interact with other ions to produce new compounds. This explain the decrease in C ions and the lower values of the Ca/P weight after radiation exposure. We also observed the incorporation of O and Mg after irradiation with 55 and 70 Gy (Table 2, $p = 0.000006$ and $p = 0.00061$, respectively). Once more, the ions released by water after radiation exposure induced the formation of a secondary nonapatitic calcium phosphate phase [20], which likely would have made the HA more susceptible to degradation [22]. Furthermore, Mg as a substituent component inhibits crystal growth and strongly influences the lattice parameters, which might have made the apatite amorphous [23]. Upon irradiation, these defects could be mobilized from the surface layer of the crystals, removing the entrapped ions and modifying the structure of the crystals [24]. This alteration may also contribute to cracks and the obliteration of dentin structure as shown by SEM images (Figure 1 B and C), as the presence of a less well-structured crystal arrangement increases the permeability and susceptibility of that substrate to cracks [25]. These structural defects can make the dentin dry and friable [14], which also impairs its mechanical resistance, which also could be related to the faster development of caries lesions in irradiated substrates. The formation of free radicals by radiolysis [21] within their structure can be present in irradiated teeth for long periods of time.

All these changes in the substrate (i.e., reduced Ca mineralization or presence of free radicals within its structure) could also negatively interfere with the adhesion of the restorative dental materials that are commonly used to treat such lesions [26]. The material of choice for root caries lesion restoration is the resin-modified glass ionomer cements, which involve in their chemical process the formation of ionic bonds between the carboxylate groups on the polyacid molecules and Ca ions in the tooth surface [27]. In addition, some adhesive systems that are based on functional monomers, such as 10-methacryloyloxidecyldihydrogen phosphate, must be used with caution, as this system promotes chemical bonding to hydroxyapatite Ca ions [28].

With respect to the changes in the organic components of dentin, they can be explained by the induction and activation of enzymes that degrade collagens by radiation exposure, such as matrix metalloproteinases [29]. When collagen type IV is degraded, an instability in the substrate occurs [4], which explains the dentin breakdown presented herein (Figure 1). Because collagen IV has a large biochemical/structural role in molecular bonding of enamel and dentin [4], irradiation of root dentin could negatively interfere with the adhesion process. Our findings underscore the importance of additional studies in this area.

In our study, X-ray diffractograms were used to analyze changes in the crystalline structure of the specimens. When X-rays interact with a crystalline substance, such as dental substrates, a diffraction pattern is obtained. Figure 2 shows a remarkable disorganization of dentin apatite crystals after irradiation with 70 Gy. However, irradiation did not induce a reduction in dentin crystallinity based on the XRD analysis, as the pattern of dentin before and after irradiation produced peaks that were similar in their intensity and position. These data indicate a comparable level of crystalline domains among the specimens before and after irradiation (Figure 2). It is important to note that a new peak was observed between 20 and 25 degrees after 70 Gy irradiation, but not after 55 Gy. This could be explained by the interaction between the high-energy X-ray irradiation and intrafibrillar mineral in root dentin and water which might have influenced its elastic behavior [30] and resulted in the observed pattern.

Based on our findings, we can state that root dentin is extremely vulnerable to the effects of radiation. A limitation of the present study should be noted. The irradiation used herein was based on intensity-modulated radiotherapy (IMRT) [31]. In a clinical situation, this method presents great advantages, mainly from the use of 360° rotation radiation therapy, which allows the primary target to receive the total amount of radiation necessary for treatment, whereas the dose to the adjacent critical structures and organs at risk is limited [7,12]. Despite the advantages of this method, it is quite expensive and in developing countries is not the method of choice [12]. Clinically, even with the use of the IMRT method to treat head and neck cancer, the teeth are located close to the targeted area and exposure of hard dental tissue cannot be prevented [12]. Because of the use of the IMRT method and its associated costs, the radiation exposure applied herein was not fractioned [32], as commonly indicated in clinical situations [12]. However, as the dose is cumulative, the final dose is the same and thus should be the effects. In addition, it was expected the same effects in the final dose compared to the fractionated one, once clinically, the flow and the quality of the saliva in a patient undergoing radiotherapy are reduced and, the mineral deposition between each dose do not recover completely.

It is also important to note that when the specimens were subjected to radiation exposure, they were completely submerged in water (5.0 mL per specimen), minimizing dehydration of the irradiated area and energy absorption, to receive the required dose. This also maintained ideal conditions for the analysis and maintained the intrinsic water content of dentin. As dentin is a highly complex, hydrated biological tissue, changes in its microstructure and composition could have influenced its mechanical properties [9]. In addition, we included similarly aged teeth in this analysis, to avoid difference in the chemical composition and microstructure of dentin.

An important finding from the present investigation is that—unlike the study of Liang et al. [33], in which a total dose of 50 Gy had little additional effect on dentin—our results showed damage in the teeth using about this same dose (Table 2 and Figure 1). However, in agreement with this same study, we suggest that irradiation around 50 Gy could be the key dose that calls for anticipated preventive action against radiation-related caries (Table 1, Table 2 and Figure 1).

Overall, this study indicates that radiation exposure changes the composition and structure of human root dentin, which may contribute to deleterious changes in its mechanical properties. We observed that a higher dose results in greater damage to the teeth. Together, the changes reported herein might increase the risk of radiation-related caries and influence the selection and performance of the dental materials used in the treatment of patients who have undergone radiotherapy. To confirm whether these changes in the hard tissue of the root are directly related to dentin root caries formation, further studies should be carried out using a biofilm model to evaluate the development of dental caries using irradiated teeth.

Compliance with Ethical Standards

Conflict of interest: Marilia Mattar de Amoêdo Campos Velo declares that she has no conflict of interest. Ana Laura Herrera Farha declares that she has no conflict of interest. Aymée Shiota declares that has no conflict of interest. Paulo Sérgio da Silva Santos declares that he has no conflict of interest. Simone Sansaverino declares that she has no conflict of interest. Ana Tarsila Fonseca declares that she has no conflict of interest. Linda Wang declares that she has no conflict of interest.

'Conflicts of interest: none'

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LEGENDS

Table 1. Element content in At% (Mean±SD) in root dentin before and after irradiation, according to the groups.

Table 2. SH analysis of root dentin before and after irradiation and percentage of surface hardness loss (%SHL), according to the radiation therapy (Mean ± SD, n=10).

Figure 1. EDX/SEM results of studied groups. (A) and (B) Non-irradiated root dentin (10,000 and 20,000 X) with absence of cracks and fractures in the dentinal structure (Score 0); (C) and (D) Irradiated 55 Gy showing presence of cracks with dentin tubules partially obliterated (10,000 and 20,000 X) (Score 1) and (E) and (F) Irradiated 70 Gy showing presence of cracks and fractures with dentin tubules obliterated (10,000 and 20,000 X) (Score 1). Arrows mean presence of cracks and fissures.

Figure 2. XRD patterns of the peaks of the elements, according to the conditions: sound dentin (before irradiation), irradiation with 55 Gy and irradiation with 70 Gy.

Table 1.

	Irradiation 55		Irradiation 70	
	Before	After	Before	After
Ca	23.87±4.3^{A}	19.51±1.43^{A}	27.71±16.85^{A}	18.94±3.79^{A}
P	11.27±1.2^{A}	11.55±1.06^{A}	11.05±2.53^{A}	11.29±2.4^{A}
Ca/P weight ratio	2.1^{A}	1.7^{B}	2.5^{A}	1.7^{B}
C	23.87±4.3^{A}	20.6±3.23^{B}	27.71±16.85^{A}	24.63±17.21^{B}
Mg	0.42±0.37^{A}	0.93±0.21^{B}	0.44±0.41^{A}	0.89±0.2^{B}
O	43.00±2.53^{A}	47.41±1.25^{B}	40.08±9.75^{A}	44.25±10.87^{B}

[a] distinct upper case letters indicate statistical significant difference among columns in the same row and in the same group ($p<0.05$).

Table 2.

	SH (KHN)		
Groups	Before irradiation	After irradiation	%SHL
Irradiation 55	58.93±2.81^{A}	51.66±2.41^{B}	12.24^{C}
Irradiation 70	61.21±4.16^{A}	47.26±8.43^{B}	22.87^{D}

[b] %SLH: percentage of surface hardness loss. Distinct upper case letters indicate statistical significant difference among columns in the same row and in the different rows ($p<0.05$).

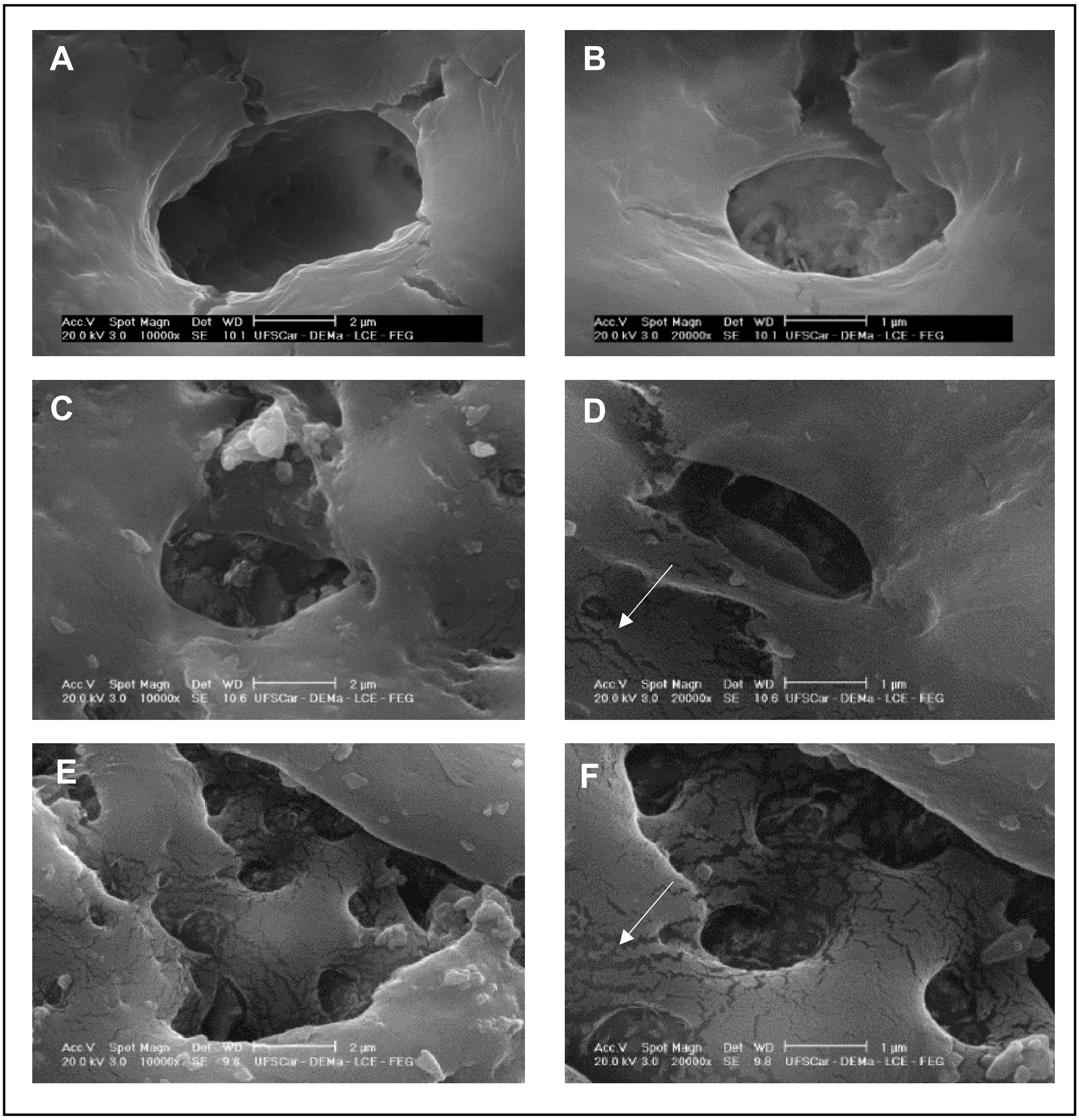
A
Acc.V Spot Magn Det WD 2 µm
20.0 kV 3.0 10000x SE 10.1 UFSCar - DEMa - LCE - FEG
B
Acc.V Spot Magn Det WD 1 µm
20.0 kV 3.0 20000x SE 10.1 UFSCar - DEMa - LCE - FEG
C
Acc.V Spot Magn Det WD 2 µm
20.0 kV 3.0 10000x SE 10.6 UFSCar - DEMa - LCE - FEG
D
Acc.V Spot Magn Det WD 1 µm
20.0 kV 3.0 20000x SE 10.6 UFSCar - DEMa - LCE - FEG
E
Acc.V Spot Magn Det WD 2 µm
20.0 kV 3.0 10000x SE 9.8 UFSCar - DEMa - LCE - FEG
F
Acc.V Spot Magn Det WD 1 µm
20.0 kV 3.0 20000x SE 9.8 UFSCar - DEMa - LCE - FEG

Figure 1.

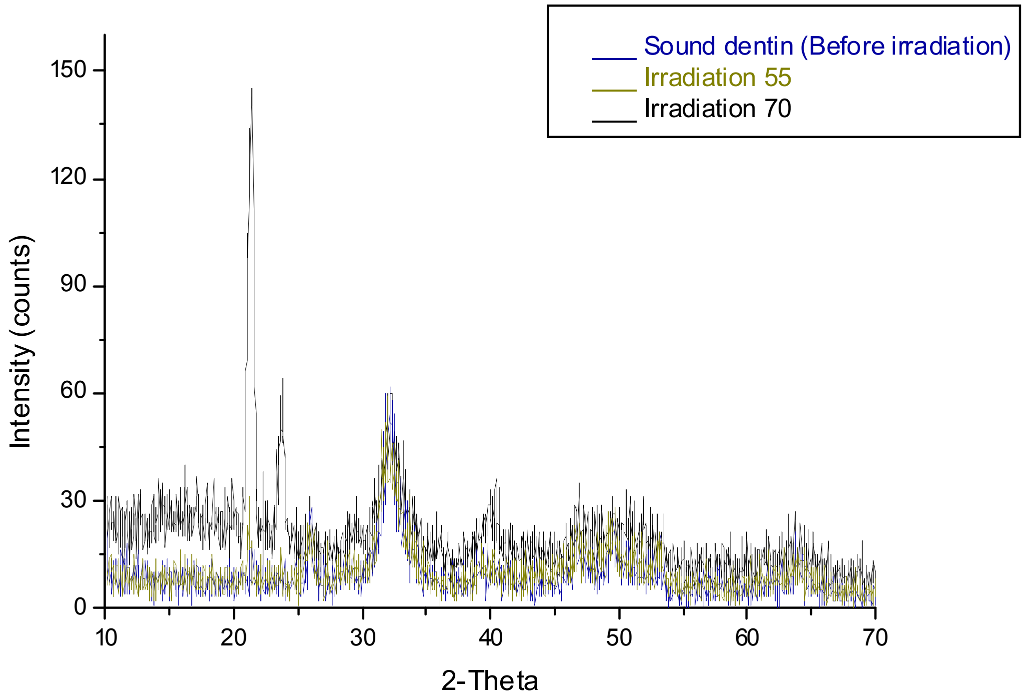
Sound dentin (Before irradiation)
Irradiation 55
Irradiation 70
Intensity (counts)
150
120
90
60
30
0
10
20
30
40
50
60
70
2-Theta

Figure 2.

3 Discussion

3 DISCUSSION

Root caries has becoming a significant dental problem in the world (BANTING, 2001). People are not only living longer but also retaining more of their natural teeth in the oral cavity, which are potentially at high-risk for developing root caries. The main idea was to understand this condition and looking for approaches to control it.

In the article 1, we have compared commercial toothpastes with 5,000 µg F/g F alone and combined with *f* TCP to verify the residual effect of the treatments and both were similar in reducing root demineralization *in vitro*. However, we still have doubts whether the results would be the same in a clinical situation. In the *in situ* study, we evaluated the same high-F toothpastes compared in the first article to a conventional and arginine-based toothpastes. All the toothpastes were NaF/silica-based, except the arginine one, which contains sodium monofluorophosphate (MFP) in its composition. We did not evaluate *in vitro* arginine-based toothpaste, once it is only hydrolysed in saliva by non-specifi bacterial phosphatases (PEARCE, DIBDIN, 2003). Therefore, arginine-based toothpastes were only evaluated *in situ*.

For sound root dentin, it was showed in article 1 that high-F toothpastes may be more indicated than conventional one in preventing root caries. In the article 2, the results confirmed these data, showing that in a clinical situation, the effects of the toothpastes were performed in the same way. Due to the critical pH for dentin dissolution is range 6.5, a decrease in the pH in the biofilm fluid will lead to demineralization (HOPPENBROUWERS et al., 1986). However, if F is present in biofilm fluid and the pH is not lower than 4.5, fluorapatite will be formed as long as hydroxyapatite is dissolved (BOTELHO et al., 2014). A higher availability of F seem to be necessary to avoid dentin demineralization. This is an interesting result, as the use of toothpastes can be conducted without monthly hygienist applications such as F varnish or other products, and this type of prevention can be less expensive than other strategies. Likewise, the addition of *f* TCP was not more effective in both articles, which is also relevant, once the introduction of biofunctional materials as *f* TCP on toothpaste is not a cost-benefit strategy.

All data might be interpreted with caution. The anticaries potential of conventional F toothpastes in reducing enamel caries is well established [Walsh et al., 2010]. In the article 1 and in a previous *in situ* study, it was also suggested that conventional toothpaste is able to decrease root dentin dental caries, when compared to non-F toothpaste (BOTELHO et al., 2014). Therefore, we speculate that only in patients that are at high-risk to develop dental caries, such as elderly population and patients undergoing radiotherapy of head and neck, high-F toothpastes must be used. On the other side, arginine-based toothpaste also showed great results in the article 2, which means that this toothpaste could be a great choice to prevent root caries in these patients, targeting the oral biofilm in association with the benefits of F. Such toothpastes are less expensive than high-F one and it is commercial available, instead of the high-F toothpastes, that must be prescribed by a professional.

The main aetiologic factor for the initiation and development of dental caries is the presence of a cariogenic biofilm and fermentable carbohydrates (RAVALD, 1986). However, in patients undergoing radiotherapy of head and neck, there is also the additional factor of reduced salivary flow because of radiotherapy or medication, which increase the risk to develop root surface caries. In the article 3, it was also showed changes on the structure, composition and mechanical properties of root dentin substrate after radiotherapy. These data are relevant since the direct effects of radiation exposure can increase the risk for radiation related caries in these patients. Therefore, in irradiated teeth, further studies might be conducted to prevent and control root caries lesions. It is possible that high-F toothpastes can be a great approach to control it, once the present results also showed good performance when it was used in sound root dentin, but this must be confirmed.

4 FINAL CONSIDERATIONS

4 FINAL CONSIDERATIONS

The present results focused on root dentin substrate and in the effects of high-F and arginine-based toothpastes to control root caries lesion. The main idea was to prevent this condition in patients at high risk to develop caries to improve their quality-of-life. It was showed *in vitro* and *in situ* that conventional toothpaste is not enough to inhibit the demineralization process and prevent the development of root caries lesion, but arginine-based toothpaste has shown better results in clinical situations. However, the best performance were showed by high-F toothpastes, even *in vitro* or *in situ*, which mean that higher F concentration might be necessary to prevent and arrest root caries lesion in patients at high-risk, such as elderly people and patients submitted to radiotherapy of head and neck. Second, it was observed that radiation exposure can alters the composition, structure and mechanical properties of root dentin. Therefore, this substrate can perform different from non-irradiated teeth, which can also influence the prevention effect of the toothpastes, but further studies are necessary.

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ANNEXES

ANNEXE 1

Título da Pesquisa: Avaliação in vitro e in situ do efeito de dentifrícios fluoretados de alta concentração de fluoreto e a base de arginina no controle da cárie radicular
Pesquisador Responsável: Marilia Mattar de Amôedo Campos Velo
Área Temática:
Versão: 5
CAAE: 44955115.9.0000.5417
Submetido em: 30/06/2016
Instituição Proponente: Universidade de Sao Paulo
Situação da Versão do Projeto: Aprovado
Localização atual da Versão do Projeto: Pesquisador Responsável
Patrocinador Principal: Financiamento Próprio

Comprovante de Recepção: PB_COMPROVANTE_RECEPCAO_636375

Título da Pesquisa: Influência da radiação na morfologia, composição e propriedades mecânicas da dentina radicular - Estudo in vitro
Pesquisador Responsável: Marilia Mattar de Amôedo Campos Velo
Área Temática:
Versão: 1
CAAE: 49812515.1.0000.5417
Submetido em: 29/09/2015
Instituição Proponente: Universidade de Sao Paulo
Situação da Versão do Projeto: Aprovado
Localização atual da Versão do Projeto: Pesquisador Responsável
Patrocinador Principal: Financiamento Próprio

Comprovante de Recepção: PB_COMPROVANTE_RECEPCAO_580355

ANNEXE 2

De: **Journal of Dentistry** eesserver@eesmail.elsevier.com
Assunto: Your Submission
Data: 5 de junho de 2017 11:56
Para: wanglinda01@yahoo.com.br, wang.linda@uol.com.br

JD

Journal of Dentistry
Ms. Ref. No.: JJOD-D-17-00545
Title: High-fluoride toothpastes combined or not with f tri-calcium phosphate reduce initial root dentin demineralization in vitro

Dear Linda,

Thank you for submitting your manuscript to the Journal of Dentistry.

Manuscripts submitted for publication are critically evaluated by the Editorial board as to their scientific importance in the area of research covered. Your manuscript was assessed and was not considered to be of sufficient priority for publication in The Journal of Dentistry. I realise that such decisions are difficult but the Journal receives considerably more manuscripts than we have room to publish, so competition for space in the journal is intense.

Although your contribution was not successful on this occasion, we hope that you will continue to consider Journal of Dentistry for future submissions.

We thank you for providing us with the opportunity to consider your manuscript.

Yours sincerely,

Professor. Christopher D. Lynch
Editor in Chief
Journal of Dentistry

ANNEXE 3

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (High-fluoride toothpastes combined or not with ƒ tri-calcium phosphate reduce initial root dentin demineralization *in vitro*) will be included in (Thesis) of the student (Marilia Mattar de Amoêdo Campos Velo) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 02 de Julho 2017.

Marilia Mattar de Amoêdo Campos Velo

Ana Laura Herrera Farha

Aymée Shiota

Heitor Marques Honório

Ana Carolina Magalhães

Linda Wang

ANNEXE 4

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (**Caries lesions inhibition and repair using high-fluoride toothpastes with or without tri-calcium phosphate and conventional toothpaste containing 1.5% arginine: an *in situ* investigation)** will be included in Thesis of the student Marilia Mattar de Amoêdo Campos Velo was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Marilia Mattar de Amoêdo Campos Velo

Ana Carolina Magalhães

Maria Angélica Silvério Agulhari

Heitor Marques Honório

Linda Wang

ANNEXE 5

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (Radiotherapy alters the composition, structure and mechanical properties of root dentin) will be included in (Thesis) of the student (Marilia Mattar de Amoêdo Campos Velo) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 02 de Julho 2017.

Marilia Mattar de Amoêdo Campos Velo

Ana Laura Herrera Farha

Aymée Shiota

Paulo Sérgio da Silva Santos

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